Characterization of the Early Stages of Diabetic Retinopathy by Vitreous Fluorophotometry

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SUMMARY

We compared insulin-dependent diabetic patients with minimal (16 eyes of 9 patients) or no retinopathy (45 eyes of 27 patients) to normal volunteers (20 eyes of 12 subjects) using a commercial vitreous fluorophotometer and different procedures for artifact correction. The influence of background autofluorescence was minimized through the use of a software program that subtracted a fluorophotometric scan obtained before administration of fluorescein from that obtained after its injection. We also compared two programs designed to minimize the contribution of the chorioretinal peak spread function to the readings in the vitreous. The fluorescein concentration in the posterior vitreous was then averaged within two different regions. We then assessed the influence of these data-processing methods on the spread of the results of the different groups. The clinical study showed that only the posterior vitreous concentration of fluorescein is relevant in the evaluation of the blood-retinal barrier. However, since there is a gradient of fluorescein concentration in the posterior vitreous, one needs a scanning device so that one can measure at a precise location in front of the retina. The posterior vitreous concentration of fluorescein was significantly increased in diabetic subjects with one or no aneurysms as compared with normals. Moreover, the eyes with minimal retinopathy, as judged by the presence of microaneurysms, had higher values than those without retinopathy. The clear differences among these three groups were not present when the midvitreous values were used. Finally, the concentrations of fluorescein in the anterior chamber were higher in the diabetic subjects with retinopathy than in the normals, but not in the diabetic subjects without retinopathy. DIABETES 1985; 34:53–59.

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Vitreous fluorophotometry was introduced in 1975 as a technique for measuring low concentrations of fluorescein in the vitreous body, thereby allowing the permeability of the blood-retinal barrier (BRB) to be assessed in healthy and diseased states.[1]

Vitreous fluorophotometry demonstrated an abnormal penetration of intravascular fluorescein into the vitreous in a variety of retinal and optic nerve diseases.[2] In diabetic patients, this occurred before angiographically demonstrable retinopathy, indicating an early alteration of the BRB.[3,4]

The potential importance of the breakdown of the BRB in diabetic retinopathy and the quantitative nature of the fluorophotometric method have generated much interest. This method is particularly attractive to the diabetologist and ophthalmologist; it allows them to detect, characterize, and quantitate the earliest stages of retinal involvement in diabetes and to monitor its evolution. An adequate means is available, therefore, to study therapeutic agents that may, eventually, stop or even reverse the disease process.

Progress has been made toward developing vitreous fluorophotometry into a practical and reliable tool for clinical investigation. The artifacts have been identified[5] and quantitated under conditions similar to those found in clinical applications.[5,6] Improved instruments in the form of commercial units (Fluorotron Master, Coherent, Palo Alto, California)[7] and research laboratory units have been introduced.[6] Moreover, different ways of analyzing the data have been suggested,[8] and protocols have been developed.[9]

In this study, we compared different procedures of data processing to obtain an optimal protocol. Vitreous fluorophotometry was then used to evaluate the BRB in insulin-dependent, juvenile-onset diabetic patients showing minimal or no retinopathy and to compare the results with those of a control group.

MATERIALS AND METHODS

A major problem in this type of study has been associated with a lack of standardization in the grading of diabetic retinopathy.

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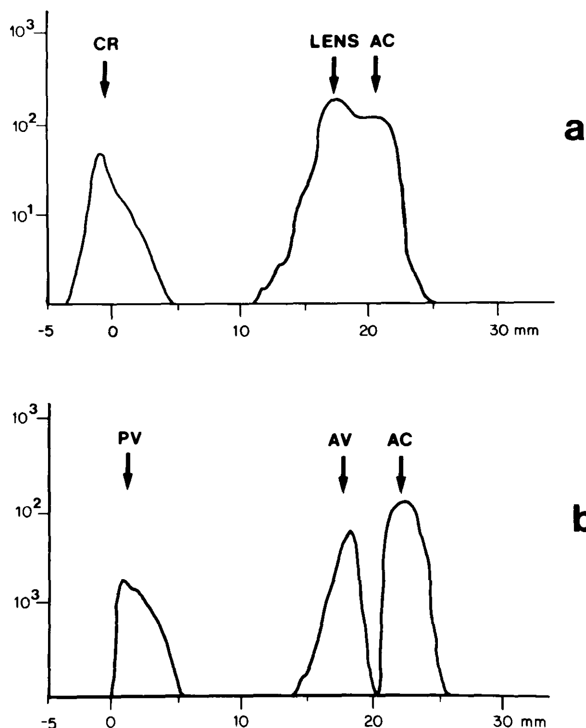

