FUNDUS REFLECTOMETRY, AN EXPERIMENTAL STUDY

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
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Blut ist ein ganz besondrer Saft

Ein vollkommner Widerspruch bleibt gleich
geheimnisvoll für Kluge wie für Toren.

uit Goethe's Faust

aan Fro,
Gösta, Melissa en
Claire

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CONTENTS

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CHAPTER I

INTRODUCTION

When considering the many questions that still arise concerning the haemodynamics of the posterior segment of the eye, distinction must be made between the retinal and choroidal vasculature. Although the retinal circulation is still to be investigated more intensively, there exists a fair amount of information on this item. Photographic methods (HICKAM & FRAZER, 1966) make it possible to give fairly accurate estimates of the diameter of the larger retinal arteries and veins and their arteriovenous oxygen differences. The mean circulation time in the retinal vessels can be calculated (BULPITT & DOLLERY, 1971) and so can alterations in this time under influence of a raised intra-ocular pressure (DOLLERY et al., 1968).

Not so for the circulation in the choroid. Covered by a layer of dense pigment, it is hardly accessible to fluorescein angiographic research. Also cardiogreen, which enables the use of longer wavelength light, which is absorbed to a lesser degree by the pigment epithelium, does not give satisfying information (BROWN & STRONG, 1973).

Many research workers could not resist the temptation to tap vortex veins in animals (BILL, 1962a; 1962b; BEST et al., 1972; NAKAMURA & GOULSTINE, 1973; SCHLEGEL & LAWRENCE, 1969) to catheterize the long posterior ciliary artery (BILL, 1963a), to place probes (BILL, 1963b; NIESEL & KONSTAS, 1959), or to dehydrate the sclera (GREAVES & PERKINS, 1952) in order to measure flow, record pressures or study the behaviour of choroidal vessels under the microscope. Unfortunately all these methods are apt to disturb the physiological conditions of the eye.

Others used radioactive isotopes (BETTMAN & FELLOWS, 1956, 1958a, 1958b,

1962; PILKERTON, BULLE & O'ROURKE, 1964; FRIEDMAN, 1970; FRIEDMAN & CHANDRA, 1972; CHANDRA & FRIEDMAN, 1972; ALM & BILL, 1973a, 1973b; WEITER et al., 1973a, 1973b; STRANG, WILSON & JOHNSON, 1974; WILSON et al., 1973) in order to give estimates of choroidal bloodflow and volume. Continuous measurements are practically impossible even with these advanced techniques. The situation now is such that any contribution to the resolution of the problem is welcome as it might replace or complete earlier less reliable experiments. Most of the available data refer to the situation at one moment only. We thought it essential to study the circulatory relationships in the eye with a continuous measurement. Because disturbance of the physiology of the eye, by for example catheterizing vessels or applying detectors, may easily result in changed haemodynamics, it was thought essential as second condition not to touch the eye if possible.

Fundus reflectometry was chosen as the technique best answering to our purpose. This method, also named fundus densitometry or fundus spectophotometry, has been a fascinating method of examining certain aspects of the human and animal eye, since Rushton (1955) brought this technique to a high level of development for his work on visual pigments.

He designed a system of light-bundles of different colours which entered the eye through the dilated pupil. The amount of light returning was measured. BROADFOOT, GLOSTER & GREAVES (1961) were the first to use alternating light-bundles of different wavelength ranges. They found, when measuring the intensity of different wavelengths of light that returned from the fundus, a change in these intensities under varying conditions of the experimental animal. They were able to demonstrate in albino rabbits and to a certain extent in humans (GLOSTER, 1967), the effect of a decreased oxygen saturation, of higher intra-ocular pressure and of some drugs. These authors made use of the changing relationship in the amount of Hb and HbO_2 present in the vascular layers and of the fact that Hb and HbO_2 have a different spectral absorption characteristic.

TROKEL (1964, 1965) described an elegant densitometric method to calculate the bloodflow in the choroid of albino rabbits by means of a dye-dilution technique. However, as at the end of each experiment dye was injected in the vitreous, each animal could serve for one experiment only.

Measurements of the blood layer thickness were done by PEREGRIN & DODT (1969) in albino rabbits. They obtained highly reproducible results. Their method made use of subtracting the logarithm of the intensity of the returning light from that of the light returning from a bloodless eye. This one bloodless eye was considered to be the standard for all subsequent experiments. They

were able to demonstrate the influence of pulsewave and respiration on the blood layer thickness of the choroid.

DE COCK elaborated a more modern technique (BAKKER, DE COCK et al., 1973), derived from that of RUSHTON & BROADFOOT. He introduced fibre optics and a contact lens to provide greater versatility. Monochromatic light of a half intensity of 1,2 nm, enabled the use of exactly the desired wavelength. Although reflectometry is not necessarily a superior method compared with other methods of examining the choroid, we felt reflectometry might give more information than previous investigations, just by using more precise techniques. The subject of this study was therefore to investigate whether reliable continuous measurements of qualitative and quantitative changes in blood layer thickness and blood oxygen saturation were possible, without disturbing the physiology of the eye.

Fundus reflectometry as elaborated by OOSTERHUIS (OOSTERHUIS, BAKKER & VAN DEN BERGE, 1970; OOSTERHUIS & BAKKER, 1973), where the fundus of the eye is used as a background against which the passage of dye can be registered, will not be dealt with. Rabbits with their merangiotic retinal vasculature (restricted to the area of medullated nerve fibres in the upper quadrant of the eye) are suitable experimental animals for investigation of the choroidal circulation. Since in pigmented rabbits a much lower percentage of the incident light returns from the fundus of the eye than in albino rabbits, the latter are preferable for most experiments.

In reporting the results of our investigations, we found it better, for readability's sake, to divide the material into chapters, each dealing with a limited part of the subject. As a consequence of this several concepts will be mentioned at a stage where they may still be unfamiliar to the reader. A glossary has been added to help overcome this difficulty.

CHAPTER II

MATERIALS AND METHODS

Two monochromatic light bundles were produced by one light source (Xenon arc, Osram 150 W/L), which has a relatively flat emission spectrum between 500 and 780 nm (see block diagram; Fig. 1). A voltage stabilizer (Philips, PE 1402/11) was used to secure a steady voltage. By the use of a mirror, both bundles were made to run parallel (see Figure 2). They passed a chopper which produced alternating lightpulses of 0.1 msec duration at 2700 ct/sec. Each bundle passed a monochromator (Hilger and Watts, D 292, reciprocal dispersion 7 nm/mm slitwidth), one of which was motordriven. The high frequency

