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SWELLING OF THE RAT RETINA INDUCED BY METABOLIC INHIBITION*

BY

CLIVE GRAYMORE

Department of Pathology, Institute of Ophthalmology, University of London

ASHTON, Graymore, and Pedler (1957) tentatively suggested that external pressure may be a causative factor in vaso-obliteration, as observed in the kitten retina following the intravitreal injection of sodium iodoacetate or sodium fluoride. They commented upon the possibility that treatment by such established metabolic inhibitors, by denying the cells the energy they normally derive from carbohydrate catabolism, might lead to a breakdown in the mechanism responsible for regulating cell size.

Opie (1949) was the first to observe that certain tissues, after isolation from the rat, imbibed fluid unless they were bathed in solutions which were distinctly hypertonic. This apparent hypertonicity of such cells to their natural environment, could be effectively abolished by certain toxic agents (Opie, 1950). Other workers have since confirmed this phenomenon in tissues such as liver, kidney, spleen, and brain, and have concluded that there is some definite relationship between cell size and the efficiency of the energy metabolism of the cell. Isolated tissues, even in a balanced saline medium, have been observed to swell under a variety of conditions leading to a reduced efficiency in energy production or capture (Stern, Eggleston, Hems, and Krebs, 1949; Aebi, 1950; Robinson, 1950a, b).

This work led to the concept that living cells were not in osmotic equilibrium with their environment, and that their osmotic pressure was maintained at a higher level, as a steady state, by a process drawing on metabolic energy to pump water outwards through the cell membrane. This hypothesis and the experimental evidence which led to its formulation have been adequately reviewed (Robinson and McCance, 1952; Robinson, 1954).

In the light of the suggestion of Ashton and others (1957) that retinal swelling induced by iodoacetate and fluoride may conceivably be a causative factor in vaso-obliteration, it was thought that it would be worth while investigating the *in vitro* effect of these inhibitors on the imbibition of water by the rat retina, to determine whether this phenomenon does, in fact, occur in retinal tissue.

Method and Materials

Variations in the water content of isolated rat retinae were determined after incubation with and without iodoacetate or fluoride. The water content was

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assessed by calculating the wet-weight:dry-weight ratio of the tissue at the end of the period of incubation.

Animals.—The animals used were female albino rats aged about 3 months and weighing 180–200 g.

Incubation Medium.—A standard Krebs-Ringer-bicarbonate buffer was employed (Krebs and Henseleit, 1932), the solution being gassed throughout with 5 per cent. CO_2–95 per cent. O_2. 200 mg. per cent. "analar" glucose was added as substrate. For the preparation of tissues this buffer was chilled in ice.

Iodoacetate.—Iodoacetic acid was recrystallized from a sample obtained from British Drug Houses and was neutralized before use. The final concentration employed in the flask was 0·001 M.

Sodium Fluoride.—This was also obtained from British Drug Houses and was used in a final concentration of 0·01 M.

Both solutions were made up in such a concentration as to necessitate the addition of 0·1 ml. to the experimental flasks. 0·1 ml. of glass-distilled water was added to the control flasks.

Removal and Weighing of the Retina.—In order to obtain results of sufficient accuracy to be able to detect small variations in the water content of the tissue by simple weighing procedures, two conditions must be fulfilled:

(1) Removal of the retina should be accomplished rapidly, involving as little cellular fragmentation as possible. Ideally, the retina should be removed in its entirety. This will minimize irreversible changes in the metabolic pattern.

(2) A technique must be devised which will minimize errors inherent in the weighing of small quantities of tissue. The fragility of the retina is a complicating factor in both these procedures.

(*1*) *Removal of the Retina.*—Several recognized procedures were tried, but that to be described was developed in this laboratory and found to be the most effective. In view of its wide application this will be presented in some detail.

After enucleation, the eyes were transferred to a small plastic cutting block (Fig. 1, overleaf). A convenient block measures 6 × 3 × 0·5 cm. A series of pits of suitable dimensions was drilled in the surface. Those in the block used in the present investigation had diameters ranging from 3 to 6 mm. and their depths lay within the limits 1 to 3 mm. A fine drainage canal was drilled through to the base of the block from each well.