FIGURE 1. (A) Vitreous fluorophotometry scan performed in a normal volunteer 60 min after i.v. injection of fluorescein (14 mg/kg). CR: chorioretinal peak, AC: anterior chamber. The vertical scale represents fluorophotometric readings equivalent to concentrations of fluorescein given in ng/ml. (B) Scan of Figure 1A after background correction. PV and AV represent the posterior and anterior vitreous, respectively.

After maximal dilatation of the pupils, four red-free photographs were taken, one centered on the disc and one on the macula in each eye. A 20% solution of sodium fluorescein was injected intravenously in a dosage of 14 mg/kg. Pictures of the field centered on the fovea (right eye) were taken before the appearance of the dye and at approximately 1-s intervals from the appearance of the dye to the completion of the capillary phase. The second eye (left) was photographed immediately after. Late pictures were taken of both eyes. Only fields 1 and 2 of the standard photographic fields (Diabetic Retinopathy Study) were photographed. One stereo pair of the macular field was obtained.

Since it is accepted that microaneurysms are one of the earliest lesions recognized by fundus photography and fluorescein angiography, we based our grading system on them. We defined microaneurysms as hyperfluorescent spots, larger than capillary size, that either became visible in the early capillary phase of the angiography or appeared as stained spots surrounded by fluorescent halos in the later frames.

Included in the study were 36 patients with an established diagnosis of insulin-dependent diabetes mellitus (IDDM) and 12 normal subjects. The diabetic subjects were classified into two groups according to the degree of retinopathy: no retinopathy (zero or one *doubtful* microaneurysm present) and minimal retinopathy (up to five microaneurysms). Diabetic subjects with more than five microaneurysms or with proliferative retinopathy (characterized by the development of new vessels) were excluded from the study.

All patients and subjects underwent visual acuity testing and were examined by direct ophthalmoscopy and fluorescein angiography (posterior pole) before undergoing vitreous fluorophotometry. A biomicroscopic vitreous evaluation was performed, and anyone exhibiting any degree of vitreous detachment was excluded from the study. Subjects with blood pressure of >90 mm Hg diastolic or 160 mm Hg systolic, cloudy ocular media, or a refraction correction of more than 5 diopters were also excluded.

Forty-five eyes of 27 diabetic subjects, 17–47 yr old (mean ± SD, 31.4 ± 10.5), exhibited no retinopathy; sixteen eyes of 9 patients, 26–47 yr old (mean ± SD, 38.4 ± 7.4), were classified as having minimal retinopathy. Twenty eyes of 12 normal subjects, 15–49 yr old (mean ± SD, 31.0 ± 12.0), served as controls. Duration of diabetes was 7.8 ± 4.7 yr for the no-retinopathy group and 10.5 ± 8.5 yr for the diabetic subjects with minimal retinopathy.

Vitreous fluorophotometry examination. Vitreous fluorophotometry measurements were made with the Fluorotron Master. Since the instrument has been described elsewhere,[7] we will review only its principal features. The Fluorotron Master consists of two major components: a data acquisition system, specifically designed for fluorophotometry, and a computer. The fluorophotometric unit delivers an exciting beam of blue light to the eye and detects the fluorescence emitted from a small probing volume. A contact lens

