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COMMUNICATIONS

OX VITREOUS HUMOUR. 1.—THE RESIDUAL PROTEIN*

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WHEN a vitreous humour is removed intact from an eye and put on a filter or hung from a clamp, a viscous fluid drips out of it until finally all that remains is a thin membranous wisp of material. Robertson and Duke-Elder (1933) describe the vitreous humour as " a gel composed of a meshwork of elastic fibrillae suspended in a viscous fluid," and we may suppose that the membranous stuff left after filtration forms the elastic fibrils of the intact vitreous body. This insoluble residue was first studied by Young (1894) and Mörner (1894), who considered that it was collagen because it dissolved in boiling water and the solution set to a gel on cooling. Mörner (l.c.) termed it the residual protein, and it has since been

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known by this name. Duke-Elder (1930) described it as like gelatin, but Meyer (1945) classified it as an insoluble mucoid of unknown composition. The analyses of the insoluble residue show that it is a protein. Krause (1934) found 14·16 per cent. N and gave analytical figures for arginine, histidine, lysine, cystine, tryosine and tryptophane, all of which were present in the material analysed.

The experiments described in this paper bring forward further evidence in support of the view that the residual protein of vitreous humour contains a protein like collagen and describe the liquefying effect of preparations containing the enzyme collagenase on the vitreous body. These results are considered in relation to the well known acid and alkaline shrinkage of the vitreous humour, and are used to formulate a theory of the way in which the residual protein is laid down.

It is difficult to collect sufficient of the insoluble residue from the vitreous humour to identify it by analysis of the constituent amino-acids. One ox vitreous humour with a volume between 12-15 ml. yields only 2-3 mg. of insoluble residue. We have, therefore, attempted to identify this residue through its general properties, by amino-acid chromatography of hydrolysed material, by X-ray analysis and by enzyme analysis. The material appears to be fairly uniform, judged by solubility tests and enzyme digestibility, but we realise that the results we have obtained do not in any way prove that the insoluble residue of the vitreous humour consists of a single substance.

METHODS

Material. The experiments have been done using ox vitreous humours obtained within ½-2 hours after death of the animal. The humours were removed from the eye by making a complete equatorial cut through sclera, choroid and retina and allowing the vitreous humour to fall away from the retina, while still attached to the anterior half of the eye. It was then gently cut and pressed apart from the ciliary body and lens. The posterior lens capsule sometimes adheres to the vitreous humour, but can be removed with forceps.

Preparation of residual protein from vitreous humour. The insoluble residue may be separated from the soluble constituents by suspending the humour in saline and allowing the soluble substances to diffuse out into the surrounding fluid. Friedenwald and Stiehler (1935) found that the vitreous humour retained its form during prolonged washing in saline and that when it was finally filtered, the filtrate contained no organic material. We found that if an ox vitreous humour were left in the ice chest in saline

which was changed every few days, all detectable protein and hyaluronic acid had diffused out of the humour within 8-12 weeks. Such washed humours are more flaccid than when fresh and we have taken the insoluble residue remaining after their filtration as the residual protein. Salt may be removed from this by a few days' wash in distilled water.

The insoluble residue may also be prepared by filtration of the fresh vitreous humour followed by washing. This might seem the quicker method, but we found that very prolonged washing, by suspension in saline or water, was necessary to remove the small amount of soluble material remaining after the first filtration of the fresh humour.

Total nitrogen. Total nitrogen was estimated in 5-10 mg. samples frozen dried over P_2O_5. The samples were incinerated with 2 ml. H_2SO_4 + 0·5 g. catalyst (40 g. K_2SO_4, 10 g. $CuSO_4$, 0·17 g. SeO_2) incineration being continued for 5-8 hours after clearing. The ammonia was estimated by Markham's (1942) method.

Total carbohydrate. This was estimated by the orcin colorimetric method described by N. W. Pirie (1936).

Hexosamine. This was estimated by the method described by Elson and Morgan (1933).

Amino-acid chromatography. The method of Consden, Gordon and Martin (1944) was used.

GENERAL PROPERTIES OF INSOLUBLE RESIDUE

The residues from vitreous humour are grey and fibrous, showing considerable tensile strength and some anisotropy, particularly when stretched. When dried, each residue is a dark grey horny string. The colour seems to be due to adsorbed uveal pigment, cotton fibres and dust from the air and from the washing solutions. We found that the total nitrogen of such residues was near 12 per cent. and that they contained no hexosamine and from 3-7 per cent. total carbohydrate. Further purification of the residual protein while it was in this insoluble state was difficult owing to its capacity to adsorb other insoluble materials, such as cotton fibres, etc. From the general properties one may say that the vitreous residue appears to be made up largely of fibrous protein, but we felt that for further purification it was essential to get the protein, or proteins, into solution, even if this involved some degree of hydrolysis. We have, therefore, made a soluble preparation from the vitreous humour residues.