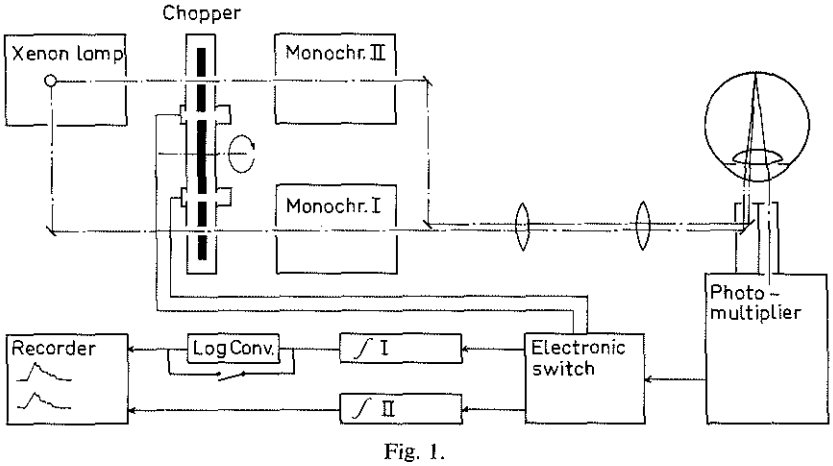


Fig. 1.
Block diagram of the set-up

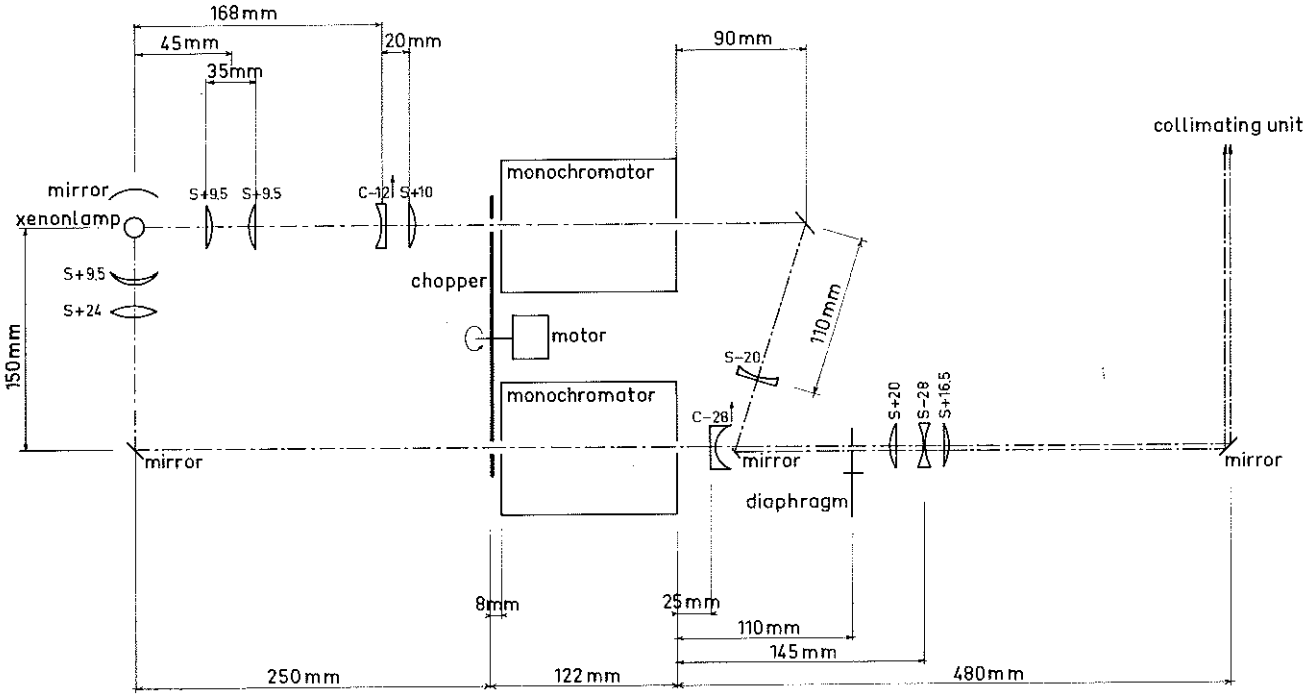


Fig. 2. Diagram of the optics, used in the set-up

of chopping produced a rapid enough response to follow any changes in reflection that were of interest (GAMBLE, HUGENHOLTZ, MONROE, POLANYI & NADAS, 1965). The two monochromatic bundles were aligned by means of two surface mirrors. Both bundles passed together through an optical system which produced a nearly parallel narrow bundle. This was aimed at the pupil, where it passed cornea and lens with a size of 1×2 mm. On the fundus a lightspot was produced of about half this size. In order to minimize reflections from lens and cornea, use was made of the fact that in an emmetropic eye the ingoing and outgoing lightbundles run parallel. Only the light coming back from the fundus of the eye where the lightspot produced by the ingoing light functioned as lightsource, was allowed to reach the photosensitive window of the photomultiplier (EMI 9592 B) (see Fig. 3). This was effected by two 1×1.5 mm diaphragms in the path of the outgoing light with a distance of 20 mm in between. The size of the dia-

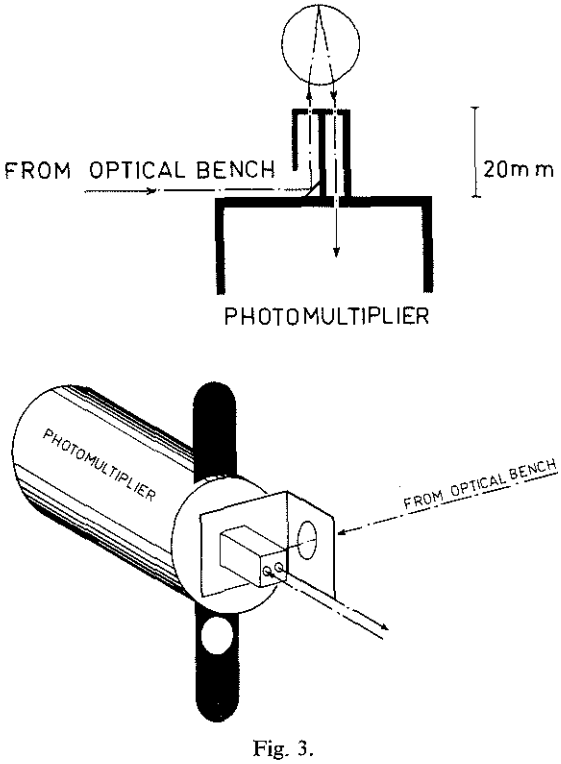


Fig. 3.
A: Diagram of the collimating unit
B: Collimating unit mounted on the photomultiplier

phragm was determined by the available amount of light. When a piece of white paper was kept at less then 50 mm distance from the collimating unit, illustrated in Figure 3, no reflections from the incident light could be registered, illustrating the small divergence of the entrance bundle to the eye. This technique drastically reduced the influence of reflections from cornea and lens on the measurements. The photocurrent was linear with the intensity of the received light. It represented both lightbundles separated only in time. Split on guidance by a photosensitive device in the chopper, each channel was integrated, amplified and written on a self-balancing potentiometric recorder (watanabe). Each channel was darkcurrent and background illumination–corrected by subtraction of the signals received during a lightpulse and the subsequent darkperiod. Subtracting both corrected signals enabled the registration of the difference between the intensities of the two lightbundles as measured by the photomultiplier. A logarithmic converter could be inserted in one of the channels.

The experiments were performed in a darkroom, using male and female rabbits (2.5–3.5 kg). After induction and intubation, a gas mixture of 25% O_2 and 75% N_2 was given, Pavulon® was used as a muscle relaxant. Positive pressure ventilation was applied (Amsterdam Infant Ventilator) using a constant volume of gas per minute. Body temperature was kept constant by a warm water mattress. The E.C.G. was controlled on an oscilloscope. The eye to be examined was atropinized. When the pupil was sufficiently dilated, the combination of lightsource, chopper, monochromator and optics, mounted on a stand with joystick, was brought near the animal, with the photomultiplier at a few millimeter distance from the eye. The eye was kept moist by a dropper connected with a bottle of saline.

When arterial shunts were used, a polythene tube (inner diameter 0.5 mm) was fixed in the cut ends of the common carotid artery. The ends were connected with a transparent polythene tube of a blood transfusion set (outer diameter 3 mm). By pressing the tube between two sheets of Perspex, a cuvette was formed of a depth varying from 0.0–0.4 mm (Fig. 4).

The set-up as described for the in vivo measurements was found preferable to the one we used in earlier experiments (BAKKER, DE COCK et al., 1973), where fiber optics were used to guide the light to and from the eye. As the distance between the end of both lightguides near the cornea was very small, much reflection against cornea and lens was measured as well. With the use of lenses and parts at our disposal at the time the light energy was improved by trial and error. This became necessary for the application of fundus reflectometry in vivo in pigmented eyes. For that reason also the former chopperdisc with 59 holes of 1.8 mm diameter was replaced by a disc with 57 slits of 15 × 3.1 mm.