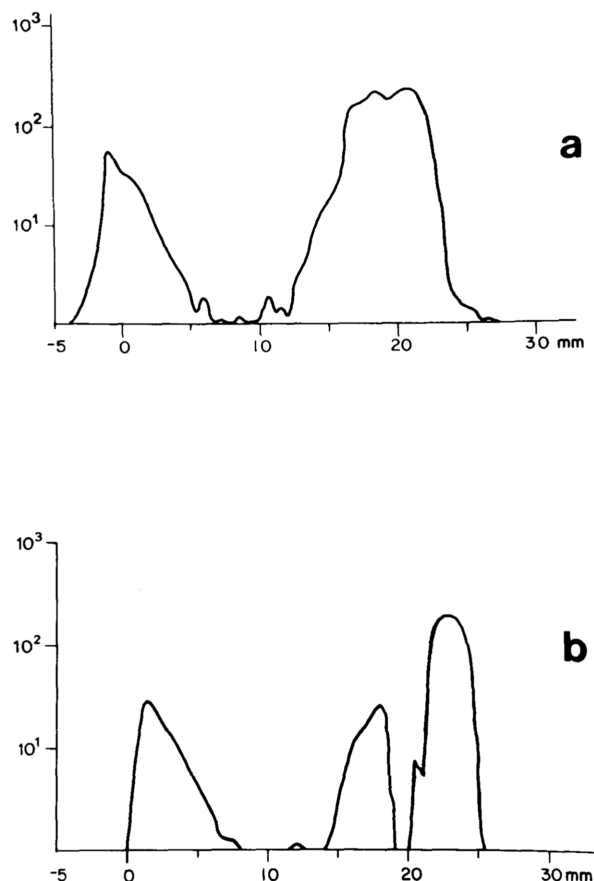

FIGURE 2. (A) Vitreous fluorophotometry scan performed in a diabetic patient with no visible retinopathy 60 min after i.v. injection of fluorescein (14 mg/kg). (B) Scan of Figure 2A after background correction.

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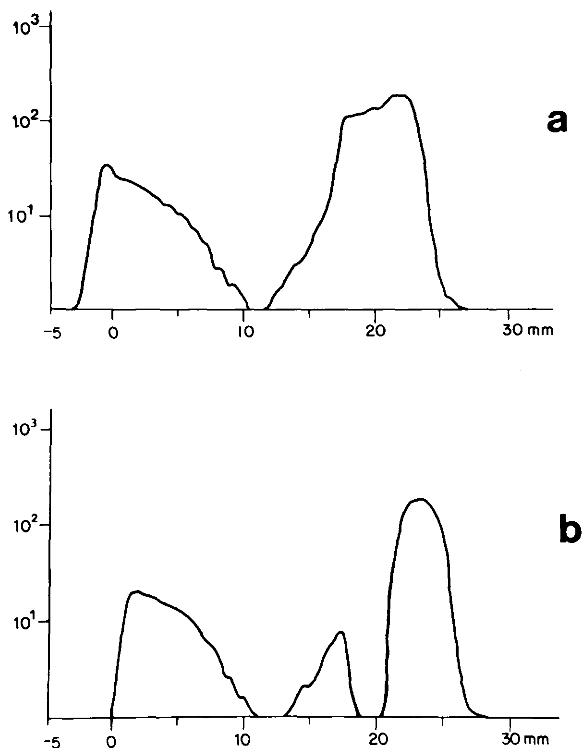

FIGURE 3. (A) Vitreous fluorophotometry scan performed in a diabetic patient with minimal retinopathy 60 min after i.v. injection of fluorescein (14 mg/kg). (B) Scan of Figure 3A after background correction.

is not necessary. The probing volume is moved posteroanteriorly across the eye in 25 s by the displacement of an internal lens, and the amount of fluorescence from the retina to the cornea is measured at predetermined intervals. In this study, we used 4 steps/mm with an acquisition time of 130 ms/step.

The computer directs the operation of the measuring system: it controls the scan, acquires the data, and calibrates the readings. When the scan is completed, it is displayed on a monitor (Figures 1A, 2A, and 3A); data may be saved on magnetic diskettes, printed on paper, and/or processed further.

The Fluorotron Master has been evaluated and compared with other instruments.[7] It was found to have, in vitro, a low limit of detection (0.43 ng/ml), using an acquisition time of 100 ms/step. The in vivo reproducibility, evaluated by the standard deviation of repetitive measurements obtained in eyes with low vitreous levels of fluorescein, was 12%.[7]

Before a scan was performed, the subject's pupils were dilated with 10% phenylephrine and 1% cyclopentolate. We considered it important to use a cycloplegic agent to paralyze the accommodation to prevent any variation in the optics of the eye that are coupled to that of the instrument. The subject was then seated and asked to fixate on a target viewed through the optical system. The operator viewed the eye, aligned it, and initiated the automatic scan. Scans were taken before the administration of a 14-mg/kg i.v. injection of fluorescein, 3 min after injection, and 1 h later.