Preparation and properties of soluble protein from vitreous humour residues. Ox vitreous humours were washed as free as possible of soluble material by suspension in saline for long periods followed by filtration. The residues from about 50 humours were dissolved in 60 ml. HCl pH 1·8 on a boiling water bath. The cloudy solution was centrifuged and the small black sediment discarded. The supernatant was brought to pH 4 and the small flocculent precipitate that

came out was centrifuged off and has not been further investigated. It seems probable that this is an "artificial mucin," similar to those prepared by Meyer and Smyth (1937) and formed in this case from hyaluronic acid and any of the proteins present. The supernatant fluid from this precipitate was half saturated with ammonium sulphate by addition of an equal volume of saturated solution. An immediate copious precipitate came down. This was centrifuged out, re-dissolved in water and dialysed against distilled water in the ice chest. No further precipitate came out when the supernatant fluid from half saturation was wholly saturated with ammonium sulphate.

After several days' dialysis in the ice chest the solution of the precipitate got by 50 per cent. saturation with ammonium sulphate was frozen and dried. The preparation was a white fibrous material and had a total N 13·0-13·5 per cent. Further purification by dissolving in warm water, bringing the solution to pH 4, centrifuging and precipitation from the supernatant fluid by half saturation with ammonium sulphate gave a product that formed a firm jelly in the dialysis sac and had no ash, 14·8 per cent. N, no hexosamine and 6 per cent. total carbohydrate. We found that the vitreous humour residual protein lost 3·3 per cent. of its total nitrogen when taken into solution in dilute HCl. This loss probably represents loss of the amide group of glutamine, as Thierfelder and v. Cramm (1919) have shown that glutamine peptides are unstable under such conditions. If we correct for the loss of N on solution in acid the total nitrogen of the soluble product from ox vitreous humour residual protein is then 15·3 per cent.

The characteristic insoluble proteins of the animal body are collagen, elastin and keratin, with reticulin as a rather nebulous substance of unknown composition. The method of preparation and the general properties of the soluble stuff from ox vitreous humour residue suggests that the original material is like collagen and that it dissolves to form gelatin. Solubility in dilute acid is a property of collagen and precipitability by half saturation with ammonium sulphate and ability to form a gel are properties of gelatin. Yet the final value of 15·3 per cent. N is low for typical collagen or for gelatin. Bergmann and Stein (1939) found that collagen from ox Achilles tendon had 18·6 per cent. N. Few collagens have been analysed, other than those from skin and tendon; Mörner (1894) gave N 17·03 per cent. for ox cornea collagen and we have found that collagen prepared from ox cornea by the method of Bergmann and Stein (l.c.) had N 16·1 per cent. uncorrected for ash.

Both histologists and chemists accept cornea collagen as a typical collagen, so we felt that it would be valuable to compare it in some detail with the vitreous humour material. We have, therefore, prepared a soluble gelatin from ox cornea collagen and have compared its general properties with those of the soluble material from vitreous humour. We have also compared the amino-acid chromatograms of ox cornea gelatin and vitreous humour residues with that of commercial gelatin.

We found that we could prepare a soluble protein from ox cornea collagen by the same method as we had used with vitreous humour residue. This had no ash or hexosamine, total N 15·5 per cent. and 6 per cent. total carbohydrate, and in appearance and

properties was similar to the preparation from vitreous humour. If we correct the total N for the nitrogen lost on solution in dilute acid, the final figure is 16·0 per cent. total N.

AMINO-ACID CHROMATOGRAM OF OX VITREOUS HUMOUR RESIDUE AND OF CORNEA GELATIN COMPARED WITH COMMERCIAL GELATIN

Collagen and gelatin have a characteristic amino-acid composition. Bergmann and Stein (l.c.) found that ox Achilles tendon collagen contains 26·2 per cent. glycine and 17·1 per cent. proline and Schneider (1940) found 10·8 per cent. hydroxyproline in cowhide collagen. No other protein so far analysed has such a high simultaneous concentration of these three amino-acids, so that a qualitative analysis, such as is given by an amino-acid chromatogram, may be used to help identify a protein suspected of being related to collagen or gelatin.

The method of partition chromatography on filter paper as a qualitative method of protein analysis was introduced by Consden, Gordon and Martin (1944). The principle is that if a drop of a solution of mixed amino-acids is put on a strip of filter paper and a water saturated solvent is then allowed to flow through the paper by capillary action, the amino-acids will be carried down the paper at different rates, depending on their solubilities in the two phases; water saturated with solvent and solvent saturated with water. They will thus be separated one from another. The presence of the separated amino-acids on the paper can be detected by the usual colour reaction with ninhydrin. A mixture of known amino-acids may be used as a marker solution. Each mixture will give a characteristic pattern of spots which ideally will be separated from one another on the filter paper and one can, therefore, compare one protein hydrolysate qualitatively with another by this technique.

The soluble preparations from ox vitreous humour and cornea were compared with a commercial gelatin (total N 17·0 per cent.) and with a mixture of glycine, proline and hydroxyproline made up in the proportions in which they occur in collagen. Three to five mg. of each preparation was dissolved in 1·0 ml. 6 *N* HCl and hydrolysed in sealed tubes at 110° overnight. The solutions were then dried *in vacuo* over H_2SO_4 and NaOH and all remaining HCl was removed by dissolving the residues again in 1 ml. H_2O and re-drying *in vacuo*, as before. This was repeated 5-6 times, to be certain of removing all acid.

When partition chromatograms are run on mixtures of amino-acids, the pattern which results when the paper is sprayed with ninhydrin and then heated is characterised by the following features:—

1. The presence of spots at different positions along the length of the run. The exact position of each spot depends upon the nature of the amino-acid component (Consden, Gordon and Martin (l.c.)), and to a certain extent also upon the amount of amino-acid contained in it (Fisher, Parsons and Morrison, 1948).

