CXLI. STUDIES ON THE VITREOUS BODY. II.

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IN an earlier paper [Duke-Elder *et al.*, 1935] direct experiments were described showing that the vitreous body (henceforth called simply the vitreous) does not take up water *in vitro* under the influence of soaps and changed p_H . As the clinical picture of primary chronic glaucoma strongly suggests that a swelling of the vitreous is closely related to the raised intra-ocular pressure the work was continued with a view to showing whether such a change in volume of so dilute a gel can reasonably be expected on physico-chemical grounds.

So far two possible theories of the mechanism of volume increase have occurred to us depending on different conceptions of the mode of origin of the vitreous. First, if it is assumed that the vitreous is formed by a continuous process of swelling from an initial xerogel similar to the process of swelling of dry gelatin in water, then, considering it as at its swelling maximum at normal p_H , it seems possible that it could swell further if the p_H were altered in a direction favourable to increased hydration.

All previous experiments designed to show whether the vitreous swells have tacitly assumed the truth of the latter proposition. However, although the colloid chemical literature contains many studies on the swelling of gels in the presence of foreign substances, no one, it seems, has studied the influence of these substances on a gel already at its swelling maximum. The procedure in all cases has been to swell a quantity of the xerogel at one p_H , say, and then to take another lot of the xerogel and swell it at a new p_H and so on. On consideration it is by no means evident that a swollen gel will respond to a change in its environment to the same extent or in the same way as the dry xerogel, owing to the intervention of peptisation which is particularly to be expected when the micelles are already strongly hydrated.

If, on the other hand, the vitreous is formed by a sol to gel change another theory must be presented to show how it can swell¹. In this case it is suggested that a minute fraction of the vitreous is peptised owing to the presence of some lyophilic substance; if, then, the procedure is reversed whereby the comparatively protein-rich fluid is converted into its gel form, the presence of the adsorbed hydrotropic substance enables the protein to orient more water than previously. The net result is an increase in volume. If this were to proceed over a number of years the effect would be to produce the pressure symptoms characteristic of chronic glaucoma. The process may be illustrated in a crude way by placing a lump of 10 % gelatin gel in a small volume of water. Addition of acid produces eventual peptisation; neutralisation of the acid converts the sol back into a gel of larger volume than before. (The objection that the increased pressure will expel fluid after the peptisation of a portion of the vitreous is overcome by postulating a unidirectional permeability of the surrounding membranes. This is not a

¹ Unpublished experiments of our own on gelatin clots together with theoretical considerations lead to the conclusion that a dilute clot, as opposed to a swollen gel, will not increase in volume directly when its environment is changed.

further *ad hoc* assumption, as some such state must be the case or the vitreous would break down under the unbalanced stress.)

This paper is divided into three parts according as the experiments described throw light on the possibility of a gel at its swelling maximum swelling further on changing the p_H suitably (Part I), on the structure of the vitreous and hence its mode of origin (Part II), and on the possibility of peptising the residual protein (Part III).

EXPERIMENTAL.

Part I. Swelling of fibrin.

Pure fibrin, prepared by the combined methods of Fischer [1908] and Schuldenzucker [1928], was swollen at different p_H values in the acid range in a Schmidt apparatus [1931] which permits measurements of water uptake to within 0.002 ml. In Fig. 1 the water uptake per g. of dry fibrin is plotted against p_H . It is evident that by allowing fibrin to swell to its maximum at p_H 6 and then changing the medium to one of p_H 2 the conditions necessary to show whether a gel at its swelling maximum can take up further water by changing the p_H favourably will be achieved.

Fig. 1.

Fig. 2.

To this end the Schmidt apparatus was modified as in Fig. 2. A bulb B with a tap T_1 fits on the side-tube by a ground joint. With T_2 closed the bulb is filled with concentrated HCl and T_1 is closed. Fibrin is placed on the porous plate, P , and when the water uptake, as measured by the movement of a meniscus in the capillary in communication with the chamber, C , has reached its maximum the tap T_1 is opened allowing acid to diffuse in, thus lowering the p_H of the fluid in contact with the swollen fibrin.

In Fig. 3 the water uptake is plotted against time. For convenience the initial part of the curve is not shown and the scale of ordinates above 2.8 ml. is half of that below this value. Obviously the swollen fibrin adjusted itself to the changed p_H . If the acid had not diffused in, the water uptake would have reached a final value of 2.80 ml., whereas, owing to the changed acidity, it rose to a maximum of 3.18 ml.

Fig. 3.

Fig. 4.

Swelling of gelatin. Gelatin was allowed to swell at p_H 4.8, the isoelectric point and hence the p_H at which swelling is minimum, and at p_H 1. Pieces of gelatin which had already swollen at p_H 4.8 were transferred to the solution of p_H 1 and their subsequent swelling followed.