Once the fluorophotometric data were saved on magnetic diskettes, they could be processed to minimize the influence of artifacts and to provide numerical values for the amount of fluorescein that had penetrated into the vitreous and anterior chamber.

The basic correction procedure to minimize artifacts has been described elsewhere.[8] First, the preinjection scan was subtracted from all subsequent scans after aligning the chorioretinal and lens peaks. In this study, we have used the anterior lens peak as the landmark. This subtraction minimizes the contribution of natural fluorescence, mainly that of the lens. Second, we corrected for the spread function of the chorioretinal peak, namely, the influence of the fluorescence in the choroid-retina on the readings taken in the vitreous. We tested the results of two previously outlined methods:[8]

(1) Individualized spread function. The chorioretinal peak spread function was obtained from a 2- to 3-min scan after subtraction of the preinjection scan. The early chorioretinal peak was aligned with that of the 1-h scan, its amplitude was adjusted to the same value, and it was then subtracted.

(2) Standard spread function. The chorioretinal peak spread function was obtained from the average of scans obtained from 25 normal eyes 10 min after injection. The procedure was otherwise identical to that mentioned above.

The first procedure had the advantage of using a spread

TABLE 1
Influence of data processing programs on the mean concentration of fluorescein in the posterior vitreous

	Baseline-corrected 3-mm measurement (ng/ml)	Standard CR spread function average 2.5–6.0 mm (ng/ml)	Individualized CR spread function	
			Average 2.5–6.0 mm (ng/ml)	Average 0.5–6.0 mm (ng/ml)
Normals				
12 Right eyes	4.2 ± 1.7 (39%)*	2.6 ± 0.9 (36%)	2.5 ± 1.1 (43%)	4.2 ± 1.1 (25%)
8 Left eyes	4.3 ± 1.4 (32%)	2.7 ± 1.2 (44%)	2.4 ± 1.0 (42%)	3.9 ± 0.9 (24%)
Diabetic subjects with no retinopathy†				
24 Right eyes	8.9 ± 5.0 (56%)	6.0 ± 3.9 (65%)	5.4 ± 3.0 (56%)	9.4 ± 5.4 (58%)
21 Left eyes	7.9 ± 4.3 (54%)	5.7 ± 3.8 (66%)	5.1 ± 3.7 (72%)	9.0 ± 5.7 (64%)
Diabetic subjects with minimal retinopathy†				
8 Right eyes	15.1 ± 7.9 (52%)	13.0 ± 5.9 (45%)	10.4 ± 4.5 (43%)	17.0 ± 8.0 (47%)
8 Left eyes	18.8 ± 8.3 (44%)	15.0 ± 8.6 (57%)	11.7 ± 5.5 (47%)	21.0 ± 12.8 (61%)

CR: Chorioretinal.
*Results expressed as mean ± SD (coefficient of variation).
†See text for definition.

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TABLE 2
Influence of normalization by the plasma fluorescein concentration (mean ± SD)

	Mean posterior vitreous (0.5–6.0 mm) (ng/ml)	Posterior vitreous penetration ratio (10^{-6}/min)	Plasma fluorescein integral (10^{+6} ng × min/ml)
Normals			
12 Right eyes	4.2 ± 1.1 (25%)	2.1 ± 0.5 (25%)	1.9 ± 0.3
8 Left eyes	3.8 ± 0.9 (24%)	2.1 ± 0.5 (25%)	
Diabetic subjects with no retinopathy*			
24 Right eyes	9.4 ± 5.4 (58%)	5.3 ± 2.3 (43%)	1.9 ± 0.3
21 Left eyes	9.0 ± 5.7 (64%)	5.1 ± 2.5 (48%)	
Diabetic subjects with minimal retinopathy			
8 Right eyes	17.0 ± 8.0 (47%)	7.9 ± 3.9 (56%)	2.2 ± 0.3
8 Left eyes	21.0 ± 12.8 (61%)	9.8 ± 6.0 (61%)	

*See text for definition.

function that was obtained from the individual eye. In contrast, the second procedure used an averaged value, which had the advantage of being a smoother curve obtained by averaging over numerous scans, in contrast with the single scan of the first procedure.