2. Each spot is coloured. Most amino-acids give spots with ninhydrin which are coloured rose or pink; some, *e.g.*, alanine, are rather more blue, while proline and hydroxyproline are instantly recognised by a characteristic yellow colour.

3. The size of each coloured spot varies. It has been shown that there exists a relatively simple relationship between the area of a spot and the amount of amino-acid in it (Fisher, Parsons and Morrison (l.c.)).

One dimensional partition chromatograms were run on solutions of the hydrolysates prepared as described above from ox vitreous humour and cornea, from commercial gelatin and on a marker solution of a mixture of glycine, proline and hydroxyproline made up in the proportions in which these amino-acids occur in collagen. The dilutions were so made that the volume of each hydrolysate put on the paper was 3 μl and contained 30 μg total N. Chromatograms of the three hydrolysates and of the mixture of amino-acids were run on the same sheet so that the pattern would not be affected by such adventitious sources of variation as those due to temperature changes and length of run. The chromatograms were run in two different types of solvent, so that two different types of pattern could be obtained, since the positions of the amino-acids in a developed chromatogram relative to each other and to the solvent front depend upon the solvent used. We used either phenol with 0·7 per cent. NH_3 or a pyridine-amyl alcohol mixture (Edman, 1945).

When the chromatograms were developed very striking similarities in the pattern exhibited by the three hydrolysates were at once evident (Fig. 1). The components in all three mixtures were comparable not only in position but in shades of colour and in spot area. This was true for chromatograms developed in both types of solvent. No attempt was made to identify all the amino-acid components of the hydrolysates, *e.g.*, by running two dimensional runs or by running hydrolysates in parallel with various synthetic mixtures; it was, however, evident that all three hydrolysates contained roughly comparable amounts of proline, hydroxyproline, glycine, glutamic and aspartic acids, and alanine.

Using the one dimensional technique and dealing with complex mixtures of components it is not possible to say whether or not small amounts of other amino-acid residues are present in one of

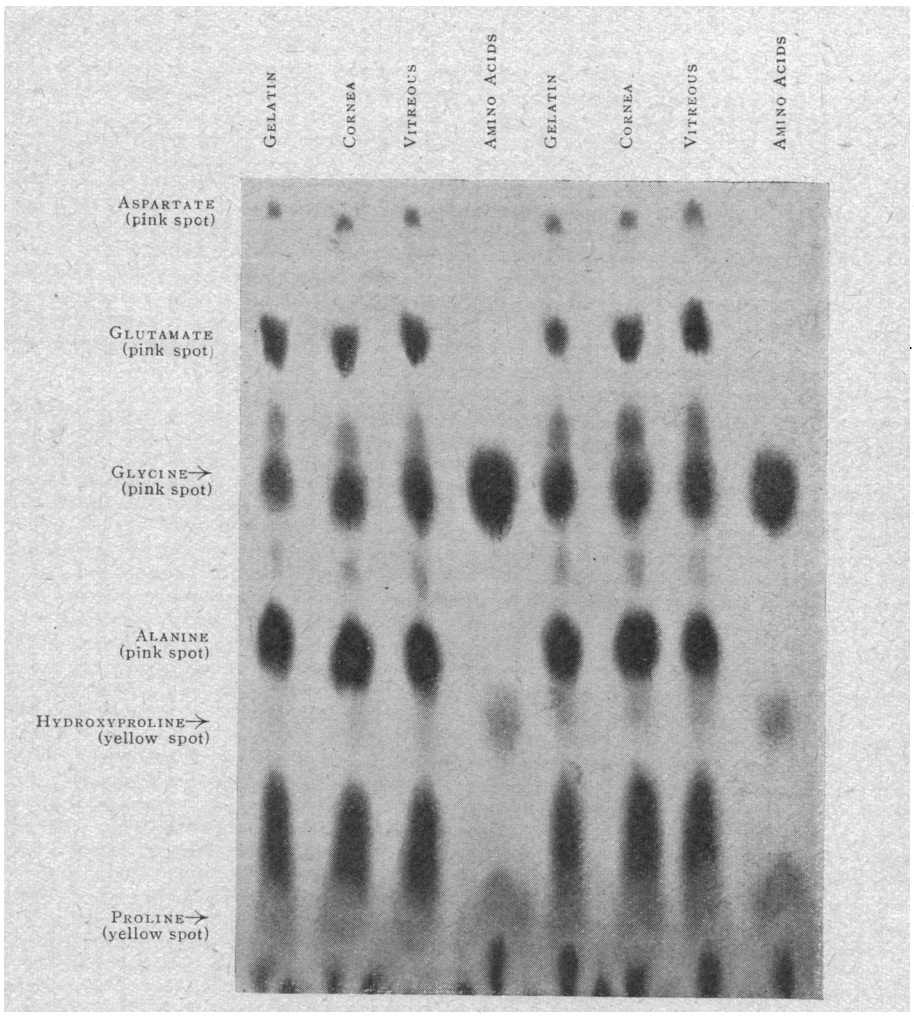

FIG. 1.