The eyes were placed anterior surface upwards, in a well having the appropriate dimensions, such that half the eye was exposed above the block, the posterior aspect of the eye fitting snugly into the pit. The eye was held in place by a plastic strip possessing a suitably proportioned hole at one end, allowing a firm grip over the cornea. Ideally the eye should be situated as shown in Fig. 2 (overleaf). The eye was cut through the equator by means of a sharp flat razor blade inserted between the block and the holder. In the position shown in Fig. 2, a blade applied along the surface of the block will section the eye immediately behind the ora serrata, the retina will then be freed from the choroid anteriorly, and its only remaining point of attachment will be around the optic disc.

The posterior "cup" of the sectional eye was then removed into a shallow vessel containing the appropriate chilled buffer, and the "cup" was inverted. The vitreous, if still adherent to the exposed retina, was removed with forceps. At this stage, the retina "parachuted" out from the inverted cup of the choroid, and a single cut across the posterior point of attachment at the optic disc freed it completely.

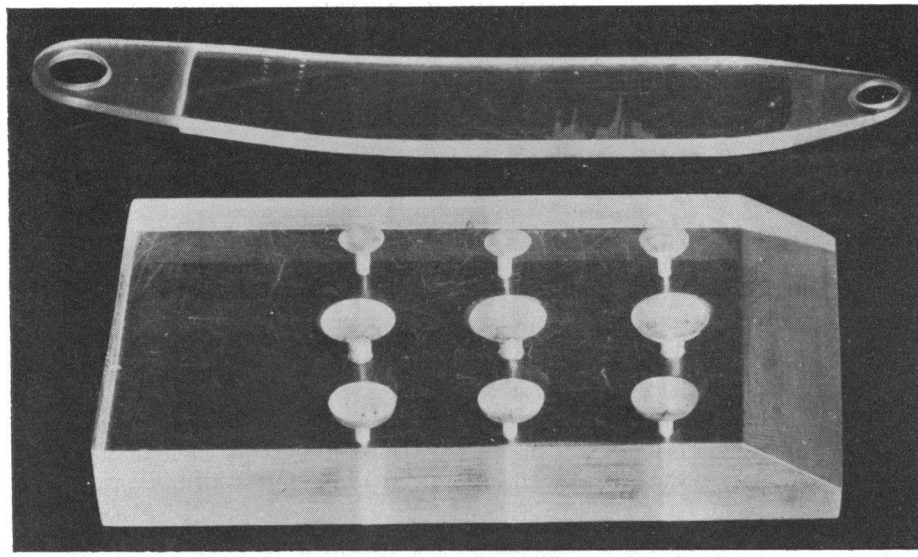

FIG. 1.—Plastic holder (A) and block (B) described in text.

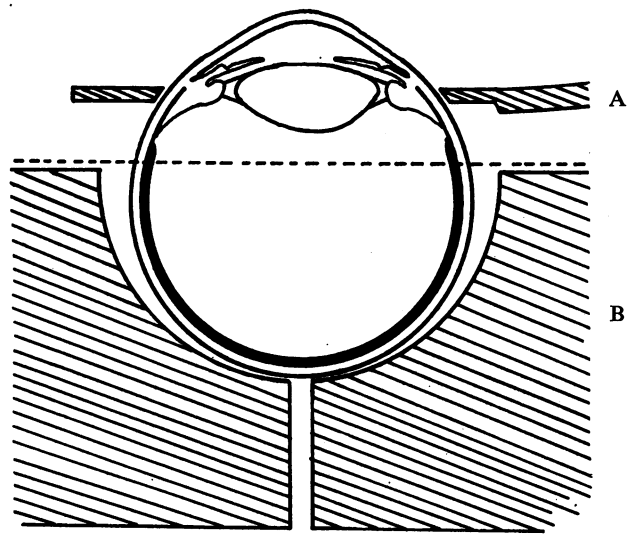

FIG. 2.—Diagrammatic representation of an eye being held in position by the holder (A) in one of the wells of the block (B). The broken line indicates the position through which the blade should pass.

The whole procedure, from the time of killing the animal to the final isolation of the retina, should occupy no more than 1 to 2 minutes.

