STUDIES ON DEVELOPING RETINAL VESSELS*

VI. HISTOLOGICAL MEASUREMENT OF FLUORIDE-INDUCED SWELLING IN THE RETINA

BY

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It is now a firmly established experimental finding that the vessels of the immature retina specifically close as a result of raising the ambient oxygen concentration. Since this was first reported (Ashton, Ward, and Serpell, 1953) subsequent research has been directed towards the elucidation of the underlying mechanism. Some progress has been made, and it has recently been reported (Ashton, Graymore, and Pedler, 1957) that when certain established enzyme inhibitors were injected through the vitreous of an immature animal on to the retinal surface, complete obliteration of the retinal vasculature occurred within a short space of time. This effect was studied in the living animal, using a direct observation technique (Ashton and Cook, 1954; Pedler, 1957), and on repetition was found to be consistent, not only in the time elapsing between injection and vaso-obliteration, but also in the order of closure of the vessels concerned. These experiments have led to the formulation of a working hypothesis that the vessel closure observed might be caused by external pressure resulting from swelling of surrounding retinal tissue.

This possibility has been discussed (Ashton and others, 1957) and further work (Graymore, 1958) has provided some support for it by showing that when the isolated retina is incubated in solutions containing sodium fluoride a significant increase in Wet Weight : Dry Weight ratio takes place. It was thought possible that such an intake of fluid might result in an actual increase in thickness which would be measurable histologically. This paper reports the results of some experiments performed to investigate this possibility.

Material and Methods

Animals from which the eyes were removed for histological study were first anaesthetized with Nembutal, and then injected intravitreally with either 0.15 or 0.2 ml. of an 0.61 per cent. solution of sodium fluoride, a concentration which was found to be isosmotic with the red cells of the animal and to have a pH of 7.1. After injection, the animals were left to survive for a known period and then killed under anaesthesia by intravital perfusion with Kolmer's fluid. Thus the eyes were considered in pairs, one eye being injected, and the other acting as a control.

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All material examined histologically was embedded in celloidin and sections cut—in the sagittal plane through the optic disc—at 10μ ; these were then stained with either Lieb's phosphotungstic-acid haematoxylin or Anderson's modification of Mallory's phosphotungstic acid method.

Where two eye sections were to be compared for measurements of retinal thickness, exactly similar staining methods and preparation were used, to prevent errors arising from differences in histological technique.

To avoid observer bias and to provide a purely arbitrary location for measurements, the following method was employed:

A perimeter chart was photographed and a series of reduced negatives made; these were then fixed over the sections to be measured (see Fig. 1) so that $AB=BC$, $DB=BE$, and the meridian AC passed through the centre point of the cornea and the optic disc.

FIG. 1.—Method of charting the meridians in which the retinal layers were measured.

A second slide was then fixed over the first, and the combined image of the specimen and chart projected onto a flat screen at a standard distance and magnification. The individual retinal layers were then measured in sixteen separate places on each section, eight to the left (L_1 , L_2 , etc.), and eight to the right (R_1 , R_2 , etc.), using a centimetre scale superimposed on the screen image. Mean values for each retinal layer were then worked out, and the results for each section expressed, first as a mean total thickness and secondly as a percentage of the total thickness made up by any one layer. The presentation of the results in this form minimizes sources of error due to differences between the eyes of the same animal and to errors, say, of magnification. In this way approximately 112 measurements were made on each section and fifty sections were examined. If the perimeter chart line fell upon a zone showing obvious histological artefact, such as fragmentation or retinal folding, the whole set of readings from that position was omitted.

Results

Measurements using the perimeter chart method (with a normal eye as a control) were recorded as shown in Table I. This represents the total number of measurements taken from one slide (T. 39), the average values appearing on the bottom line. The last two columns show the observed total retinal thickness as measured on the screen (Total 1), and the calculated thickness which was obtained by adding together the observed thickness of the individual layers (Total 2). The object of this procedure was to provide some indication of the accuracy of the method, and it will be seen that on comparison of the two columns the agreement is in fact tolerably close.