Partition chromatograms of hydrolysates of commercial gelatin, cornea gelatin, vitreous humour residual protein and a mixture of glycine, proline and hydroxy-proline. Whatman filter paper No. 1. Solvent saturated solution of phenol in water plus 0·7 per cent. NH_4OH.

the hydrolysates, but not in the others. While, therefore, there is no justification on the evidence of partition chromatography alone for identifying the nature and relative amounts of all the residues in the hydrolysates from the three sources, the evidence is sufficient to suggest that the vitreous humour protein might be a member of the collagen gelatin group.

X-RAY ANALYSIS OF RESIDUAL PROTEIN

We have taken X-ray photographs of vitreous humour residues, both in the unstretched and the stretched state. Material was prepared for X-ray analysis by combined washing and filtration. Vitreous humours were suspended in saline and left in the ice chest for several days with frequent changes of saline. This preliminary washing removed most of the soluble protein and some hyaluronic acid. The humours were next suspended and washed in successive changes of distilled water in order to remove most of the salt, which would interfere with the X-ray photograph of the protein. The humours were then filtered by suspension on a glass filter and, after a further wash, the residue on the filter was dried. This residue, collected from several vitreous humours, was photographed without being stretched. In a further experiment and in order to get some orientation of the material, the vitreous humours were only partly filtered after being washed and while still wet were bunched together with a loop of cotton and hung on a clamp to drip further. When nearly all the contained fluid had dripped out, a loop of cotton was tied round the lower end of the bunch of humours and a 50 g. weight was attached. The bunch was now hung in a dessicator and allowed to dry completely while under tension. This dried material was always deep grey or black, probably owing to the presence of uveal pigment.

About 20 mg. of dry material, as prepared above, were compressed into a round specimen and photographed on a flat plate at a specimen-to-film distance of 4·0 cm. with an X-ray beam capable of recording up to 60 A. A powder diagram was obtained whose spacings are recorded in Table I. The close resemblance of these spacings to the figures given by Astbury (1943) for collagen suggest that the protein of the vitreous humour can be classified as a collagen type.

Confirmation of this view has come from a study of stretched material prepared as already described. This method produced some degree of orientation, as was shown by the birefringence of the stretched residual protein which had been isotropic in the unstretched state. The X-ray photograph (Fig. 2), taken in this case on a cylindrical camera of radius 2·00 cm. and with the "fibre" axis of the specimen perpendicular to the X-ray beam, also demonstrates a considerable degree of orientation. The general agreement of the spacings of the X-ray reflections with those of oriented collagen fibres provides additional support for the view that the protein of the vitreous humour belongs to the collagen class.

TABLE I

SPACINGS GIVEN BY INSOLUBLE PROTEIN OF OX VITREOUS HUMOUR

Spacings obtained from unstretched specimen	*Spacings obtained from stretched specimen* (calculated from Fig. 1)	
	Equatorial	Meridian
A	A	A
2·9	2·1	2·9 (2·86; 2·91)
3·9	4·3 (4·4)	9·1 (9·5)
12·1	5·9 (5·62)	—
ca ·50	11·8 (10·9)	—

The figures in brackets are those given by Astbury (l.c.) for collagen.

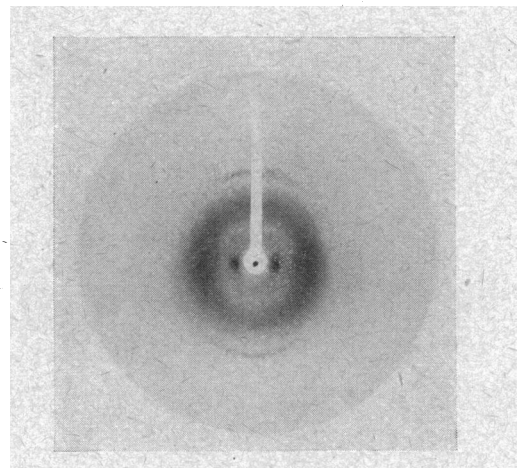

FIG. 2.

X-ray diagram of insoluble protein of ox vitreous humour. Stretched preparation showing orientation of fibres. Photograph taken with cylindrical camera, radius 3·00 cm. Cu radiation, Ni filtered.

ENZYME ANALYSIS OF VITREOUS RESIDUAL PROTEIN

The simplest test to find whether a given enzyme digests the residual protein of the vitreous humour is to see whether the enzyme takes the protein into solution. Previous workers have shown that the residual protein of the vitreous humour is, like collagen, dissolved by pepsin, but not by trypsin. We have confirmed this with our own preparations.

Through the kindness of Dr. W. E. van Heyningen we were able to test the effect of preparations of collagenase from *Cl. Welchii*. Bidwell and van Heyningen (1948) have shown that this enzyme digests only collagen and reticulin of all proteins so far tested, and this remarkable specificity makes it extremely useful for purposes of identification.

We found that a preparation of the enzyme readily dissolved the isolated residual protein of the vitreous humour. Small volumes of undiluted enzyme solution were next injected into the vitreous humour and Table II shows that collagenase preparations will liquefy the vitreous humour either in the eye or after extraction of the humour from the eye. Liquefaction was more complete after extraction, possibly because there was inevitable damage to the structure during removal from the eye, which made diffusion of the enzyme through the humour more rapid. After incubation with enzyme the vitreous humours were filtered and the residues on the filters—if any—were washed by suspension in water for some hours or days and were then dried *in vacuo* and weighed. In all experiments vitreous humours that had been injected with saline were used as controls.