After the scans were corrected for artifacts (Figures 1B, 2B, and 3B), calculations were performed to quantitate the leakage of fluorescein. We have shown that one could eventually derive from these data the permeability coefficients of the BRB.[8] However, since this procedure is still experimental, we have chosen to quantitate the presence of fluorescein by computing its average concentration in the posterior vitreous, middle-vitreous, and anterior vitreous. The anterior vitreous was defined as the region between the lens and 6 mm posterior to it. The middle-vitreous was defined as the region 7–13 mm away from the lens.

Finally, the posterior vitreous was averaged over two regions: 0.5–6 mm and 2.5–6.0 mm from the retina. The second region was previously shown to be relatively free of artifacts after correction.[7] We intended, however, to test an extended region that encompasses locations closer to the retina. This region potentially contains more information on the amount of fluorescein penetrating into the vitreous, because at 60 min a significant amount of fluorescein has not yet diffused deep into the vitreous and is still located near the retina.

To compare our findings with previous studies and reports from other centers, we have also reported the 1-h vitreous fluorescein concentration taken at a displacement 3 mm from the retina. We corrected only by subtracting the preinjection scan.

Plasma fluorescein measurements. A study has shown that the decay of fluorescein in the plasma can be expressed, during the first 2 h after injection, as a logarithmic function:[10] log[plasma conc.] = a + b log(t), where a and b are constants that can be determined by two or more measurements and t is the time after injection. We chose to perform these three measurements at 5, 15, and 60 min. A software program then determined a and b and computed the integral of the function from 3 min to t (about 60 min). The precise time of t was recorded. Basically, the integral of the plasma fluorescein concentration over time reflected the amount of fluorescein to which the BRB was exposed until the measurement was performed. The first 3 min during which fluorescein reached its maximum have been ignored here. More data on the kinetics during that time will allow us to refine the mathematical function and to upgrade the accuracy of the calculation. This did not influence our study in which we compared different groups rather than using absolute values.

Blood was drawn by venipuncture, and 5 ml was obtained and mixed with EDTA. Each sample was centrifuged at

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TABLE 3
Penetration ratio (10^{-6}/min) at different locations in the eye

	PVpr	MVpr	AVpr	ACpr
Normals				
12 Right eyes	2.1 ± 0.5	0.3 ± 0.3	1.4 ± 0.7	49 ± 26
8 Left eyes	2.1 ± 0.5	0.5 ± 0.2	1.4 ± 0.5	61 ± 25
Diabetic subjects with no retinopathy*				
24 Right eyes	5.3 ± 2.3	0.7 ± 0.5	1.8 ± 1.1	72 ± 37
21 Left eyes	5.1 ± 2.5	0.7 ± 0.7	2.6 ± 2.3	65 ± 30
Diabetic subjects with minimal retinopathy				
8 Right eyes	7.9 ± 3.9	2.1 ± 2.6	3.7 ± 4.7	95 ± 53
8 Left eyes	9.8 ± 6.0	1.8 ± 1.9	3.3 ± 4.2	100 ± 44

PVpr, posterior vitreous penetration ratio; MVpr, middle-vitreous penetration ratio; AVpr, anterior vitreous penetration ratio; and ACpr, anterior chamber penetration ratio.
*See text for definitions.

