

THE EFFECT OF HYALURONIDASE INJECTION ON THE VITREOUS HUMOUR OF THE RABBIT*

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THE effect of hyaluronidase injections on the slit-lamp appearance and the composition of the vitreous humour of the rabbit has been examined. The purpose of the experiments has been to see whether disaggregation of hyaluronic acid from a viscous to a non-viscous form in the vitreous humour is a permanent change and whether any visible change persists as a result of hyaluronidase action. Very little is known of the production or fate of hyaluronic acid, either in the eye or in other fluids and tissues of the body, such as synovial fluid, skin, or Wharton's jelly, where it also occurs. Meyer (1947) has suggested that there is a circulation of hyaluronic acid in the eye, the viscous hyaluronic acid of the vitreous humour being disaggregated and removed through the normal exit channels. Presumably this is a very slow process in the normal eye and we do not know whether complete disaggregation of the vitreous hyaluronic acid, such as might take place under pathological conditions, can ever be reversed. Nor has any clinical change been correlated with such breakdown. Aggregated hyaluronic acid is the viscous material that contributes to the turgor of the vitreous humour and its loss—if irreversible—might possibly lead to considerable changes in the state of the vitreous body.

von Sallmann (1948) has briefly described the effect of injections of hyaluronidase on the vitreous humour of the rabbit. He found that the enzyme caused a considerable inflammatory response and considered its use too dangerous for clinical purposes. In the experiments reported here the eyes have been watched for nearly a year after injection of hyaluronidase to see if permanent change occurred and the vitreous humours have been analysed to see whether the chemical changes could be correlated with any clinical change. The eyes were examined with the slit-lamp and ophthalmoscope, and the composition of the vitreous humour, sometimes also of the aqueous humour, was determined when the animal was killed. In a few animals the effect of hyaluronidase on intra-ocular pressure was noted. The state of aggregation of hyaluronic acid was judged by the type of mucin

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clot it gave in acid acetone. The enzyme system hyaluronidase causes a rapid disaggregation followed by a slow hydrolysis of hyaluronic acid. The primary change only has been studied, as this seemed the most important relative to vitreous humour structure. Disaggregation can be followed either by following the change in the type of mucin clot produced by acidification of the vitreous humour filtrate, or in some cases by following the reduction in viscosity of hyaluronic acid due to disaggregation. Although a viscometric method is preferable, it could not be used here as increase in protein in the vitreous humour raised the viscosity and this overshadowed any possible fall due to change in hyaluronic acid.

The total nitrogen content of the vitreous and aqueous humours was determined to give a picture of protein movement in the eye and the hexosamine content was determined in the hope that it would reflect changes in the hyaluronic acid of the vitreous humour. The change in protein—which contains small amounts of hexosamine—was however so large that hexosamine change reflected only this.

In all these experiments one eye of each animal was injected with active enzyme and the other with an equal volume of the enzyme solution after inactivation by boiling.

METHODS

Total nitrogen was estimated by micro-Kjeldahl followed by distillation and titration, using the apparatus described by Markham (1942).

Hexosamine was estimated by the method of Elson and Morgan (1933) modified for use with small volumes. 0.5 ml. or less of vitreous humour filtrate or 0.1-0.2 ml. aqueous humour were used. The samples were put in small glass bulbs of about 3 ml. capacity and having a wide neck of about four inches long. The hydrolysis, acetylation and reaction with Ehrlich's reagent were all done in these bulbs without transfer and the final volume was brought to 3 ml. Colour was estimated by a Hilger Biochem absorptiometer, using Filter No. OG.1 (Hilger). A reagent blank and glucosamine standards were carried through all stages with each estimation.

State of aggregation of hyaluronic acid. The state of aggregation of hyaluronic acid was estimated by observing whether a fibrous or flocculent precipitate was given when the filtrate of the vitreous humour was added to acid acetone (Robertson, Ropes and Bauer, 1940; Pirie, 1949).