TABLE II

EFFECT OF *Cl. Welchii* COLLAGENASE ON OX VITREOUS HUMOUR

Experiment No.	Vitreous preparation	Enzyme addition ml.	Saline addition ml.	Time at 37° hr.	Gross effect	Weight of residue mg.
1	in ox eye	0·4	—	16	½ liquid	1·4
	,,	—	0·4	,,	intact	3·4
2	in ox eye	0·2	—	24	½ liquid	1·7
	,,	—	0·4	,,	intact	3·6
3	freshly extracted	0·4	0·2	20	liquid	none
	,,	—	0·6	,,	intact	3·6
4	after six days in saline	0·4	—	22	liquid	none
	,,	—	0·4	,,	intact	—
5	residue protein fron filtered vitreous humour	0·1	0·9	24	dissolved	none

NOTE: *Cl. Welchii* collagenase preparation contained 220 Q enzyme units/ml. Eight ml. of enzyme preparation were mixed with 2 ml. antiserum containing 75 α units/ml., 70 θ units/ml. and 320 antihyaluronidase (Lister) units/ml. before use in these experiments.

The table shows that the collagenase preparation had an obvious liquefying effect on the vitreous humour and, in conjunction with this, the weight of insoluble residue remaining after filtration was smaller than in the controls. In some cases the residual protein was completely dissolved. The weights of the control residues were rather high, probably because they were only washed in water for a day or two before drying. We feared that prolonged washing would disintegrate the residues from the enzyme treated vitreous humours and so restricted the washing of all residues to short periods.

The evidence given by the various experimental methods described all suggests that the insoluble residue of the ox vitreous humour is largely made up of a protein of the collagen class. To the ophthalmologist, knowledge of the chemistry of the vitreous humour is mainly of interest if it increases knowledge of its development and of the normal and abnormal behaviour and appearance of the vitreous humour during life. The experiments reported here tell us nothing of the development of the vitreous humour, but we think that the results can be useful in formulating a more precise picture of the " meshwork of elastic fibrillae suspended in a viscous fluid," which Robertson and Duke-Elder (l.c.) considered to be the structure of the vitreous humour, as a result of their work on its physical properties. In the following paragraphs we have, therefore, attempted an interpretation of the chemical results in terms of the behaviour of the vitreous humour under various conditions and we report experiments which we believe support this interpretation.

COMPARISON OF VITREOUS HUMOUR WITH COLLAGEN GEL

The insoluble protein in the vitreous humour appears to be laid down in a state of extremely fine division, either as a very fine meshwork of fibres or as extremely thin sheets. Such a network of molecular fineness is on the borderline of what is usually considered to be the structure of a gel.

We have, therefore, compared the properties of the vitreous humour with the properties of a collagen gel. Soluble collagen was first described by Nageotte (1927), who prepared it by treating rat tail tendon with very dilute acetic acid. Most of the tendon goes into solution and collagen may be re-precipitated either as macroscopic fibres, by neutralising the solution, or as a firm gel by dialysing the solution against distilled water to remove salts and acid. Nageotte and Guyon (1931) found that rat tail tendon collagen is the only collagen that goes into solution in this way. Tendons from the ox are insoluble.

We have prepared collagen gels of varying concentration from filtered solutions of rat tail tendon and have compared these with the vitreous humour. Gels in which the concentration of collagen is 0·3 per cent. or over do not break down when put on a filter, but gels of 0·07 per cent. or lower concentrations, could be filtered in exactly the same way as the vitreous humour, the collagen being left behind on the filter as a wisp-like residue. This collagen residue showed considerable anisotropy, particularly when stretched, rather like the residue from the vitreous humour and it stained brown with silver, again like the vitreous humour residue.

There were, however, two important differences between the gel residue and the vitreous humour residue. In the first place, the vitreous humour residue showed considerable tensile strength, even when wet, while the gel residue showed little or none. A more important difference was that when the gel residue was suspended in water it swelled and finally went completely into solution, whereas the vitreous humour residue showed no sign of dissolving under such conditions and remained intact for many months.

We consider, therefore, that the structure of the vitreous humour is rather different from a collagen gel, the differences between them perhaps showing that there is a firmer, more stable arrangement in the vitreous humour, which may perhaps be considered as a network of submicroscopic fibres. This picture, if correct, must conform with what is known of the properties of collagen and collagen fibres obtained from other sources.

SWELLING OF COLLAGEN AND SHRINKAGE OF VITREOUS HUMOUR

One of the most characteristic properties of collagen is its capacity to swell, the degree of swelling varying with the acidity or alkalinity of the solution. Fig. 3 gives the swelling curve of fresh, undried ox cornea collagen (Pirie, 1947) and shows that there is maximal swelling in acid, minimal at neutrality and intermediate swelling in alkaline solutions. Highberger (1939) has found that the isoelectric point of native skin collagen is pH 7·4-7·6, which corresponds with the zone of minimal swelling of cornea collagen.