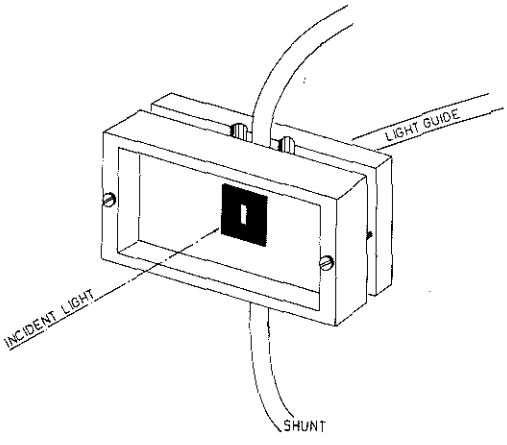


Fig. 4.
Shunt cuvette

A fundus camera (KOOYMAN, 1972) has been used with reasonable success. Shortage of light energy however forced us to find another method. Moreover not all the lens and corneal reflections could be eliminated. Polaroid filters were tried in our attempt to overcome these problems, but they appeared to have no effect on reflections from the lens.

CHAPTER III

THE APPLICABILITY OF LAMBERT-BEER'S LAW.

Lambert-Beer's law gives the relation between density (D), layer-thickness (d) in cm, concentration (c) in mMol/L and molecular extinction coefficient (ε) in $cm^2/\mu Mol$.

$$D = d.c.\varepsilon$$

This law, frequently applied in the following chapters, in principle is supposed to be valid for homogeneous solutions only. Whole blood certainly cannot fulfill this qualification. The difference in refractive index between erythrocytes and plasma is supposed to cause much scattering, which is why in the range for thicker layers the density is lower than expected. The question therefore has to be answered whether this law is valid for non-haemolysed blood experiments in vitro and also whether it may be applied in fundus reflectometry.

In order to verify these matters a wedge-shaped cuvette was used formed by two microscope slides, separated on one side by a small piece of a thick cover-glass (0.56 mm), placed 56 mm from one end of the slide (Fig. 5). The instrument was positioned at the end of the optical bench, as described in detail in chapter II. A photomultiplier was positioned just behind the cuvette, with a

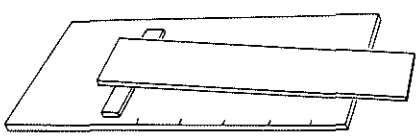


Fig. 5.
Wedge-shaped cuvette

10 mm diafragm. Measurements were done through the cuvette filled with water, with haemolysed blood and non-haemolysed blood in various concentrations.

Figures 6 and 7 give the density/layerthickness curve for haemolysed human blood of respectively 1.5 and 9.45 mMol Hb/L. A straight line illustrates the linearity of the relationship between density and layer thickness. Equivalent curves for non-haemolysed non-flowing blood are presented in Figures 8 and 9.

While for blood of Hb 9.8, a layer thickness of 150 μ would be the upper limit for applicability of Lambert-Beer's law, for Hb 1.96 we find that 400 μ is the ultimate acceptable thickness. The limit is not determined solely by the amount of erythrocytes in the layer. For a noticeable deviation the straight line according to Beer's law, the product of concentration and layer thickness is different, being 0.15 and 0.06.

This implies that in transmission through blood for which the concentration x layer thickness is a certain product, that solution for which the concentration is lower shows more aberration from Beer's law.

It may be concluded that Lambert-Beer's law is applicable for non-haemolysed blood in layers of up to 150 μ. Others advocate the application of Beer's law for layers up to 0.1 mm (POLANYI & MEHIR, 1962). For the rabbit fundus the blood layer measured usually does not exceed 30 μ, but it is twice traversed by the light, in one direction by collimated light, and in the other by more diffused light, reflected by the sclera.

Whether Lambert-Beer's law is valid in measurements in the rabbit eye is difficult to demonstrate. The stasis effect of the erythrocytes, which can be considered as a multiplication factor for the returning light, is expressed as a constant deviation of the density. This seems plausible because of the restricted

Fig. 6.
The relation between optical density (D) and layer thickness, measured at wavelength 548 nm, in haemolysed blood of 1.5 mMol Hb/L.

Fig. 7.
The relation between optical density (D) and layer thickness, measured at wavelength 506 nm in haemolysed blood of 9.45 mMol Hb/L.

Fig. 8.
The relation between optical density (D) and layer thickness, measured at wavelength 506 nm in whole blood of 9.8 mMol Hb/L.

Fig. 9.
Relation between optical density (D) and layer thickness, measured at wavelength 548 nm, in whole blood of 1.96 mMol/L.

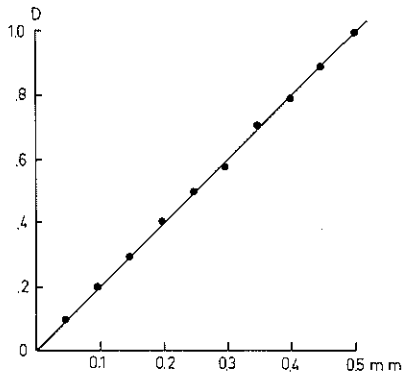


fig. 6

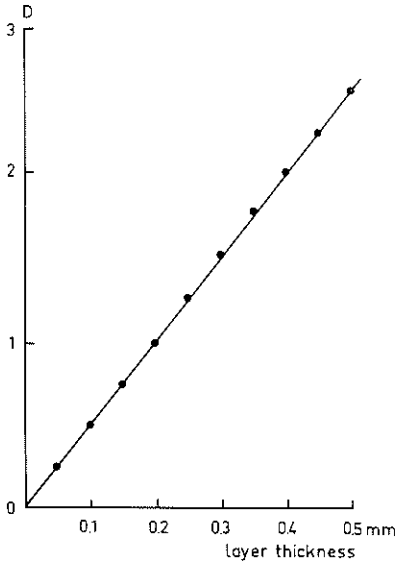


fig. 7

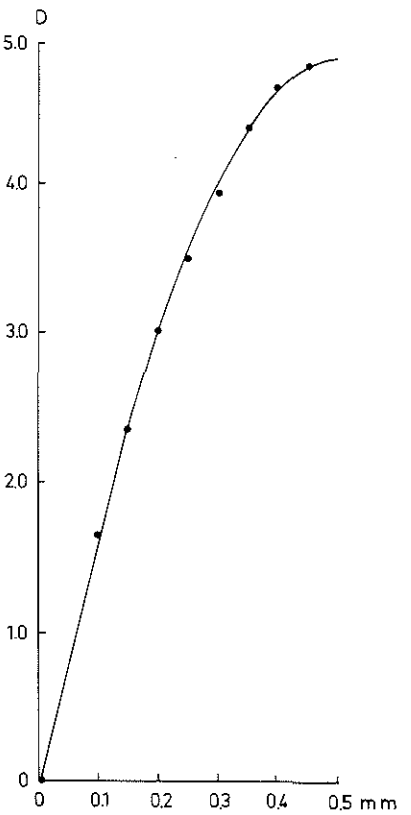
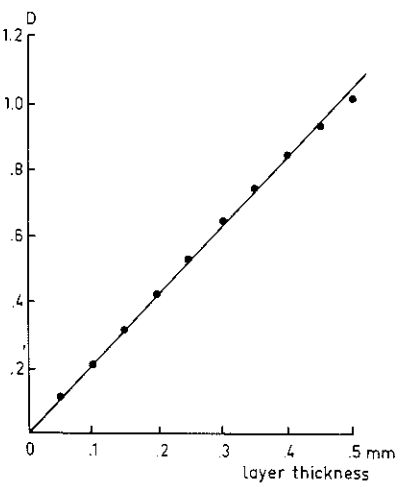


fig. 8

fig. 9



wavelength range in which considerable changes in diffusion etc. are not probable.