(*2*) *Weighing*.—Conventional methods cannot be applied so readily to the retina. The tissue is remarkably fragile and any attempt to dry the excess moisture from its surface by the accepted technique of light mopping with filter paper will result in the tissue adhering to the paper. The following method overcame these difficulties and provided an accurate and rapid means of weighing.

Small squares of filter paper (Whatman No. 50) were prepared having the approximate measurements 5 × 5 mm. The simplest procedure is to cut these simultaneously from a block of paper, thus ensuring a reasonable uniformity of size. (A cork borer was also found to provide a convenient cutting tool which would yield discs of paper of matching size.)

These papers were immersed in the buffer to be used, and were placed on a large flat filter paper for 15 seconds, care being taken to ensure adequate contact. Preliminary experiments had revealed that this treatment was sufficient to drain away all excess buffer from the papers and that under these conditions the papers retained exactly the same proportion by weight of the buffer. The percentage loss of weight of these papers was determined after 2 hours heating at 110° C. This was found to be constant.

To weigh the tissue, the retina was removed from its medium and laid flat on a square of paper which had been previously weighed after saturating with buffer as above. The paper was transferred to the drying sheet for 15 seconds, and re-weighed. The wet-weight of the retina was obtained by subtraction. The retina plus paper was then dried for 2 hours at 110° C., and the dry-weight of the retina was calculated taking into account the weight loss of the paper. Excellent reproducibility was obtained by this method.

General Procedure.—Animals were killed by dislocation of the cervical vertebrae and the eyes were enucleated as rapidly as possible. The retinae were removed as described and transferred to square Warburg flasks, containing 6 ml. ice-cold gassed incubating medium, to which had been added sodium fluoride, sodium iodoacetate, or glass-distilled water. The flasks were attached to their manometers and gassing with 5 per cent. CO_2–95 per cent. O_2 was continued. They were incubated for one hour at 37° C. Shaking was somewhat slower than that normally employed (70 per minute) to preserve the integrity of the tissue as far as possible. At the end of the period of incubation the retinae were carefully removed and their wet-weight and dry-weight were determined as described.

Time-Course.—In the experiments designed to test the rapidity with which iodoacetate-induced swelling occurs, all retinae were incubated in normal medium for 30 minutes before zero time. Some retinae were then removed and their ratios determined, whilst iodoacetate was added to the remainder. This served to eliminate transient changes which might arise as a result of removing the retina, immersing in chilled buffer, etc. Iodoacetate-treated retinae were then removed at the time intervals indicated.

Results and Discussion

Results were calculated as the ratio Wet-weight:Dry-weight at the end of incubation.

This procedure was considered preferable to that of weighing before and after incubation and expressing the result as increase in weight, since it eliminates errors due to tissue disintegration and loss.

As the Table (overleaf) shows, the Wet-weight:Dry-weight ratio of the control retinae was 6·4, whereas retinae which were incubated in the presence of iodoacetate or fluoride yielded values of 9·6 and 9·66 respectively, representing an increase of approximately 50 per cent. over the controls. In both cases the difference is statistically significant.

TABLE

MEAN WET-WEIGHT:DRY-WEIGHT RATIOS OF RETINAE AFTER INCUBATION FOR 1 HOUR WITH GLUCOSE SUPPLEMENTED KREBS-RINGER-BICARBONATE

Inhibitor present where stated

Inhibitor	Number of Retinae	Wet-weight:Dry-weight
None	5	6·40 ± 0·34
0·001 M. Iodoacetate	6	9·60 ± 0·50 p = 0·001*
0·01 M. Fluoride	6	9·66 ± 0·66 p = 0·003*

* Indicates that the difference between this result and that of the controls is statistically significant.

Both iodoacetate and fluoride are known to be powerful inhibitors. The *in vitro* fluoride inhibition of glycolysis has been demonstrated in a variety of tissues and the retina has been shown to be particularly sensitive (Dickens and Greville, 1932). Iodoacetate is an effective thiol enzyme poison, combining with the SH groups of proteins, glutathione, cysteine, and coenzyme A. It thus exerts a widespread influence on glucose breakdown. Phosphoglyceraldehyde dehydrogenase is particularly sensitive (Rapkine, 1938). It produces *in vivo* a characteristic rod cell degeneration in the rat, which has been attributed to glycolytic inhibition (Noell, 1952).