Coignet's leaf gelatin was cut into approximately equal squares and after drying over conc. H_2SO_4 placed in a dish containing 2l. of the solution in a room maintained at constant temperature (20.4°). At intervals pieces were removed, blotted with filter paper, and placed on weighed pieces of filter-paper which had been previously dried over H_2SO_4 . The filter-paper and swollen gelatin were weighed and placed in a vacuum desiccator. After 24 hours drying was completed over H_2SO_4 and they were weighed again. The weight of H_2O taken up per g. of dry gelatin was plotted against time, Fig. 4.

Although a cruder technique than that of Jordan-Lloyd [1927] this has the advantage that the H_2O content of the swollen gel as it is in its final state is determined, whereas Jordan-Lloyd, by weighing the initial quantity of dry gelatin and then that of the wet gelatin remaining, is able to tell us nothing more than the change in weight of a piece of gelatin after placing it in a given medium. A medium very favourable to swelling dissolves a considerable portion of the gelatin so that by her technique the swelling of gelatin would appear to fall off with increasing acidity. This is definitely not the case.

In Fig. 4 I and II are the swelling curves of gelatin in solutions at p_{H} 1.1 and 4.8 respectively. The upper branch of curve II represents the change in the rate of swelling on transferring the gelatin from a medium of p_{H} 4.8 to one of p_{H} 1.1. It is at once evident that there is an immediate adjustment to the changed p_{H} .

Part II.

Goedbloed [1934, 1] described ultramicroscopic changes in the vitreous on treatment with KCNS solutions, which he interpreted as being due to an increased hydration of the micelles. If such were the case a change in the elastic properties of the vitreous on treatment with this substance would be expected. To test this two techniques were used, first the resistance of the treated vitreous to the emulsification of Hg by ultrasonic waves was compared with that of a control, and secondly the technique of Freundlich and Seifriz [1923] and Seifriz [1924] for the measurement of the elastic properties of gels, as modified by Robertson and Duke-Elder [1933, 1] to suit the peculiarities of the vitreous, was used.

Ultrasonic waves. When H_2O and Hg are submitted to ultrasonic waves, an emulsion of Hg in H_2O is immediately formed, the whole mixture taking on a dark grey colour [Wood and Loomis, 1927; Bull and Söllner, 1932]; if the H_2O is replaced by a jelly the elastic properties of the latter hinder the emulsification of the Hg so that after a given time there is a fairly well-defined boundary between the clear jelly and that into which Hg particles have penetrated. The height of this boundary gives a rough idea of the elasticity of the jelly.

Ox vitreous was placed in 1.0 *M*, 0.5 *M* and 0.14 *M* solutions of KCNS and in isotonic saline for 24 hours at 20° . The samples were transferred to test-tubes and 1 ml. of Hg added, and the tubes were held in succession over the vibrating column of oil above the quartz plate of the ultrasonic apparatus. The heights of the emulsified Hg columns were compared.

There was no detectable difference in the heights of the columns of the vitreous treated with KCNS and of those treated with normal saline, so that it is concluded no very gross change in the elastic properties of the vitreous are caused by KCNS.

Nickel particle technique. Vitreous is placed in a rectangular glass cell, 1 cm. edge, and a nickel particle about 50μ diameter is picked off a slide under the microscope and stabbed into the vitreous. The particle is focused and its extension due to an applied magnetic field measured by a micrometer eye-piece. Portions of vitreous treated with KCNS, K_2SO_4 and dilute HCl at p_{H} 3 were studied together with normal untreated vitreous. The results are shown in Figs. 5 and 6.

Curve 1, Fig. 5, is the typical extension-time curve for normal untreated vitreous, characterised by a rapid initial elastic displacement followed by a viscous creep approaching a fairly constant velocity.

Before describing the remaining curves we must say a few words in regard to the limitations of the technique and the results expected should hydration or dehydration of the colloid particles be achieved by the solutions mentioned. Firstly, a direct comparison of the absolute displacements of one given particle in different lots of vitreous in a given time and with a fixed field strength is difficult, as a particle of the size necessary for its recovery from the first vitreous is very irregular and its extension will depend on the direction of motion. Furthermore, as the vitreous is not stuck firmly to the cell walls there will be a certain amount of "give" which will vary with the quantity of vitreous in the cell and the depth of the particle in it. On the other hand the curve I, Fig. 5, is very typical and can be reproduced from vitreous to vitreous without difficulty. As far as the changes expected on treatment of the vitreous with the reagents mentioned are concerned, from the work of Leick [1904] and Kramer [1926] a decrease in elasticity is to be expected with K_2SO_4 and an increase with KCNS. This should be reflected in a diminution in the viscous creep, characterised by the upper part of the curve, in relation to the initial elastic displacement, in the case of K_2SO_4 , and the reverse should hold for KCNS. (Once again no work has been done on the effect of such solutions on gels already formed; workers in this field have added the substance to be examined to the sol which was then allowed to set, and the elastic modulus measured.)