No controlled variation in layer thickness or blood concentration is possible. Consequently, the molecular extinction coefficient is the only factor of which the variation can be used, by measuring over a wavelength range where blood has different ε-factors. When there are difficulties in obtaining blank, bloodless values, necessary to calculate the optical density, it was possible to measure the difference of two densities (i.e. the difference between two log R values). Details can be found in chapter VII, where the same procedure is used to demonstrate a stasis effect in the eye (see Fig. 26). As far as the D/ε curve reflects the influence of layerthickness, Lambert-Beer's law is applicable in the albino rabbit eye.

CHAPTER IV

SOME CONSIDERATIONS ON THE ORIGIN OF RETURNING LIGHT

Light reflected from the eye, or more precisely in our experiment, light returning to the photomultiplier, can arise from various sources in the eye.

a) Reflections occur at any plane of transition, where the light passes over to a tissue or material with a different refractive index. The type of reflection is rather stable. It is not influenced by variations in vascular layer thickness, nor oxygenation. This includes not only the transitions between air and cornea, aqueous and lens, lens and vitreous, but also between all the individual layers in the cornea and between separate lens fibres. Also minor opacities in the vitreous may contribute. In fact even the vesselwalls and other tissue in the retina and choroid might be sources of reflection.

b) Light, after passing through the vascular layer, is reflected by the sclera and passes the vascular again (OOSTERHUIS, BAKKER & VAN DEN BERGE, 1970) (Fig. 10). This reflected light is comparable with the transmitted light used in transmission oximetry, for which the relation with oxygen saturation is given by the formula:

$$\text{oxygen saturation} = S_{O_2} = a - b \frac{D_{\lambda \text{ detecting}}}{D_{\lambda \text{ isobestic}}}$$

where a and b are constant factors depending on the method used (VAN KAMPEN & ZIJLSTRA, 1968). As at an isobestic wavelength, the density of a certain blood layer is constant, irrespective of the oxygen saturation, the S_{O_2} is linearly related with the density at the detecting wavelength. As D equals log incident light minus log transmitted light and the intensity of the incident light will be kept constant, the changes in S_{O_2} are linearly related with the changes in log transmitted light. For measurements in the eye, the same will be valid if only trans-

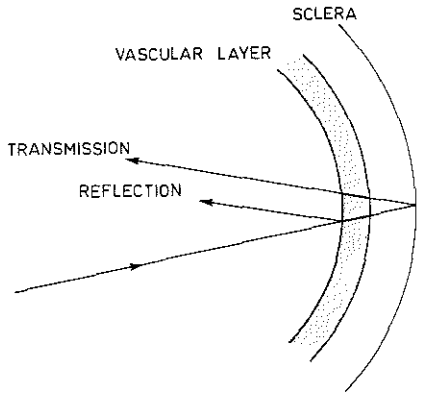


Fig. 10.
Scheme indicating the difference between transmission and reflection from the erythrocytes.

mitted light is measured, a condition which to a high degree is fulfilled in albinotic eyes, although even in the most ideal recording some reflections can be registered.

c) The amount of transmitted light is not only reduced by absorption. Part of the incident light is reflected directly by the erythrocytes. These reflections probably occur mainly at the cell walls. In our case the light so reflected may pass the pupil again and will then be measured by the photomultiplier. This light has passed some part of the total bloodlayer, depending at what postion in the bloodlayer the reflecting erythrocyte was situated. The light carries information after its passage through the blood, concerning the SO_2, but will give a false impression concerning layer thickness. For this type of reflections also a linear relationship exists between SO_2 and the log $R_{detecting}$.

ZIJLSTRA based his method on reflection oximetry on this phenomenon (ZIJLSTRA, 1958). As illustration of the afore-mentioned relationship it may be related that MOOK et al. (1968) demonstrated a nearly linear relationship between the oxygen saturation and Log R^{640}/R^{880}, using reflection oximetry with intra-arterial fibre optics. As 880 nm in those experiments was considered to be isobestic, this means that the saturation is related linearly with:

$$\log \frac{R_{detecting}}{R_{isobestic}}$$

For a given total haemoglobin $R_{isobestic}$ will be constant. Consequently the oxygen saturation is linearly related to log $R_{detecting}$. ZIJLSTRA noticed in

reflection oximetry, while working in vitro with blood of a given oxygen saturation and concentration and using a certain wavelength, that a layer thickness of about 3–4 mm at least was necessary to arrive at optimal reflection. Using a layer thickness of 1 mm, blood with a total Hb concentration of 3 mg% gave hardly any reflection, whereas higher concentrations gave reflections which were considerably reduced, compared with thick layers.

Summarizing we can state:

1. The amounts of returning light as described under (b) and (c) although of completely different origin, indicate parallel variations in oxygen saturation.

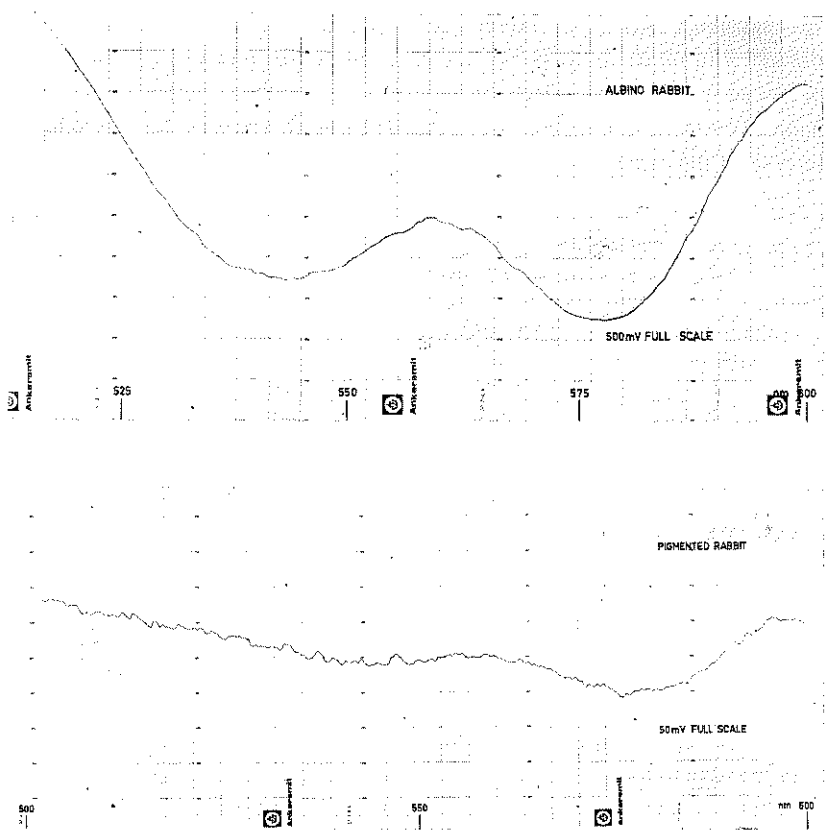


Fig. 11.
Linearly recorded energy of light returning from the fundus of an albino and of a pigmented rabbit eye, over wavelength range 500–600 nm.

2. With incident light of a constant quality, the light, returning from the eye, consists of a non-variable part, originating from reflections at various planes of transition, and a part of the bloodcorpuscles themselves, that may be considered as light passed through a blood film of twice the vascular layer thickness. This part varies with the density of the vascular layer. While in practice this goes for albinotic eyes, for pigmented eyes the conclusions made in the last paragraph are at least doubtful. To illustrate this, the R curves of an albinotic and a pigmented rabbit eye are presented in Figure 11. When the layerthickness was calculated in the log R curves, in the way as will be treated later, this gave respectively 28 and 13 μ. In order to find a reason for this improbable difference, the following experiment was performed.

In enucleated pigmented rabbit eyes a scleral window was prepared. No difference in returning light was noticed, whether the image on the retina was projected on the window area, or just beside it. In albino rabbits on the other hand a considerable difference was noticed (Table 1). The slitwidth was different in each of the experiments.