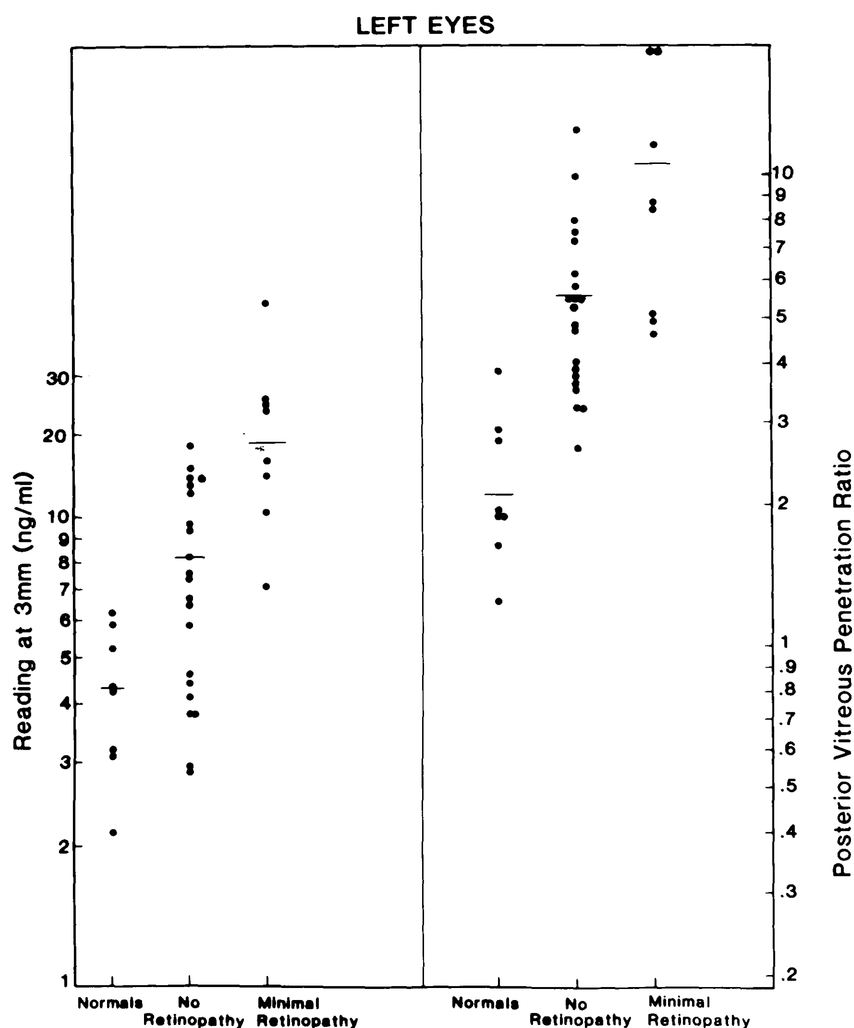

FIGURE 4. Scattergram of the blood-retinal permeability in the left eyes of the different groups. Left: Fluorescein concentration at 3 mm from the retina, 1 h after injection of 14 mg/kg, in normals, diabetic subjects with no retinopathy, and diabetic subjects with minimal retinopathy (see text for definition). Right: Posterior vitreous penetration ratio in the above three groups.

800 × *g* for 15 min and the supernatant was collected and diluted 400 times in phosphate buffer (pH 7.4, 0.2 M). The fluorescein concentration was then measured with the fluorophotometer.

RESULTS

Correction using individualized versus standard spread functions. The results have been summarized in Table 1. There was no significant difference among the results of the two spread functions in the three groups. The average posterior values were similar in their means as well as in their standard deviations.

Extended (0.5–6.0 mm) versus limited (2.5–6.0 mm) region of averaging. The extended region of averaging yielded higher values (columns 2 and 3, Table 1). This was due to the fact that, 1 h after injection, the fluorescein was not homogeneously distributed and its concentration increased with proximity to the retina. The comparison between the standard deviations showed that, by averaging within the extended region, the coefficient of variation was similar in the different groups with 30% except for the normals, in which it appeared to have been improved by >40% in both eyes.

Normalization to mean plasma fluorescein concentration. The 1-h integral of the plasma fluorescein level (Table 2) was similar in all three groups. We divided, for each eye, the mean posterior concentration by the plasma integral, thus obtaining a ratio that we called the posterior vitreous "penetration ratio." It quantitates the amount of fluorescein that has penetrated into the posterior vitreous for a given amount of fluorescein that has circulated over time in the bloodstream.

The results (Table 2) showed no noticeable change in the coefficient of variation of the values of the normals and those with minimal retinopathy. Some improvement was noticed in the results obtained in diabetic subjects with no retinopathy.

Fluorescein concentration at different locations in normal and diabetic subjects. For the posterior vitreous, we used the mean of the readings between 0.5 and 6.5 mm from the retina and divided it by the plasma level, thereby obtaining the posterior vitreous "penetration ratio." We also used the readings at 3 mm from the retina after correction for autofluorescence. The results are shown in Table 3 and plotted in Figures 4 and 5.