Fig. 5.

Fig. 6.

Curves II and III of Fig. 5 are those for vitreous treated with 0.5 M KCNS and K_2SO_4 respectively. They are representative of a number of curves determined similarly, but it should be mentioned that a few curves were obtained not conforming to these types; the reason for these irregularities, which were not observed in normal vitreous, will be brought out later. The displacements in the different curves are in no way comparable with one another as different particles were used. It is evident that 0.5 M K_2SO_4 has no appreciable effect on the extension-time curve of ox vitreous, whilst 0.5 M KCNS has the reverse effect to that expected on the supposition that it increases hydration, the extension subsequent to the first 10 secs. being very small compared with that during this time. The absence of effect by 0.5 M K_2SO_4 is very surprising as a considerable contraction in volume was observed. The curves for isotonic

solutions of these salts (not shown) were in general similar to those for normal vitreous.

The curves obtained with vitreous treated with dilute HCl at p_H 3 (Fig. 6) throw a little light on the problem. Curve I was obtained by applying a small magnetic force; increasing the field with conditions otherwise the same gave curve II which is quite normal. It is evident that the viscosity of the medium has increased under the influence of the acidity, so that a limiting force has to be exerted to allow the creep to proceed. A similar phenomenon was observed with K_2SO_4 but not so regularly.

Baurmann [1924] showed that between p_H 3 and p_H 2 a precipitate fall in the volume of the vitreous occurs; this change is accompanied by the precipitation of the mucoprotein in the gel. If we assume that the volume change is due to the collapse of the vitreous structure, as opposed to a semi-reversible dehydration, then at p_H 3 we should expect the undermining of the vitreous structure to have already begun, and hence the changes in the elasticity could be put down to this cause. The decreased stability of the vitreous, which would tend to lower the elasticity, would at the same time increase the viscosity of the intermicellar fluid and the two effects would compensate each other to varying extents. If this is true, the irregularities in the shapes of the curves obtained by treating the vitreous with KCNS, K_2SO_4 and dilute HCl mentioned earlier are explained, as it is only to be expected that a body in a state of very unstable equilibrium will respond extravagantly to minute changes in its environment, thus prejudicing the reproducibility of results obtained on it.

In view of the results obtained on the extension-time curves, it was important to see whether the absolute values for the extension of a given particle varied greatly according as the vitreous was treated with K_2SO_4 , KCNS or dilute HCl. The only way of achieving this end was to treat a large number of pieces of vitreous successively and analyse the results statistically.

Two cells were filled at the same time to the same height with normal and treated vitreous. A particle was placed in one, and its extension in a fixed magnetic field lasting for 5 secs. was measured. Immediately the particle was recovered and placed in the other cell, and its extension under the same conditions measured. This was repeated for 12 to 13 pairs and the results are tabulated in Table I.

Table I.

Solution	d	n	s	t	P
0.5 <i>M</i> KCNS	+4.34	13	2.56	3.3	<0.01
0.5 <i>M</i> K_2SO_4	-1.10	12	4.21	0.9	0.3-0.4
1.10 ⁻³ <i>M</i> HCl	-2.30	13	2.53	3.3	<0.01

In Table I d is the mean difference in extension for normal and treated vitreous; a positive value means that the treated vitreous gave a larger extension than its control. n is the number of pairs of determinations, t is the ratio of the mean difference to its standard error [Fisher, 1928], and P is the probability that such a value of t will occur by chance. It appears that in 0.5 *M* KCNS the extension is greater and in dilute HCl at p_H 3 less than the control whilst the difference with K_2SO_4 is insignificant. Here again the results are more in conformity with the supposition that the vitreous stability has been upset than that a reversible syneresis has occurred, or in the case of KCNS a reversible hydration. The relation of these results on the elasticity of the vitreous to its mode of origin will be dealt with in the discussion later.

As the volume decrease of the vitreous on treatment with dilute HCl appeared to be correlated with the precipitation of its mucoprotein [Goedbloed, 1934, 2]

we decided to determine in what way the presence of a second protein influenced the stability of a protein clot.

Pure de-ashed gelatin was dissolved in phosphate buffer at p_H 7 to 10 % and 5 ml. lots were transferred to test-tubes. 2 ml. of a protein solution in phosphate buffer at the same p_H were added to one group and 2 ml. of the phosphate solution to another which served as controls. After equilibrating in a bath at 38° pairs were transferred to another at 20° and the course of setting followed by dropping in lead shot at intervals. When a shot failed to penetrate the surface the solution was considered to have set. As Fraas [1894] has shown that at room temperature complete equilibrium is only attained after 24 hours, the test-tubes were transferred after setting to the ice-chest overnight and the melting times of the clots determined by placing them in the bath at 38°. The results are shown in Table II; positive signs indicate degrees of increased stability of the clots as evidenced by a more rapid setting or a more protracted melting.