The efficiency of these inhibitors was checked during several of the runs by measuring the CO_2 evolution manometrically in the presence or absence of the inhibitor. Changes in tonicity due to the addition of the inhibitor cannot be responsible for the differences observed in the ratios, as the same volume of glass-distilled water was added to the control flasks. It would appear therefore that there is a definite correlation between the inhibitory properties of iodoacetate and fluoride and the swelling of the retina. This supports the concept that constancy of cell size can be maintained only when an adequate source of energy is available. The manner in which these two processes are coupled is still a matter for speculation. It has been suggested that hypothetical energy-driven "pumps" located in the mitochondria may be responsible (Bartley, Davies, and Krebs, 1954).

Mudge (1956) points out that there is still some controversy as regards the postulate that the cell is hypertonic to extracellular fluids during life (Conway and McCormack, 1953). Experimental evidence clearly indicates, however, that swelling does occur when the metabolism is inhibited, even though the nature of this swelling is unknown. It may be isosmotic (Mudge, 1956).

These results may also explain an observation by Terner, Eggleston, and Krebs (1950), in investigating the role of glutamic acid in the transport of

potassium in brain and retina, that retinae incubated in bicarbonate saline under aerobic conditions with glucose and glutamate increased in weight by about 10 to 15 per cent. Retinae incubated anaerobically without a substrate lost about 15 to 30 per cent. of their initial wet-weight. They ascribed the weight loss to tissue disintegration. This fragility, and the opacity they noted, may well have been a result of cellular oedema. Fig. 3 shows the rapidity with which iodoacetate-induced swelling occurs. After 15 minutes the shortest time interval observed, swelling is almost maximal.

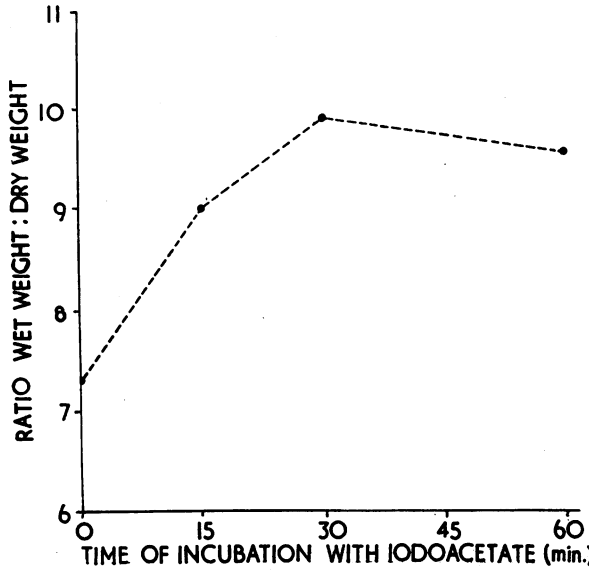

FIG. 3.—Changes in the Wet-weight : Dry-weight ratio during incubation of the retinae with 0·001 M. iodoacetate in Krebs-Ringer-bicarbonate. Each point represents the mean of three determinations.

Conclusions

Although these results clearly indicate a relationship between retinal oedema and inhibition of metabolism by iodoacetate and fluoride, it is too early to assess the importance of this phenomenon as a causative factor of vaso-obliteration. Observations on fluoride-induced vaso-obliteration in the retina of the kitten do provide some evidence for an accompanying retinal oedema, notably the development of a milky-white opacity as the vessels close, and a reversal of vascular closure by injecting hypertonic solutions into the vitreous (Ashton and others, 1957). These workers also point out that intracellular or extracellular oedema can be demonstrated histologically in the inner layers of the kitten retina subjected to oxygen, sodium fluoride, or sodium iodoacetate treatments, but the possibility that these are artefacts is stressed. Further work is yet required to elucidate this complex problem.

Summary

(1) Simple procedures for the removal and weighing of rat retinae are described.

(2) The Wet-weight:Dry-weight ratio has been determined in rat retinae after incubation in Krebs-Ringer-bicarbonate supplemented with glucose.

(3) The ratio was determined in retinae in which the metabolism was inhibited by sodium fluoride or sodium iodoacetate.

(4) The large and statistically significant difference which was observed between these ratios is discussed in the light of present concepts.

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