If we apply these results to the vitreous humour we see that the fibres in it will be *minimally* swollen under normal conditions of neutrality, but will swell with any change, either towards acidity or alkalinity. This is exactly the reverse of the effect of acidity or alkalinity on the size of the vitreous humour as a whole. Many investigators have shown that there is very marked shrinkage of the vitreous humour in either acid or alkali and we found that the same is true for vitreous humour residues washed free from the

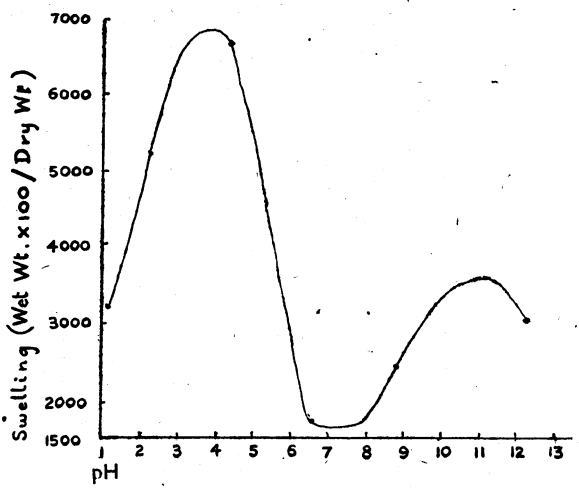

FIG. 3.

Swelling curve of ox cornea collagen.

soluble contents. A vitreous humour residue may have a wet weight of 10-15 g. at neutrality and one of 1-2 g. after suspension in $\frac{N}{100}$ HCl.

We think that shrinkage of the vitreous humour and of the isolated vitreous humour residues in acid and alkali may be explained on the assumption that the fibres in it are arranged in a network. Küntzel (1926) showed that collagen fibres from rat tail shortened in length as they swelled in acid, the shortening being directly proportional to the degree of swelling of the fibre. This was confirmed by Jordan-Lloyd and Marriott (1935). If we consider a loose network of collagen fibres, swelling of these fibres will cause a shortening of each individual one. If the nodes are fixed, the shortening of each fibre will cause an over-all contraction of the whole structure because the lateral swelling of the fibres simply fills up the interfibrillar spaces which originally are very large compared to the volume of the fibres.

We suggest that the shrinkage of the vitreous humour in acid or alkali is due to the swelling and shortening of the fibres within it. Some evidence in favour of this view may be got from a study of the effect of salts on the volume changes in acid and alkali of the vitreous humour and of the isolated residues. Loeb (1920) showed that acid swelling of gelatin was depressed by salts, the degree of depression at any particular pH and salt concentration depending

upon the valency of the anion. Alkaline swelling of gelatin was also depressed by salts, the depression depending on the valency of the cation.

If salts act in this way on the fibres of the vitreous humour we should expect that the volume of the vitreous humour, which we suggest is *inversely* related to the volume, or degree of swelling of the fibres within it, will be greater in acid plus salts than in acid alone. We should also expect that salts with di- or tri-valent anions will be more effective in reducing the shrinkage of the vitreous humour in acid than salts with monovalent anions.

A great deal of work on swelling and shrinkage of vitreous humour has already been done, in some of which the effect of salts was examined. A difficulty in assessing these results is that the fresh vitreous humour contains both soluble and insoluble constituents and when suspended in acid or alkali the soluble constituents —if not precipitated—will diffuse into the surrounding fluid, thus altering its composition. The amount of salts diffusing out of the humour may appreciably change the concentration in the solution unless the volume of solution is very great or it is renewed after some time.

EFFECT OF SALTS ON VOLUME OF WASHED VITREOUS HUMOUR RESIDUES

We have examined the effect of salts on acid and alkaline swelling of the washed vitreous humour residues. By using such washed residues we have avoided any complication due to precipitation of vitreous mucin by acid, and lack of equilibrium owing to outward diffusion of soluble constituents, including salts. The results we obtained were the same as those for fresh vitreous humours, but we found that the final volume was much more rapidly obtained and, in general, the valency effect was more clearly shown.

Each washed residue or fresh vitreous humour was put in a stoppered flask containing 200 ml. of the appropriate solution and left on the bench for 24-48 hours. The volume of the vitreous residue or fresh vitreous humour was determined both before and after suspension in fluid by placing in a measuring cylinder of appropriate size and reading off the volume. The pH of the solutions was determined electrometrically before and after immersion of the vitreous preparations in them.

Table III shows the percentage decrease in volume of washed vitreous residues after immersion in different salt solutions at either acid or alkaline pH. The first experiment shows that in acid the monovalent anion Cl^- has practically no effect on vitreous shrinkage, the divalent anion SO_4^{--} diminishes vitreous shrinkage considerably and the trivalent anion $Fe(CN)_6^{---}$ in a much lower concentration almost abolishes the shrinkage. Experiment two shows

TABLE III

EFFECT OF SALTS ON ACID AND ALKALINE SHRINKAGE OF WASHED VITREOUS HUMOUR RESIDUES

Expt.		HCl at pH 2·0			
	Salt added	None	$\frac{M}{128}$ NaCl	$\frac{M}{128}$ Na_2SO_4	$\frac{M}{1024}$ $K_3Fe(CN)_6$
1	Per cent. decrease in volume	82	79	36	14