TABLE I
MEASUREMENTS MADE ON ONE SECTION FROM A NORMAL EYE

T 39		Stratum Opticum	Inner Plexiform Layer	Inner Nuclear Layer	Outer Plexiform Layer	Outer Nuclear Layer	Rods and Cones	Total	
								1	2
Left	1	3.50	2.00	1.50	1.25	2.75	1.50	12.25	12.50
	2	3.25	2.00	1.50	0.75	2.75	1.25	11.75	11.50
	3	4.25	2.25	1.25	1.00	2.75	1.50	13.00	13.00
	4	2.25	2.25	1.00	1.25	3.00	1.50	11.25	11.25
	5	2.75	1.75	1.25	1.50	3.00	1.50	11.00	11.75
	6	1.50	2.00	1.25	1.25	3.00	1.50	10.50	10.50
	7	1.50	1.50	1.00	1.25	2.50	1.25	9.00	9.00
	8	1.00	1.00	0.75	1.00	1.50	1.00	6.00	6.25
Right	1	4.00	1.50	1.50	1.00	2.50	1.25	12.00	11.75
	2	1.75	1.50	1.00	1.00	2.75	1.00	9.00	9.00
	3	1.25	1.50	1.00	1.00	2.25	1.25	8.25	8.25
	4	1.50	1.25	1.00	0.75	2.25	1.00	8.00	7.75
	5	2.00	1.50	0.75	0.50	2.50	1.25	7.50	8.50
	6	1.25	1.00	0.75	0.75	1.75	1.00	6.50	6.50
	7	1.00	0.75	0.50	0.75	2.00	0.75	5.50	5.75
	8	1.25	0.75	1.00	0.50	1.50	0.50	5.25	5.50
Mean		2.13	1.53	1.06	0.97	2.42	1.19	9.17	9.30

Tables II to V (overleaf) are all constructed from mean values; thus each line of figures on these Tables denotes the mean values from one complete set of measurements as in Table I.

Table II shows the mean results obtained from one experiment. In this, one eye of a 15-day-old kitten was injected intravitreally with 0.15 ml. of an 0.61 per cent. solution of sodium fluoride. The animal was then allowed to survive for 60 minutes, and was finally killed by intravital fixation. T. 9, 11, 14, 16 are the mean values of four sections from the control eye; T 10, 12, 13, 15 are the values of four sections from the eye injected with fluoride. The last column on the right shows the proportion of the total retinal thickness made up by the stratum opticum (nerve fibres and ganglion cells) together with the inner plexiform layer. It will be seen that the retina treated with fluoride has undergone an overall increase in thickness. This is perhaps

TABLE II
MEAN VALUES FROM EYES OF A 15-DAY-OLD KITTEN KILLED 60 MIN.
AFTER INJECTION

Eye	No.	Stratum Opticum	Inner Plexiform Layer	Inner Nuclear Layer	Outer Plexiform Layer	Outer Nuclear Layer	Rods and Cones	Total	SO & IP %
									Total
Control	T 9	2.18	1.32	1.67	0.65	5.12	0.88	12.12	28.88
	T 11	2.25	1.41	1.96	0.46	5.55	0.45	12.21	29.98
	T 14	2.10	1.35	1.57	0.65	5.28	0.82	11.77	29.31
	T 16	2.06	1.63	2.06	0.56	5.73	0.42	12.04	30.65
Treated with Fluoride	T 10	2.30	2.00	1.75	0.89	4.61	1.41	12.98	33.13
	T 12	2.11	2.04	1.96	0.75	5.25	1.14	13.39	30.99
	T 13	2.02	2.39	1.73	0.98	5.09	1.61	13.77	32.03
	T 15	2.07	2.45	1.79	0.98	5.02	1.46	13.75	32.87

more readily seen in Fig. 2 (opposite), which compares two slides from Table II in block form. The ordinate scale is the thickness in centimetres as measured on the screen, and the individual retinal layers are indicated along the abscissa. This diagram shows that the increase is predominantly made up by the inner plexiform layer and the visual cells. This difference is significant (*vide infra*).

Table III shows similar values obtained from the eye of an 8-day-old kitten, using the same dose of sodium fluoride, but with the difference that the animal survived for 105 minutes after injection. It is clear from the last column that the corrected difference between the eyes is greater than in the previous experiment.

TABLE III
MEAN VALUES FROM EYES OF AN 8-DAY-OLD KITTEN KILLED 105 MIN.
AFTER INJECTION

Eye	No.	Stratum Opticum	Inner Plexiform Layer	Inner Nuclear Layer	Outer Plexiform Layer	Outer Nuclear Layer	Rods and Cones	Total	SO & IP %
									Total
Control	T 17	1.66	1.34	1.86	0.67	4.00	0.53	10.20	29.41
	T 19	1.59	1.44	1.77	0.64	4.05	0.63	10.27	29.50
	T 21	1.60	1.23	1.80	0.58	4.13	0.50	9.90	28.59
	T 22	1.48	1.27	1.78	0.65	3.92	0.62	10.00	27.50
Treated with Fluoride	T 18	1.73	2.50	1.93	0.61	4.39	1.25	12.32	34.33
	T 20	2.17	2.25	2.03	0.63	4.64	1.39	12.89	34.29
	T 23	2.10	2.23	1.88	0.69	4.27	1.38	12.60	34.37
	T 24	2.02	2.13	1.88	0.54	4.61	1.36	12.45	33.33

Fig. 3 (opposite) shows that the increase in thickness is most apparent in the inner layers; however, in this experiment, both the ganglion cells and the nerve fibres have contributed to the increase as well as the inner plexiform layer. Again the rods and cones are significantly affected.

FIGS 2-7.—Alteration in thickness of individual retinal layers after intravitreal injection of sodium fluoride (see Tables II-V).