TABLE 1

Energy of light returning from enucleated rabbit eyes with and without scleral window. The energy of the incident light was different in each experiment.

albino rabbit eye nr	with sclera mv	without sclera mv	%
1	1 140	600	53
2	636	455	72
3	865	380	44
4	905	365	40
		mean	52%
pigmented rabbit eye nr			
1	575	672	117
2	610	580	95
		mean	106%

That the light returning from albinotic eyes with a scleral window was so large was due to the fact that any minor change in the curvature of the posterior pole had a tremendous influence on the amount of direct reflections. During these experiments large variations in returning light energy could be provoked just by pushing careful against the slightly bulging choroid. Furthermore the deformed bulbus gave a considerable higher amount of corneal reflections.

Although the experiments with and without scleral window are not completely comparable as the change of position of the bulbus and the lowered intraocular pressure may be reasons for a different proportion of corneal reflection, the results were repeatedly so convincing that we may conclude that in pigmented eyes most of the returning light has never reached the sclera. In recordings over a wavelength range in pigmented eyes we nevertheless discern information on the bloodlayer.

Until now, we have assumed that reflections from erythrocytes, if at all existent, make up only a very small part of the total amount of returning light, due to the shallow blood layer concerned. Whether these reflections really do play a relevant role was estimated by means of the following in vitro experiment.

A wedge shaped cuvette (see Fig. 5), with a depth from 0 to 0.5 mm was filled with water. The cuvette was placed on black felt, soaked in glycerin, without any airbubbles in between. Light from the optical bench was aimed at the cuvette under an angle of 45° (see Fig. 12). The photomultiplier was mounted immediately behind the last surface mirror of the optical bench and aimed in the direction of the incident light. The diaphragm in the photomultiplier window was set at a diameter of 10 mm. The wavelength was chosen at 600 nm. The returning light was measured to be 4 mv. Measurements were performed over the range 0.1 to 0.5 mm layerthickness. The energy of the reflected light was calculated by subtracting the 4 mv found in the waterfilled cuvette, apparently the result of reflection against the glass surface, from the total number of millivolts. The black felt was removed and replaced by a sheet of opale acrylate (Vinkoplast 060) 0.27 cm thick. The degree of reflection for wavelength 600 nm for this material, as found in several measurements, surpassed the reflection of human sclera by only 6%. The total light now measured, consisted of twice

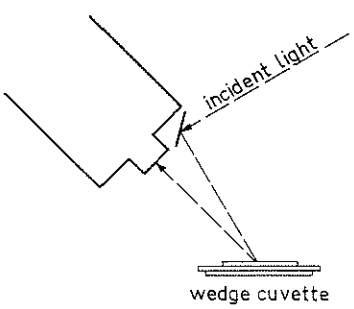


Fig. 12.
Position of incident light and photomultiplier in an experiment described on page 17.

transmitted light, plus light reflected by the erythrocytes and 4 mv glass reflection. In order to find the intensity of the transmitted light the total was reduced by both last factors as registered at a corresponding layer thickness with black felt instead of opale acrylate.

By inserting a thicker crossbar in the wedge cuvette the thickness range could be increased fivefold to make it 0 to 2.5 mm. In both kinds of cuvettes registrations were made with blood of Hb 2 mMol/L and 10 mMol/L.

Figure 13 gives the values of transmitted and reflected light, plotted against the product of layerthickness and concentration. As was the case in the experiments to prove the validity of Lambert-Beer's law for non-haemolysed blood, we noticed that for blood of Hb 10 mMol/L the transmission was higher than for

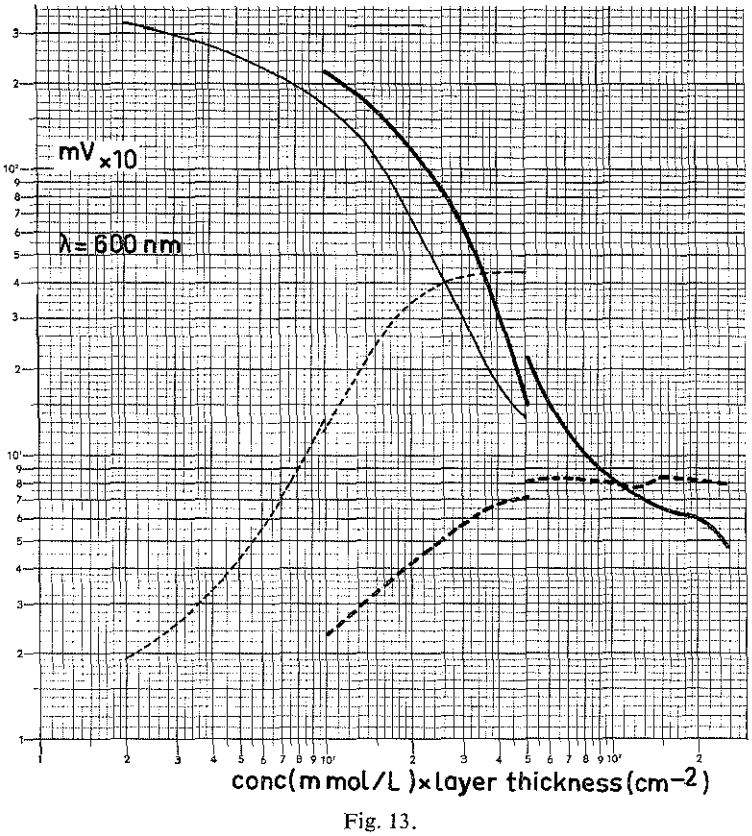


Fig. 13.
Relation between transmitted light (drawn lines) and light reflected from erythrocytes (interrupted lines) for whole blood of 2 mMol Hb/L (thin lines) and 10 mMol Hb/L (thick lines).

blood of Hb 2 mMol/L, as long as the product concentration × layerthickness remained constant. While for a blood layer of 500 μ of Hb 2 mMol/L, the percentage of reflection is 8% of the total returning light, for a 100 μ bloodlayer of Hb 10 mMol/L this percentage is just 1%. As the bloodlayer in rabbits is anyway under 100 μ, the amount of reflection from the erythrocytes can be expected to be negligible.

If indeed in pigmented animals the only light returning would originate in the erythrocytes, no returning light would be measurable if the blood was haemolysed.

In order to verify this, the following experiment was performed: In two albino and two pigmented rabbits the head was perfused, while the eye was examined with the fundus reflectometer (see Chapter II). To do this the common carotid artery was canulated and ligated proximal to the canule. The jugular vein was opened and the animal perfused with Macrodex. When a bloodless recording had been made, the head was perfused with haemolysed bovine blood, alternated after rinsing with Macrodex with nonhaemolysed blood. The perfusion pressure was the same for both kinds of blood and so was their concentration. When during the experiment the pupil became too narrow a limbal incision was made and a slip of iris pulled into the wound. The same procedure could be repeated at the other side of the eye. Afterwards the anterior chamber was restored by means of a needle in the anterior chamber, connected with a bottle of saline.

The results of these complicated experiments, that were performed in two albino rabbits and two pigmented rabbits will not be described in detail. We could draw the following conclusions:

- the choroidal blood layer was filled for only 50% in both perfusions.
- no difference was noticed between perfusion with haemolysed and nonhaemolysed blood. This confirms the experiment just described.
- when the logarithmic recorded registrations were compared with a bloodless registration, the blood density could be calculated. For haemolysed blood these values, measured at isobestic wavelengths were mutually related according to their respective ε-values. Not so for the whole blood, where all values were higher than was expected by approximately 0.03 log unit at each wavelength. Probably this was due to the stasis effect, which depends on the presence of erythrocytes (see Chapter VII).

Since we still did not know where the returning light did come from, the following experiment was performed, in two albino and two pigmented rabbits.