Table II.

Protein solution added	Change in stability	
	Setting time	Melting time
1 % ovalbumin	—	+
0.5 % agar-agar	—	+
2.0 % agar-agar	+ + +	+ + +
1.0 % serum globulin	+	—
0.75 % isinglass	—	+ +
1.5 % isinglass	—	+ +
2.0 % caseinogen	— —	— —

It is evident from Table II that a second protein, or in the case of agar-agar a carbohydrate, may enter into the intimate structure of a gelatin gel. Hitherto, in the vitreous, the residual protein has been considered as the determining factor in the degree of hydration of the vitreous. The results given in this paper lead to the view that the vitreous stability and hydration are bound up with the physical states not only of the residual protein but also of the mucoprotein and perhaps of the albumin and globulin.

Part III. Attempts at peptisation of the residual protein.

The vitreous was submitted to ultrasonic vibrations¹, as a class of gels, the thixotropic gels, are converted into their sol form by this treatment. No liquefaction of the vitreous was obtained in this manner. Solutions of KCNS of strengths varying from 0.5 *M* to a saturated solution, a variety of soaps, and thiourea, in the heat and the cold, were tried on the separated residual protein without any evidence of peptisation. Mörner's treatment [1894] by which the protein was heated with H₂O at 105° under pressure gave a clear solution, which, however, failed to set on cooling as described by him. Repeated attempts using this treatment failed to give a jelly, and it must be concluded that this vigorous treatment gave breakdown products.

DISCUSSION.

In accordance with the results of Part I, it is probable that if the vitreous were formed by a continuous process of swelling without the intervention of a sol stage, then the presence of soaps in the surrounding tissues would favour an increased volume of the vitreous (as suggested in the first paper of this series).

The results of Part II, however, in so far as they show that the effects of acid, KCNS and K₂SO₄ tend to lower the stability of the gel, point to the vitreous having been formed as a clot from an aqueous solution of the proteins,

¹ We are indebted to Prof. Freundlich and Dr Söllner for performing the experiments involving the use of ultrasonic waves described in this paper.

since gels formed by a swelling process alone exhibit a certain degree of reversibility in their hydration and dehydration phenomena. When, furthermore, the similarity in the elastic and ultramicroscopic properties of the vitreous and dilute plasma clots is considered [Robertson and Duke-Elder, 1933, 2] the balance of evidence points to the vitreous having been formed as a clot. (The histological study of embryonic vitreous development tells us very little about its physical mode of origin. In a preparation using Zenker's solution as a fixative streaks are seen emerging from the epithelium of the optic cup and later from the glial elements of the retina.) The experiments with gelatin clots show that it is likely that the mucoprotein and albumin and globulin of the vitreous enter intimately into the structure of this body.

The unsuccessful attempts at peptising the residual protein described in Part III lend a certain support to the idea of a swelling origin of the vitreous, and militate against the theory that the raised intra-ocular pressure in glaucoma is brought about by a cyclical process of peptisation and clotting. Attention should be drawn here to the statement by Goedbloed [1934, 1] that KCNS in concentrations of 2.5 *M* and greater causes a complete solution of the vitreous. Such is not the case; we have observed repeatedly that, whilst at 2 *M* concentration and greater there is a decrease in the volume of the vitreous culminating at a concentration of 2.5 *M* in its shrinkage to a minute fraction of its original volume, the effect is not one of solution but one of dehydration as the residual protein is to be seen invariably floating on the top of the solution. We may add that the context of Goedbloed's statement leaves no doubt that it was his intention to convey the idea that the residual protein completely dissolved. The same author [1934, 1, 2] denies that the vitreous is a gel on the grounds that the phenomena associated with it are not reversible. There is no point in discussing the matter at length; we can only say here that his exclusion of the vitreous from the category of gels on this count simultaneously rules out practically all substances hitherto considered as belonging to this class.

SUMMARY.

The physico-chemical mode of origin of the vitreous body, the possibility of its swelling *in vivo*, and factors in its stability and structure are investigated.

The subsequent behaviour of swollen gels on altering the p_H is described.

The changes in the elastic properties of the vitreous after treatment with solutions of K_2SO_4 , KCNS and dilute HCl are examined, leading to the conclusion that a decrease in stability, as opposed to reversible changes in hydration, is the consequence of these treatments.

The influence of proteins and agar-agar on the setting and stability of gelatin gels is studied.

It is concluded that the mucoprotein enters intimately into the vitreous structure and a possible mechanism of an increase in the volume of the vitreous based on a cyclical process of peptisation and clotting is suggested.

All attempts at peptising the vitreous or its isolated residual protein were without success.

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