A statistically significant difference in the posterior vitreous penetration ratio was found between diabetic patients and

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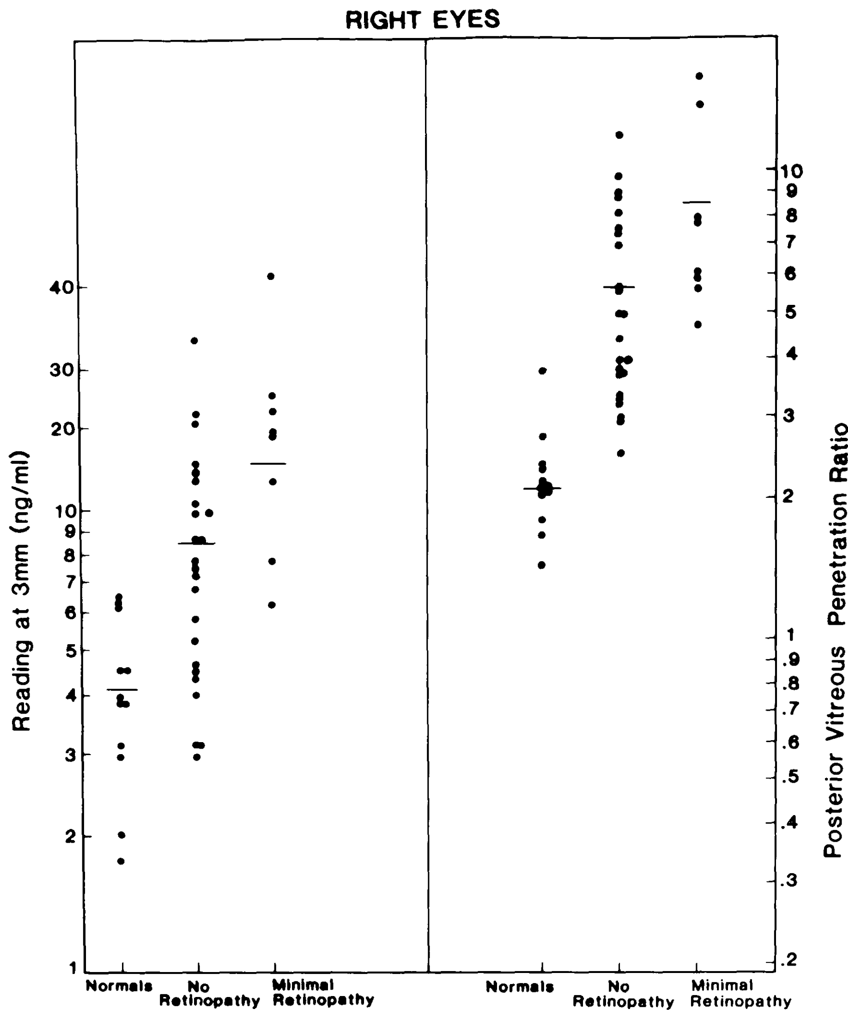

FIGURE 5. Scattergram of the blood retinal permeability in the right eyes of different groups.

normal volunteers (Table 3). The difference was apparent between normal volunteers and diabetic patients with no retinopathy ($P < 0.0005$ for both eyes). A difference was also present between diabetic patients with no retinopathy and those with minimal retinopathy ($P < 0.01$ and $P < 0.05$ for right and left eyes, respectively).

In the middle and anterior vitreous (Table 3), there was no significant difference ($P > 0.05$) between the normals and the diabetic subjects with no retinopathy except for the anterior vitreous of the left eyes ($P < 0.01$). There was no difference between the normals and the diabetic subjects with minimal retinopathy at both locations ($P > 0.05$).

In the anterior chamber (Table 3), there was no difference in the right eyes between normals and diabetic subjects with no retinopathy ($P > 0.05$), but there was a difference in the left eyes ($P < 0.025$). There was a significant difference ($P < 0.025$) in both eyes of the diabetic subjects with minimal retinopathy as compared with normals.