After perfusion with Macrodex a registration of the returning light was made

to ensure that the eye was indeed bloodless. Then 2 ml 5% Evans Blue was injected in the common carotid artery. At wavelength 590 nm the ε-value of Evans Blue is approximately 25.000. Even a shallow layer of 16 μ will transmit only 1%. Within a few seconds the dye passes the vesselwall and fills the whole of the choroidal tissue, without penetrating, however, through the pigment epithelium. After the dye was injected the intensity of the returning light decreased immediately. Within 30 seconds an injection of the same dye followed into the vitreous by means of a preplaced needle connected with a syringe. Some seconds later again also dye was brought into the anterior segment of the eye by means of a second needle fixed through the cornea into the anterior chamber. The results of these experiments are summarized in Table 2. For albino I, pigmented rabbits II and III in this experiment the same slitwidth was used. For the other animals more light was allowed to enter the eye.

TABLE 2

Reduction of the intensity of the light returning after injection of 5% Evans blue solution in respectively the common carotid artery, the vitreous and the anterior chamber in rabbit eyes perfused with Macrodex.

	Alb I		Alb II		Pigm I		Pigm II		Pigm III	
	mv	%	mv	%	mv	%	mv	%	mv	%
before dye	265	100	650	100	350	100	55	100	35	100
after dye in carotid art.	3.5	1,3	7	1,0	190	50	15	27	8	23
after dye in vitreous	<1	<0,4	<1	<0,2	5	1,4	1,5	2,7	1	3
after dye in ant. chamber	<1	<0,4	<1	<0,2	<1	<0,3	<1	<2	<1	<3

DISCUSSION

The very fact that the density of the choroid together with retinal pigment epithelium is between 2 and 3 over the range of 500–600 nm, supports the conclusion from the experiment with the scleral windows, for if the light indeed did reflect against the sclera the transmission would be 10^{-4} to 10^{-6}. For albino rabbits the density of the choroid is approximately 0.5 which allows a transmission of 10^{-1}. Assuming that the bloodlayer and retina will be of about the same density, this makes clear that the difference in returning light of about 8 times, which we find in practice, cannot be explained by transmission as far as the sclera, through choroidal tissue of different density.

There seem to be two possibilities to explain the apparently small bloodlayer information in pigmented rabbits.

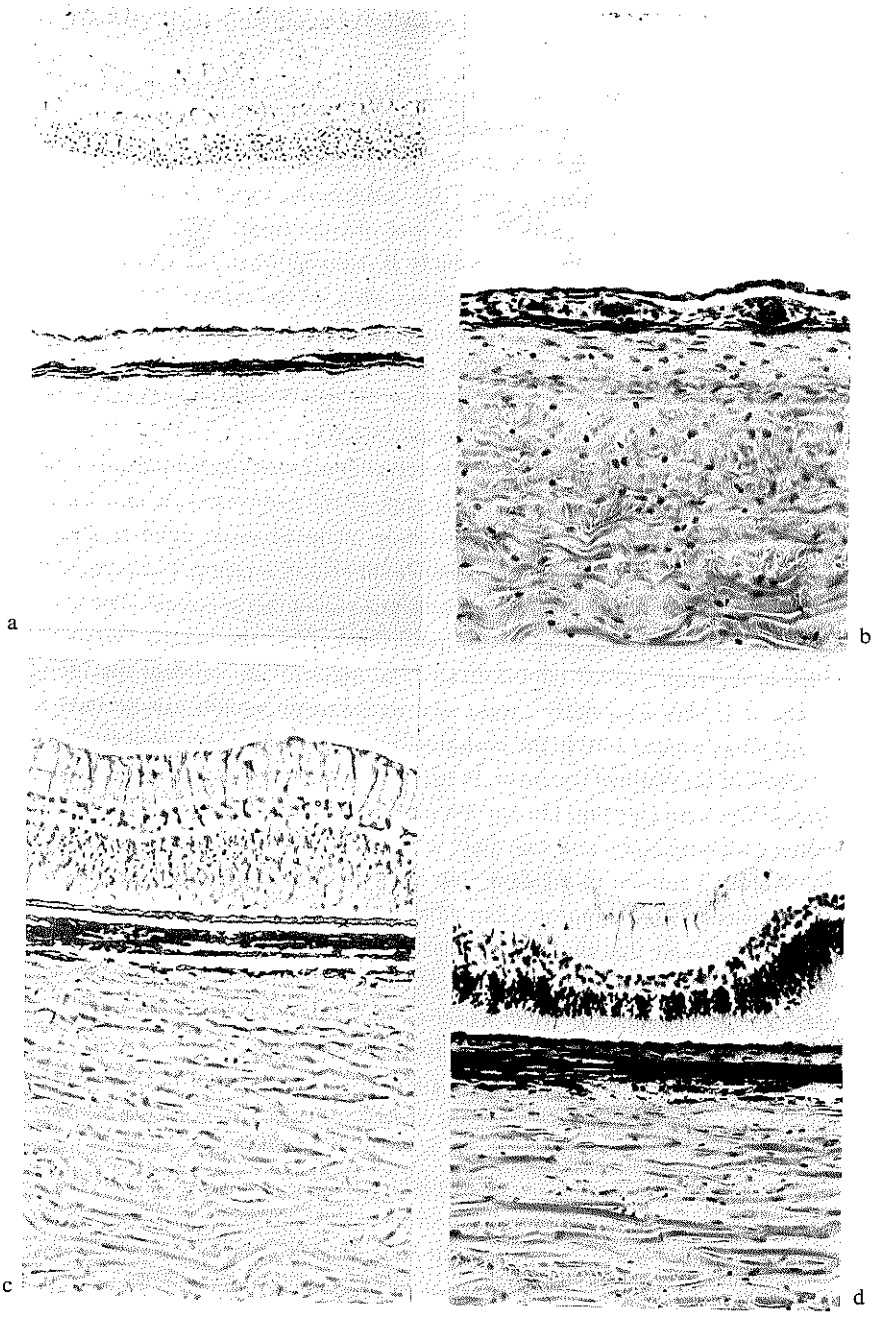
A. Or the bloodlayer as measured is very shallow indeed, which would mean that the light was reflected by some medium about halfway in the bloodlayer and *B.* the information we receive is diluted by reflections. Due to the controlled effectiveness of the collimating unit, the reflections would originate in the posterior part of the eye, but in front of the vascular layer.

The dye experiments support possibility B, because when the vascular layer is virtually impenetrable to light, still about 40% of the original light returns. This must be reflected by some structure in front of the vascular layer. It is however not certain whether before the dye injection the light that passed this structure and came back with information on the bloodlayer, traversed the whole blood layer or only part of it (possibility A).

The most conspicuous distinction between albinotic and pigmented eyes is the presence of pigment in the latter. In sections of the posterior pole of a lightly and a heavily pigmented eye, the disposition of the pigment could be clearly discerned (see Fig. 14). The retinal pigment epithelium is clearly demarcated as a single layer of uniformly pigmented cells in a regular arrangement. The uveal pigment is often concentrated in the posterior part of the lamina vasculosa, which is therefore named lamina fusca. The pigment lies between the blood vessels. It is very irregularly spread and in the same section can be seen to occupy either a small zone in the posterior side of the lamina vasculosa or nearly the whole of the width of this layer. In between both pigment zones is a relatively pigment free area (see Fig. 14a and 14c), consisting of the entirely pigment free lamina choriocapillaris and that part of the lamina vasculosa which contains only a limited amount of pigment, and is of varying thickness. These findings are consistent with the reports from PRINCE & EGLITIS (1964).

It appears likely that the light is reflected by pigment, partly by the pigment epithelium resulting in dilution of the information and partly by the uveal pigment, giving information on the traversed bloodlayer, i.e. the choriocapillaris and possibly part of the blood in the uveal bloodvessels.