		HCl at pH 1·8					
	Salt added	None	$\frac{M}{64}$ $CaCl_2$	$\frac{M}{64}$ $AlCl_3$	$\frac{M}{64}$ K_2SO_4	$\frac{M}{1024}$ K_2SO_4	$\frac{M}{1024}$ $K_3Fe(CN)_6$
2	Per cent. decrease in volume	94	92	92	50	85	30

		NaOH at pH 11·1		
	Salt added	None	$\frac{M}{512}$ NaCl	$\frac{M}{512}$ $CaCl_2$
3	Per cent. change in volume	−78	−63	+18

		NaOH at pH 11·4				
	Salt added	None	$\frac{M}{256}$ KCl	$\frac{M}{256}$ K_2SO_4	$\frac{M}{256}$ $CaCl_2$	$\frac{M}{32}$ K_2SO_4
4	Per cent. decrease in volume	61	77	56	23	36

that calcium chloride and aluminium chloride do not depress shrinkage in acid, which is in favour of the view that this effect in acid is dependent on the valency of the anion not the cation. It also shows that although $\frac{M}{64}$ K_2SO_4 depresses shrinkage, $\frac{M}{1024}$ has little effect, while $\frac{M}{1024}$ $K_3Fe(CN)_6$ a salt with a trivalent anion, has a greater effect than $\frac{M}{64}$ K_2SO_4. Experiments three and four show that in alkali $CaCl_2$ reduces shrinkage—in fact, in one case there is slight expansion—while KCl and NaCl have no effect. In

alkali, therefore, shrinkage is depressed according to the valency of the cation of the added salt.

We found that if those vitreous residues which had been immersed in $K_3Fe(CN)_6$ or K_2SO_4 solutions were washed free of salts and then put in HCl of pH2 they shrank further until they equalled in size the vitreous residues that had been placed directly in acid. This shows that the salts that depress vitreous shrinkage have no permanent effect. The dry weight of fully shrunken residues whose volume was between 1-2 ml. was the same as the dry weight of vitreous residues held at neutrality, showing that the shrinkage was not due to solution of the residue by the acid.

These experiments were repeated using fresh vitreous humours. We found that one ox vitreous humour could change the pH of 200 ml. dilute acid or alkali by as much as two units and it was necessary to transfer the humours to fresh solutions after 24 hours, both in order to obtain the required pH and also to dilute the salts which had diffused out of the vitreous itself.

TABLE IV

EFFECT OF SALTS ON ACID AND ALKALINE SHRINKAGE OF FRESH VITREOUS HUMOUR

		HCl at pH 2·2			
Expt.	Salt	None	$\frac{M}{128}$ NaCl	$\frac{M}{128}$ K_2SO_4	$\frac{M}{1024}$ $K_3F3(CN)_6$
1	per cent. decrease in volume	71	76	62	26

		NaOH at pH 11·2				
	Salt	None	$\frac{M}{256}$ KCl	$\frac{M}{256}$ $CaCl_2$	$\frac{M}{32}$ KCl	$\frac{M}{32}$ $CaCl_2$
2	per cent. decrease in volume	70	62	8	52	8

The shrinkage of fresh vitreous humour in acid or in alkali was also depressed by the addition of salts, in acid the depression was related to the valency of the anion and in alkali to the valency of the cation. Table V shows that $\frac{M}{4}$ KCl has about the same effect as $\frac{M}{32}$ K_2SO_4 or $\frac{M}{1024}$ $K_3Fe(CN)_6$.

TABLE V

EFFECT OF DIFFERENT CONCENTRATIONS OF KCl, K_2SO_4 AND $K_3Fe(CN)_6$ ON VOLUME OF FRESH VITREOUS HUMOUR IN HCl AT pH 1·8

Molarity of salt	Final volume of vitreous humour ml.		
	KCl	K_2SO_4	$K_3Fe(CN)_6$
0	1·5	1·5	1·5
$\frac{M}{4}$	8·0	—	—
$\frac{M}{32}$	5·0	8·0	—
$\frac{M}{64}$	—	5·0	—
$\frac{M}{128}$	2·5	2·5	—
$\frac{M}{512}$	3·5	3·0	—
$\frac{M}{1024}$	3·5	4·0	7·5

All vitreous humours had an initial volume of 14—15 ml. The final volumes were measured after 64 hours. The humours were transferred to fresh solutions after 24 hours, to avoid effect of salts diffusing from vitreous itself.

Salts, therefore, depress *shrinkage* of the vitreous humour, just as Loeb (l.c.) found they depressed *swelling* of gelatin. We think it is most likely that they are having the same effect in both cases, depressing the acid and alkaline swelling of a protein. The results support the view that the insoluble vitreous residual protein is laid down as a meshwork of fibres, each of which will swell and shorten in acid or alkali and so cause shrinkage of the whole vitreous humour.

Discussion

Slit-lamp examination of the vitreous humour during life or after extraction shows that it is not optically empty, but that it may contain sheets or membranes of reflecting material. Friedenwald

and Stiehler (1935), using the slit ultra-microscope to examine the extracted vitreous humour of the ox, observed fairly regular bright reflections which they interpreted as due to very thin sheets of the residual protein. The bright reflections ran roughly parallel to the surface of the vitreous humour and were separated from one another by spaces of 0·5-3 microns. Friedenwald and Stiehler considered that the thickness of the sheets was of the same order of magnitude as the wave length of light and they thought that cohesion between sheets might form the visible opacities of the degenerating humour. Vogt (1941), who has studied the vitreous humour in the living human eye, speaks of the scaffolding of the vitreous body and states that it is predominantly lamellar in structure, that is, membranous, but that a fibrillar structure in the membranes can often be made out. Vogt's very beautiful pictures of vitreous opacities fit with the idea that these are formed by cohesion between membranes or fibres.