DISCUSSION

The first part of this study was devoted to the testing, in diabetic and normal eyes, of different procedures that were proposed earlier.[8] Fluorophotometric readings are increased artifactually by spread functions of the chorioretinal and lens signals. We corrected for the lens fluorescence spread function by subtracting the preinjection scan from the later scans. We found that, with the Fluorotron Master, the chorioretinal spread function can be corrected either with a spread function obtained from a particular eye or with one obtained by averaging the spread functions of normal eyes. The two corrections yielded similar results for both the mean and the coefficient of variation.

We also tested whether the averaging region in the posterior vitreous could be enlarged by extending it closer to the retina, namely, from 0.5 mm to 6 mm. Judging from a trend in the coefficient of variation consistent in both eyes, the results shown in Table 1 indicate that the enlarged region did not appear to alter the results for the diabetic subjects and may have improved those obtained in normals.

When data obtained at one point (3 mm from the retina) and corrected only for the lens fluorescence spread function are compared with processed data corrected for chorioretinal spread function and averaging, the difference is not striking. This is due to the fact that the chorioretinal spread function with the Fluorotron is relatively small, even in the vicinity of the retina. Therefore, the correction algorithm had no marked influence on the variability of the data except for the normals, in whom the artifact is more pronounced relative to the fluorescein concentration in the posterior vitreous.

Finally, the variability of the penetration ratio, which ac-

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counts for the plasma concentration of fluorescein, was compared with the spread of the results when no correction was made for the plasma (Table 2). We found less spread in the results of diabetic subjects with no retinopathy; in normals and diabetic subjects with minimal retinopathy there was no significant difference.

These results indicate, in our opinion, that when vitreous fluorophotometry is performed under controlled conditions, results are reliable whether or not one corrects for the fluorescein concentration in the blood. This is mostly due to the similarity in the plasma level of fluorescein in all the groups (Table 2). However, should the injection be incomplete or only partially in the vein, or should there be a physiologic disturbance in the blood hemodynamics, the results may be unreliable without the correction for the plasma concentration. To detect and correct for such situations, we suggest that investigators obtain uncorrected as well as corrected data.

A comparison of Tables 1 and 2 shows that, compared with a measurement taken 3 mm from the retina, attempted data enhancement with the use of an integrated reading (0.5–6.0 mm) and algorithms that correct for the chorioretinal spread function and for the variability in plasma fluorescence does yield a reduction of the coefficient of variation of 29% in normals and of 22% for diabetic subjects with no retinopathy, but a deterioration of 23% in the retinopathy group. These findings indicate that data enhancement is beneficient in cases with low penetration of fluorescein in which the values in the vitreous are low and, thus, more sensitive to artifacts and local fluctuations in the fluorometric reading.

We confirm previous reports, which show that an alteration of the BRB in diabetes can be detected by posterior vitreous measurements, taken 1 h after the i.v. injection of fluorescein, in patients with minimal or no retinopathy (as judged by the presence of aneurysms).[1,3,11] Such results cannot be obtained by using midvitreous locations for fluorescence measurements, as is well demonstrated in Table 3. If measurements are performed at 1 h, significant differences in fluorescein penetration between normals and diabetic subjects without retinopathy are found in both eyes only in the posterior vitreous. These findings stress the fundamental importance of using adequate instrumentation capable of scanning the posterior vitreous and obtaining results free of artifacts due to spread function of fluorescence peaks.

In diabetic eyes, the highest fluorescein concentrations in the vitreous are found closest to the posterior pole. This clearly supports the view that increased posterior vitreous fluorescence values in diabetic subjects result from an early breakdown of the BRB.

The increased fluorescein concentration found in the anterior chamber in diabetic subjects with minimal retinopathy indicates that the blood-aqueous barrier may also be affected at this stage.

In conclusion, the comparison between the normal and the diabetic subjects showed that vitreous fluorophotometry is a useful method to detect, quantitate, and characterize the alteration of the BRB that occurs in diabetes even before microaneurysms are detected by ophthalmoscopy or fundus fluorescein angiography.

Using an adequate methodology, vitreous fluorophotometry clearly separates diabetic patients with few aneurysms or none from normal controls. Clinical vitreous fluorophotometry measurements may prove to be useful for assessing metabolic control in diabetes, for monitoring longitudinal ocular changes, and for predicting development of diabetic retinopathy.

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