Preparation of hyaluronidase. The enzyme was prepared from rabbit testis by the method described by Madinaveitia (1941). The preparation was taken as far as the precipitation with NaCl and then dialysed against 0.9 per cent. NaCl. The dialysed solution was sterilised by filtration through a collodion membrane of a.p.d. 0.82 μ .

Test of hyaluronidase activity. The disaggregating effect of the enzyme on the hyaluronic acid of the ox vitreous filtrate was used as a test of enzyme activity. Two ml. ox vitreous filtrate were mixed with 0.1 ml. enzyme at 30°. Samples were precipitated at intervals in 3 vol. acid acetone and the time taken for the enzyme to change the character of the precipitate from a compact fibrous one to a cloud-like precipitate was taken as an indication of the activity of the enzyme.

Method of injection of enzyme into vitreous humour. Dutch rabbits of either

sex and between four months and one year in age were used. The animals were anaesthetised with intravenous nembutal and the eyes were cocainised. The enzyme or boiled enzyme solution was injected a little behind the equator, care being taken to avoid the lens, which is large in the rabbit. A 26 gauge (American) needle on a 0.25 ml. syringe was used for injection. Enzyme solutions containing 0.15-0.25 mg. protein/ml. were used.

Preparation of aqueous humour and vitreous humour for analysis. In some experiments aqueous humour was removed from the animal during life. The animal was anaesthetised and aqueous humour removed by inserting a small glass capillary pipette through the cornea. About 0.2 ml. fluid ran into the pipette without suction.

At the end of the experiment the rabbit was killed either by a blow or with nembutal. The eyes were removed, carefully cleaned of all external tissue, rinsed in saline and then dried. The aqueous humour was then removed, the eyes dissected equatorially and the vitreous humour pulled away from the retina. The anterior half of the eye, together with the attached vitreous humour, was then put on a small glass mesh filter and allowed to drip into a centrifuge tube in the ice chest. Filtration usually took about $\frac{1}{2}$ -1 hour. The filtrate was then centrifuged to remove pigment and cells and the clear supernatant fluid analysed.

RESULTS

Reaction of Vitreous Humour to Injection.—Injection of the active enzyme preparation caused a prolonged inflammatory reaction in the eye. This reaction also occurred after injection of the heated enzyme, or of saline, but was usually much less severe. I found that the reaction to the heated enzyme—a reaction that is presumably unspecific in nature, due to trauma or introduction of foreign protein—could be reduced if the volume of injected fluid were kept as small as possible. If 0.1 ml. of heated enzyme diluted $\frac{1}{5}$ with saline were injected, the reaction of the eye was worse than to 0.02 ml. undiluted enzyme. Injection of 0.1 ml. into a rabbit's vitreous humour raises the tension to over 100 mm. Hg on the Maclean tonometer for a few minutes and it may be this sudden rise of tension that is responsible for the inflammatory response. The rise after 0.02 ml. injection is not noticeable.

Reaction to Active Enzyme.—Within two hours after injection, either of 0.02 ml. or 0.1 ml. enzyme preparation, the aqueous humour showed a marked flare. The fundus and vitreous humour appeared normal both to ophthalmoscopic and slit-lamp examination. After 24 hours the aqueous humour contained many circulating cells and a dense flare. The fundus was usually quite normal, but the central area of the vitreous humour was hazy. In three days the aqueous humour still showed a flare, but the number of cells was diminishing. The vitreous humour was usually full of brightly refractile particles, probably cells, sometimes attached in clumps to the back surface of the lens and scattered throughout the visible anterior part of the humour. The retina usually appeared normal, but in a few cases patches of

exudate appeared in the lower part of the fundus. From this time the anterior chamber gradually cleared and was usually normal in from six to fourteen days. The vitreous humour cleared more slowly and reached a steady state in about a month's time, when there would be a few cells visible. The normal rabbit's vitreous humour shows very little in the slit-lamp beam. The humours of the injected rabbits did not return to this optically empty state, but showed a few refracting streaks running usually vertically or at an angle of 45°. The vitreous humour did not appear grossly changed in any case.