How pigment is able to reflect light is not clear. Inspection with the ophthalmoscope in dark eyes, does not, in our experience reveal more reflections than slightly pigmented or albinotic ones. No definite conclusion could be made from these two examined eyes, as to which of the two pigment layers, is less pigmented in eyes, which are less dark. We had the impression that the pigmentation was evenly decreased. As a result of these observations it is evident that any fundus-reflectometric measurements in pigmented eyes can only be qualitative.


a
b
c
d

HUNOLD & MALESSA (in press) reported reflectometric measured optical densities in humans. They give very low figures compared with the results of our transmission measurements (see Chapter IX). This may be explained by the presence of reflection from the pigment layers in the eye.

Fig. 14.

Sections of a lightly pigmented rabbit eye, (a) and (b)

(a) Posterior pole, demonstrating the unicellular layer of retinal pigment epithelium and the irregularly spread uveal pigment in the lamina fusca (unstained, ×150; neg. nr. PA 11999).

(b) Posterior pole, demonstrating the presence of large bloodvessels in a poorly pigmented part of the lamina vasculosa. The lamina choriocapillaris is not discernable as a separate layer (haematoxylin and eosin, ×150; neg. nr. PA 12000).

Sections of a darkly pigmented rabbit eye, (c) and (d).

(c) Posterior pole demonstrating the retinal pigment epithelium and the uveal pigment spread over most of the width of the lamina vasculosa (unstained, phasecontrast, ×150; neg. nr. PA 12002).

(d) Posterior pole, demonstrating areas in which the zone between the retinal pigment epithelium and the zone of highest concentration of the uveal pigment, consists of part of the lamina vasculosa together with the choriocapillaris. Insignificant amounts of pigment are visible in the sclera, accompanying the larger vessels. (neg. nr. PA 11995).

(technician : Mr. P. VAN DER HEUL)
(photographs: Mr. J. K. VAN DIJK)
(Department of Ophthalmic Pathology, E.U.R.).

CHAPTER V

IS THERE A MEAN BLOODLAYER THICKNESS?

The described technique of fundus reflectometry uses a light-bundle aimed at the pupil of the eye. The lightspot thus produced on the fundus is visible in enucleated eyes from the outside as a 2 × 1½ mm lightspot, but the exact measurements are difficult to assess due to the diffusing properties of the sclera.

Indirect ophthalmoscopy enables us to observe the lightspot in the living eye. To do this a small mirror was placed on the collimating unit, which deflected the outgoing light approximately 90 degrees. The investigator, standing aside, could have a good view of parts of the fundus. By reducing the light intensity of the ophthalmoscope, a lightspot of about 1 × 1½ mm became visible. This method enabled us to estimate not only the size of the spot by comparison with the papil diameter, but also its exact location.

The measurements were performed over this 1 × 1½ mm area. The question arises whether information obtained by such reflectometry represents a mean value of the whole illuminated surface. If there should be even a small area with a considerably thinner bloodlayer, this would result in a disproportionately large transmission of light. It would be very difficult to prove the absence of spots with a lower blood density. It seems plausible however that a good enough approximation of a homogeneous layer is present. This is based on the following observations:

1. Scleral tissue has a good diffusing surface. Therefore even if the first passage of light had been relatively unhindered by the presence of blood, the way back is very likely to be through the bloodvessels.

2. When a normal pigmented fundus is inspected with the ophthalmoscope no bloodless area can be perceived. The pigment layer guarantees sufficient diffu-

sion of the light for the bloodvessels of the choroid to give the appearance of an equal red field. In albino rabbits it is possible to discern isolated bloodvessels. These are surrounded by a diffuse area, which is nearly as dense. When during fundus reflectometry experiments in albino rabbits the eye was slightly moved, no difference in energy of the returning light was noticed. This implies that when moving within a certain area of the fundus, the same layer thickness is measured.

3. Descriptions of the anatomy of the choroid in humans and rabbits (ASHTON, 1952; RUTNIN, 1967; PRINCE, 1964) all point at the abundance of capillaries in the choriocapillaris and the dense network of arteriolae and venulae in the choroid.

Consequently we assume for practical purposes that the choroid contains blood vessels in a regular and dense pattern in such a way that no places of comparatively low bloodlayer thickness exist.

CHAPTER VI

DEMONSTRATION OF BLOOD OXYGEN SATURATION CHANGES IN THE VASCULAR LAYER OF THE RABBIT EYE.

INTRODUCTION

We wished to repeat some of the experiments described by BROADFOOT, GLOSTER & GREAVES in 1961, equipped with a sensitive and quickly reacting machine, using monochromatic light. In order to study the difference between reflectometric results in albinotic and pigmented fundi, rabbits of both kind were used. Continuous registrations were made of returning light variations influenced by the optical density of the bloodlayer in the choroid. During the experiment this density varied due to fluctuations in oxygen saturation and layer thickness, both provoked by changes in oxygenation.

As the measurements took place over an area where we assume arterial and venous bloodvessels to be thoroughly mixed, the outcome represents the variation in the mean oxygen saturation. ELGIN (1964) reported arterio-venous oxygen differences in dogs of 3 % only. This figure is smaller than we expect our error to be.

ISOBESTIC AND DETECTING WAVELENGTHS

According to Lambert-Beer's law, variations in the density of the vascular layer are dependent on changes in concentration (c), layer thickness (d) and molecular extinction coefficient (ε). For an isobestic wavelength where $\varepsilon^{Hb} = \varepsilon^{HbO_2}$, the density of blood of a varying oxygen saturation is dependent, as long as the layer thickness and blood velocity remains constant, only on the sum of

the concentrations of Hb and HbO_2, which is the total haemoglobin concentration. For non-isobestic wavelengths, where the extinction coefficients for Hb and HbO_2 are not equal, a change in concentration of Hb and HbO_2 results in a change of density, while the total Hb remains constant. Figure 15 gives a scheme of this principle applied to our two wavelength method, where the

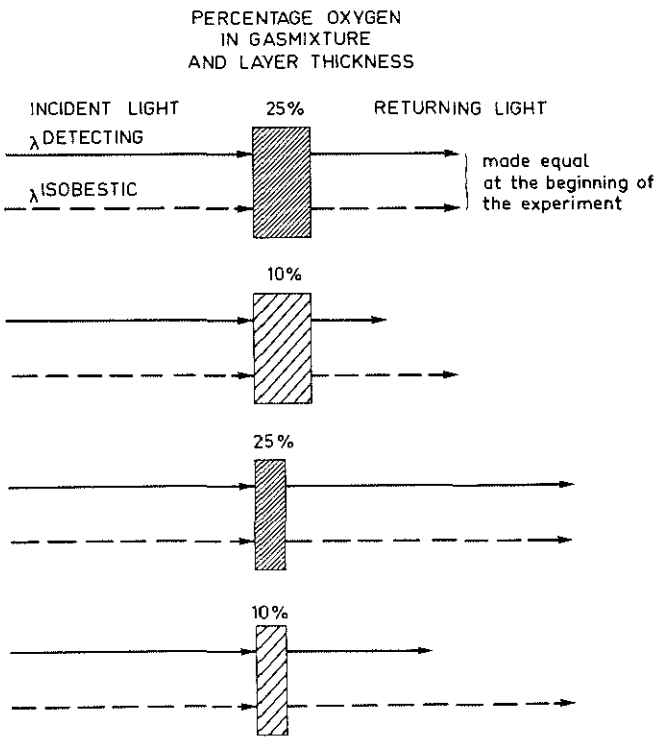


Fig. 15.
Scheme indicating the influence of changes in bloodoxygen saturation and layer thickness for light bundles with minimal and maximal difference in extinction coefficient for Hb and HbO_2.

vascular layer of the eye is considered to behave like a filter of variable thickness and colour. A change in oxygenation will produce changes only in the detecting

channel. However, when the layer thickness is for example, reduced, as in vasoconstriction, both channels will measure more returning light. This increase is dependent on the densities of blood for the wavelengths of both isobestic and detecting channels.