The vitreous humour of the ox appears to be a more rigid structure than that of any other animal so far examined. Meyer, Smyth and Gallardo (1938) have found that ox vitreous humour contains a great deal more hexosamine than the humours of other animals. This indicates that the hyaluronic acid content of ox vitreous humour is greater and probably explains its greater rigidity. In spite of such quantitative differences between animals, there appears to be a qualitative similarity, both in the slit-lamp appearance and in the chemistry of the vitreous humours of different species.

If the experimental evidence given here is accepted the picture of the structure of the vitreous humour becomes more complex. It cannot be considered as uniform, but must be made up of at least two " structures," the collagen-like network and the hyaluronic acid and protein jelly. Liquefaction of the humour follows enzymic hydrolysis of the network and therefore one may say that this network is essential for maintenance of a normal vitreous humour. Yet it does not form the humour, being minimally swollen at neutrality and occupying only a very small part of the total volume. It seems to us that it is the relation of the network to the jelly which is of first importance and must be taken into account in any theories of the causes of vitreous swelling and vitreous degenerations.

SUMMARY

1. X-ray photography, amino acid chromatography and enzyme analysis provide evidence that the residual protein of the ox vitreous humour is largely a collagen type.

2. Enzyme preparations containing collagenase liquefy the ox vitreous humour.

3. The bearing of this result on the conception of vitreous humour structure is discussed.

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REFERENCES

ASTBURY, W. T. (1943)—Adv. in Enzymol., Vol. III, p. 63.

BERGMANN, M. and STEIN, W. H. (1939)—*Jl. Biol. Chem.*, Vol. CXXVIII, p. 217.

BIDWELL, E. and VAN HEYNINGEN, W. E. (1948)—*Biochem. Jl.*, Vol. XLII, p. 140.

CONSDEN, R., GORDON, A. H. & MARTIN, A. J. P. (1944)—*Biochem. Jl.*, Vol. XXXVIII, p. 224.

DUKE-ELDER, W. S.—*Brit. Jl. Ophthal.*, Monograph Suppl., No. 4.

EDMAN, P. (1945)—*Arkiv. f. Kemi Mineral. Och. Geol.* 22A, No. 3.

ELSON, L. A. & MORGAN, W. T. J. (1933)—*Biochem. Jl.*, Vol. XXVII, p. 1824.

FISHER, R. B., PARSONS, D. S. & MORRISON, G. A. (1948)—In press.

FRIEDENWALD, J. S. and STIEHLER, R. D. (1935)—*Arch. of Ophthal.*, Vol. XIV, p. 789.

HIGHBERGER, J. H. (1939)—*Jl. Amer. Chem. Soc.*, Vol. LXI, p. 2302.

JORDAN-LLOYD, D. & MARRIOTT, R. H. (1935).—*Proc. Roy. Soc. Lond. B.*, Vol. CXVIII, p. 439.

KRAUSE, A. C. (1934)—Biochemistry of the Eye. The Johns Hopkins Press, Baltimore.

KÜNTZEL, A. (1926)—*Koll. Zeits.*, Vol. XL, p. 264.

LOEB, J. (1920-21)—*Jl. Gen. Physiol.*, Vol. III, p. 391.

MARKHAM, R. (1942)—*Biochem. Jl.*, Vol. XXXII, p. 790.

MEYER, K. and SMYTH, E. M. (1937)—*Jl. Biol. Chem.*, Vol. CXIX, p. 507.

MEYER, K., SMYTH, E. M. and GALLARDO, E. (1938)—*Amer. Jl. Ophthal.*, Vol. XXI, p. 1084.

MEYER, K. (1945)—*Advances in Protein Chem.*, Vol. II, p. 250.

MÖRNER, C. T. (1894)—*Zeits. f. Physiol. Chem.*, Vol. XVIII. p. 233.

NAGEOTTE, J. (1927)—*Compt. Rend. Soc. Biol.*, Vol. XCVI, p. 172.

NAGEOTTE, J. and GUYON, L. (1931)—*Arch. de. Biol.*, Vol. XLI, p. 1.

PIRIE, A. (1947)—*Biochem. Jl.*, Vol. XLI, p. 185.

PIRIE, N. W. (1936)—*Brit. Jl. exp. Path.*, Vol. XVII, p. 274.

ROBERTSON, E. B. and DUKE-ELDER, W. S. (1933).—*Proc. Roy. Soc. Lond. B.*, Vol. CXII, p. 215.

SCHNEIDER, F. (1940)—*Collegium*, p. 97.

THIERFELDER, H. & VON CRAMM, E. (1919)—*Zeits. Physiol. Chem.*, Vol. CV, p. 58.

VOGT, A. (1941)—Slit-lamp Microscopy of the Living Eye, Vol. III. Schwezer, Druck und Verlagshaus, Zurich.

YOUNG, R. A. (1894)—*Jl. Physiol.*, Vol. XVI. p. 325.