Réaction to Heated Enzyme.—Injection of 0.02 ml. heated enzyme into the vitreous humour caused no reaction in the aqueous humour. Injection of 0.1 ml. enzyme diluted 1/5 caused in general a reaction similar to, but milder than, that caused by the active preparation. In two cases the reaction was indistinguishable from that to the active enzyme. The reaction in the vitreous humour was very like that to the active enzyme, both in type and in duration. In fact, it was noticeable that the difference between the two eyes, the one injected with active and the other with inactive enzyme, was more easily seen in the aqueous humour than in the vitreous humour, the site of the injections.

Effect of Hyaluronidase on Aggregation of Hyaluronic Acid.—The state of aggregation of hyaluronic acid was measured by the method described. Even with the small amounts of material available the change in type of precipitate after hyaluronidase action was perfectly clear. The normal vitreous humour, or the vitreous humour injected with heated enzyme, gave a very small fibrous precipitate in a clear supernatant. The vitreous humour injected with active hyaluronidase gave a cloud only, which might settle to a flocculent precipitate after some time. The activity of the enzyme used was such that 0.1 ml. of enzyme diluted 1/5 added to 2.0 ml. ox vitreous humour filtrate disaggregated the hyaluronic acid in it in 30 seconds at 30°.

Earlier experiments have shown (Pirie, 1949) that hyaluronidase acted much more quickly in the filtrate of the ox vitreous humour than in the intact excised eye and this was considered to be due to the restraining influence of the fibrous protein on the diffusion of the enzyme. One can, therefore, expect that an amount of enzyme which will disaggregate hyaluronic acid almost instantaneously in a vitreous humour filtrate may take some hours to act *in vivo*. The shortest time interval investigated was four hours and I found that after this time complete disaggregation had taken place in the living eye. Eyes removed at later intervals after injection showed that the hyaluronic acid of the vitreous

humour remained in the non-viscous disaggregated form for at least a month. Thereafter it seemed to return to normal. The humours that contained disaggregated hyaluronic acid were not liquefied, but were more fragile and filtered much more rapidly than the normal humours. The results showed quite clearly that rabbits killed up to six weeks after injection of hyaluronidase had disaggregated, and rabbits killed six or more weeks after injection had normal aggregated hyaluronic acid in their vitreous humours. In no case did injection of inactivated enzyme have any effect on the state of hyaluronic acid.

Changes in Nitrogen and Hexosamine after Hyaluronidase Injection.—Nitrogen and hexosamine were estimated in both vitreous and aqueous humours when the rabbit was killed. The time of death ranged from 4 hours to 8 months after enzyme injection.

There was a 2–3 fold rise in nitrogen, from 0.27–0.7 mg./ml. in the vitreous humour 24 hours after injection of active hyaluronidase. This was accompanied by a slight rise, from 40–100 μ g./ml. in the hexosamine content. The vitreous humour injected with heated enzyme showed similar but slighter changes. Both nitrogen and hexosamine returned to normal about 28 days after injection of active or inactive enzyme.

The changes in the aqueous humour after injection of active enzyme into the vitreous were more pronounced and took place earlier than the changes in the vitreous humour which seems to show that there must be very rapid diffusion between the vitreous and the aqueous humour. Two hours after the injection into the vitreous humour, the aqueous humour nitrogen had risen 10 fold and remained between 3–6 mg./ml. for 24 hours. Hexosamine rose to 500–900 μ g./ml. After 24 hours both nitrogen and hexosamine fell and were normal 14 days after the injection. The changes in the aqueous humour of the eye injected with heated enzyme were very much slighter.