S.O. Stratum opticum
I.P. Inner plexiform layer
I.N. Inner nuclear layer
O.P. Outer plexiform layer
O.N. Outer nuclear layer
R.C. Rods and cones

Table IV shows the results from the eyes of an adult cat, one eye having been injected with 0.3 ml. 0.61 per cent. sodium fluoride, the other eye being used as a control.

TABLE IV
MEAN VALUES FROM EYES OF ADULT CAT KILLED 60 MIN. AFTER INJECTION

Eye	No.	Stratum Opticum	Inner Plexiform Layer	Inner Nuclear Layer	Outer Plexiform Layer	Outer Nuclear Layer	Rods and Cones	Total	SO & IP %
									Total
Treated with Fluoride	T 37	2.07	1.21	0.98	0.89	2.41	1.64	9.29	35.31
	T 38	2.18	1.47	0.97	0.90	2.42	1.83	9.67	37.75
Control	T 39	2.13	1.53	1.06	0.97	2.42	1.19	9.17	39.91
	T 40	1.97	1.38	1.10	0.92	2.33	1.30	8.97	37.35

Fig. 4 represents the mean values from two of these sections, and it is clear from this diagram that very much smaller differences are present; in fact, with the exception of the visual cells, these were not significant (*vide infra*).

In another experiment on an adult cat, three injections of 0.2 ml. 0.61 per cent. sodium fluoride were given, spread out over an 11-day survival period. The results from this experiment are shown in Table V and Fig. 5 and are included merely to show the limitations of the method employed, for there was such a widespread scatter throughout the measurements, that any comparison was, on inspection, impossible. The reason for this is that both long-term survival and multiple injections produce severe distortion and folding so that any consistency in measurement is lost.

TABLE V
MEAN VALUES FROM EYES OF ADULT CAT AFTER THREE INJECTIONS
SPREAD OVER AN 11-DAY SURVIVAL PERIOD

Eye	No.	Stratum Opticum	Inner Plexi- form Layer	Inner Nuclear Layer	Outer Plexi- form Layer	Outer Nuclear Layer	Rods and Cones	Total	SO & IP %
									Total
Treated with Fluoride	T 33	3.47	1.34	1.22	1.22	3.06	1.16	11.69	41.14
	T 34	2.81	1.25	1.61	0.92	2.83	1.28	10.72	37.87
Control	T 35	2.87	1.78	1.10	1.07	2.83	1.82	11.50	40.43
	T 36	1.90	1.50	1.19	0.85	2.32	1.58	9.46	35.94

These results suggested that the sodium fluoride was responsible for the observed retinal swelling; however, it was thought possible that the injection of any fluid into the vitreous might have the same result. This was tested on a 14-day-old animal which was allowed to survive for 105 minutes after the injection of one eye with 0.15 ml. normal saline, the other eye being used as a control. No significant* difference was observed between the two eyes either in the total retinal thickness or in the individual retinal layers (Fig. 6). Finally, to establish that individual sections were representative of the whole eye, duplicate sections of a normal eye were compared, and no significant difference was noted (Fig. 7).

Interpretation.—The results of these experiments show that, under certain conditions, intravitreal injection of isosmotic solutions of sodium fluoride can produce retinal swelling in the immature retina, and that this swelling is not seen in the adult. They also show that the inner layers of the retina, particularly the inner plexiform layer, are largely responsible for the overall increase in thickness and that, with the exception of the visual cells, the other layers are comparatively unaffected.

* Where the term "significant" is used, it implies a calculated difference of more than three times the standard error of the difference between the means.

Recent histochemical analyses carried out in rats (Strominger and Lowry, 1955; Lowry, Roberts, and Lewis, 1956) have indicated that the various cell strata of the retina may exhibit a preferential dependence upon different metabolic cycles. It would be tempting to correlate these results with the differential changes in thickness observed in our own experiments. There probably is such a correlation to be made, but complicating factors such as species differences make exact comparison difficult at this stage. It is probably reasonable, however, to use this finding of selective swelling as an argument in favour of enzymatic inhibition as a basis for the action of sodium fluoride in these circumstances, since if it was acting in a purely physical manner one would expect the histological changes to decrease regularly from the inner surface outwards.

It was previously reported (Ashton and others, 1957) that the same concentration of fluoride used to produce the swelling described above was capable of causing complete vaso-obliteration in the immature living retina. To incriminate swelling as the cause of the vessel closure, it is necessary to exclude changes in the vessel wall, which, in the immature retina, appears to consist only of a delicate endothelial tube lining a space in retinal tissue. In sections of retinæ treated with sodium fluoride, it can be seen that, where there is visible accumulation of fluid within the retina, the walls of the collapsed vessels in the affected area show no other apparent change.

Summary

(1) A method is described whereby the thickness of retinal sections can be measured and the results compared quantitatively.

(2) Experiments are reported wherein swelling of the inner layers of the immature retina occurred after the intravitreal injection of sodium fluoride.

(3) Swelling was not seen in the inner layers of the mature fluoride-treated retina.

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