The total nitrogen analyses showed that the influx of protein into the aqueous humour is much greater than into the vitreous. This is probably due to the fact that the increase in protein in the vitreous is due to a cell invasion, while soluble proteins derived from capillaries appear in the aqueous humour a short time after injection of active enzyme into the vitreous.

DISCUSSION

The results reported briefly by von Sallmann (1948) showing that injection of hyaluronidase into the vitreous humour causes an inflammatory reaction, have been confirmed. The main point

of interest in the long-term experiments reported in this paper lies in the fact that some time after subsidence of inflammation the vitreous humour hyaluronic acid is again in the viscous, aggregated condition.

The return to normal of the vitreous humour hyaluronic acid probably shows that production of hyaluronic acid is a continuous process in the living eye. Liquefaction of the vitreous humour in man appears to be non-reversible, but judging by experiments with ox vitreous humour reported by Pirie, Schmidt and Waters (1948), liquefaction is more directly related to destruction of the fibrous protein of the humour than to change in hyaluronic acid. Meyer (1947) has already suggested that hyaluronic acid is constantly produced and removed from the eye. The experiments reported here give some proof for this by showing that in the living animal hyaluronic acid is gradually replaced in the viscous aggregated form after it has been hydrolysed by injected hyaluronidase.

The cells which produce hyaluronic acid either in the eye or elsewhere are not known. Hechter (1948) found that, in human skin, the effect of an injection of hyaluronidase on wheal formation (spreading factor effect) is lost after 24—48 hours, showing that in this tissue hyaluronic acid is either re-formed in this time, or diffuses in from surrounding areas. It seems unlikely that diffusion into the vitreous humour from surrounding tissues takes place and easier to consider that hyaluronic acid is re-formed in the eye.

If hyaluronic acid is formed in the tissues surrounding the vitreous humour and secreted in the aggregated form, its diffusion into the humour structure must be very slow. In the ox vitreous humour I found (Pirie, 1949) that hyaluronic acid washed out of the humour very slowly indeed and that it was always found in the disaggregated state in the wash water. The humour of the rabbit contains a lower concentration of hyaluronic acid than that of the ox and is less coherent and firm a structure, so that it is possible that hyaluronic acid diffuses more easily. On the other hand, it is possible that it is produced by the cells that invade the humour after enzyme injection.

Slit-lamp and ophthalmoscopic examination showed that no marked permanent change resulted from injection of hyaluronidase. It seemed possible that disaggregation of the viscous jelly of the vitreous humour might be reflected in changes in the intra-ocular pressure. Records of intra-ocular pressure were studied in five rabbits of the series, using a Maclean's tonometer. The tension of the eye injected with inactive hyaluronidase was also recorded, as the tension of the normal rabbit eye can vary

considerably. Injection of active hyaluronidase caused a reduction in tension to about half that of the eye injected with inactive hyaluronidase, within 24 hours of injection. Tension stayed at this level (10—11 mm. Hg) for three to four days, but was back to normal again nine days after the injection. One cannot say whether this reduction in tension is due directly to hydrolysis of the viscous hyaluronic acid of the vitreous humour, or to the resulting inflammation of the eye, but as little change in tension occurred in the control eye injected with inactive enzyme, it seems probable that some of the fall in tension was due to the change in state of hyaluronic acid in the vitreous humour.

SUMMARY

1. The hyaluronic acid in the vitreous humour is disaggregated by hyaluronidase injection and remains so for at least a month. After this time aggregated hyaluronic acid is found, showing that hyaluronic acid can be produced in or secreted into the vitreous humour during life.
2. Injection of rabbit testis hyaluronidase preparations into the vitreous humour of the rabbit caused a prolonged inflammatory reaction, noticeable within two hours in the aqueous humour and after 24 hours in the vitreous humour.
3. The nitrogen contents of both vitreous and aqueous humours are increased after hyaluronidase injection.

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