In order to recognize differences in S_{O_2}, variations in density were registered. These are based on a change in ε-factor. To eliminate disturbances, amongst others variations in the light output of the lightsource, the potential representing the light energy of the detecting colour after equalising, was subtracted from that representing the returning energy of the isobestic wavelength, where the ε-value remained constant.

The most important disturbing factor however, the change in layer thickness, is only equally represented in both colours, when wavelengths are used with the same ε-values. This remains an approximation in practice, as the method is based on the change of the detecting ε-value, while the isobestic ε-value is stable. For comparatively low densities the method has technical advantages.

In practice the changes in layer thickness when the ventilation is changed from 100% to 10% O_2, proved to be less than 10% (see chapter VIII). The ε-value at the detecting wavelength can vary at the same occasion about 28% (when one assumes a change in saturation from 1.0 to 0.45 during ventilation with 10% O_2, working at wavelength 465 nm). In principle the correction in the variations of the ε. will then not exceed 10%.

As will be discussed later, the stasis effect can be considered, anyway over short wavelength ranges, as an ε-independent deviation of the density. Therefore the differential recording of the log $R_{detecting}$ and log $R_{isobestic}$ is indeed preferable. In this way variations due to blood velocity changes in the eye are largely eliminated. The recording indicates in this case a combination of changes in density mainly due to variations in layer thickness and in colour. Since originally log $R_{isobestic}$ — log $R_{detecting}$ was not recorded during the experiments, these curves have been constructed afterwards from the linear registered ones.

MOOK, VAN ASSENDELFT & ZIJLSTRA (1969) found in in vitro experiments the highest sensitivity to changes in oxygen saturation when measuring at 650 nm. As one of the conditions for the use of differential recording is that the densities of the two wavelengths to be compared are of the same order and no isobestic points of a comparable density were available, another couple of wavelengths had to be found. Also for the other reasons discussed, such a choice is preferable. We used as isobestic wavelength 506 nm ($\varepsilon = 5.11$) and as detecting wavelength 465 nm, where for reduced blood $\varepsilon = 3.52$, for oxygenated blood $\varepsilon = 7.29$.

RECORDING OF CHANGES IN REFLECTED LIGHT FROM THE EYE WHEN THE COMPOSITION OF THE GAS MIXTURE IS CHANGED.

In pigmented as well as in albino rabbits the incident light consisted of a wavelength of 506 nm (the isobestic wavelength) and 465 nm (the detecting wavelength). Registrations were made of the returning light of 506 nm direct linearly, 506 nm direct logarithmically, 465 nm direct linearly and the difference between 506 and 465 nm as linear output. All measured intensities were compensated for darkcurrent and background illumination disturbances by means of differential recording against moments on which no light passed through the chopper. The slit for wavelength 506 nm was set at 0.2 mm, in order to be sure of the isobesticity of the channel. The lower light intensity of the second monochromator made it necessary to have the slit for 465 nm set at 1.0 mm. At the beginning of the experiment the intensities of the incident light as measured by the photomultiplier, were equalized, in such a way that the signal of the difference 506–465 nm was zero. This occurred at ventilation with 20% O_2 and 80% N_2. This level in the recordings was considered as baseline. During 3 minute periods the animal was ventilated with gasmixtures containing successively 100, 15, 10, 5, 5, 10, 15, 100% oxygen, interrupted by periods of 20% O_2 of sufficient length to reach a steady state. This took usually four minutes. Figure 16 gives an example of a recording of such an experiment in an albino rabbit, Figure 17 that in a pigmented rabbit.

DISCUSSION

In the registrations of the returning light from the eye, the influence of hypoxia is clearly recognisable. For ventilation with 5% O_2 the change in transmission of blood was more pronounced when the 5% period was repeated. For the other percentages of oxygen no significant differences were seen, whether the oxygen content was step by step increased or decreased. The experiments were reproducable within the limits of accuracy of the flow meters. As can be expected, the intensity of the returning light is much smaller in pigmented animals, than in albino's. Of the same incident light, about 8 times more returned from the albino rabbit eye. Table 3 gives the percentual changes in the intensity of the returning light of wavelength 465 nm during the various states of hypoxia and hyperoxia, compared with the intensity in a 20% O_2 period.

The figures as such are not very significant, especially as layer thickness and total Hb were not taken into account. They only serve to indicate the difference between albino and pigmented rabbits in response to various degrees of hypoxia, as measured with reflectometry. Although usually pigmented rabbits are not

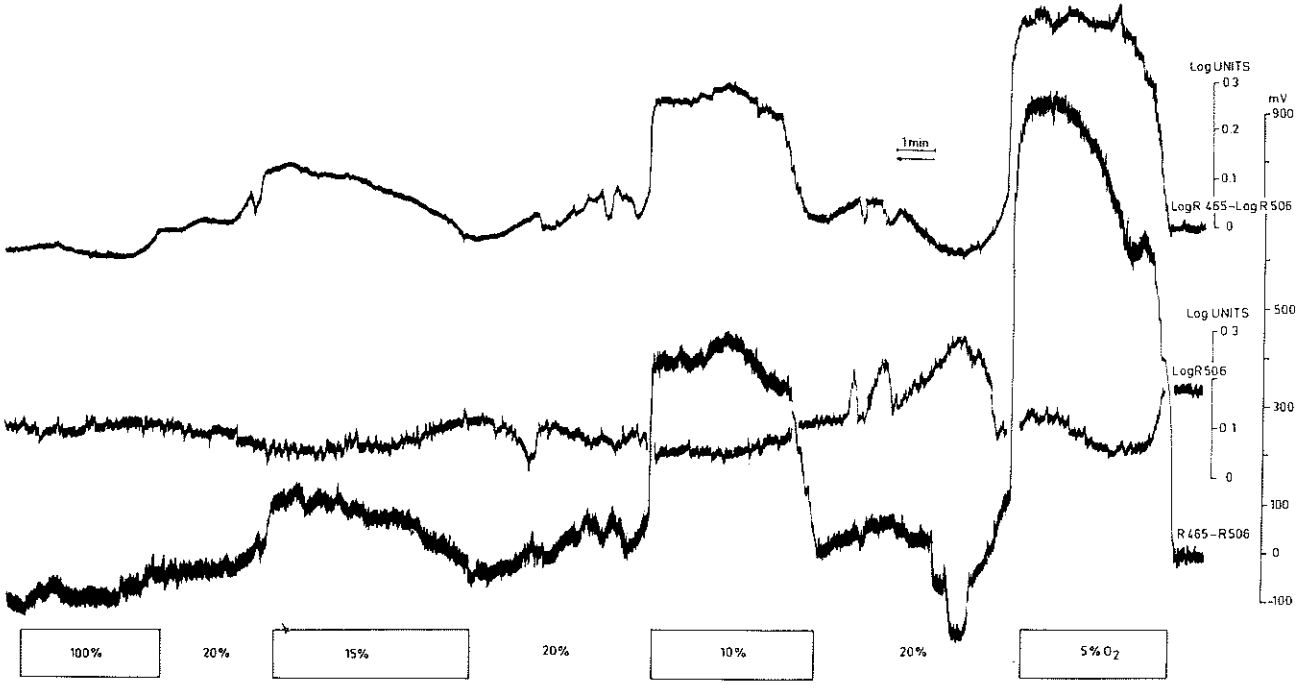


Fig. 16.

Energy returning from the eye of an albino rabbit, measured at wavelength 465 and 506 nm, recorded respectively as log R_{506} and $R_{465} - R_{506}$, during ventilation with a varying mixture of O_2 and N_2O. The values of log R_{465} — log R_{506} have been calculated from the registrations of R_{465} and log R_{506}.

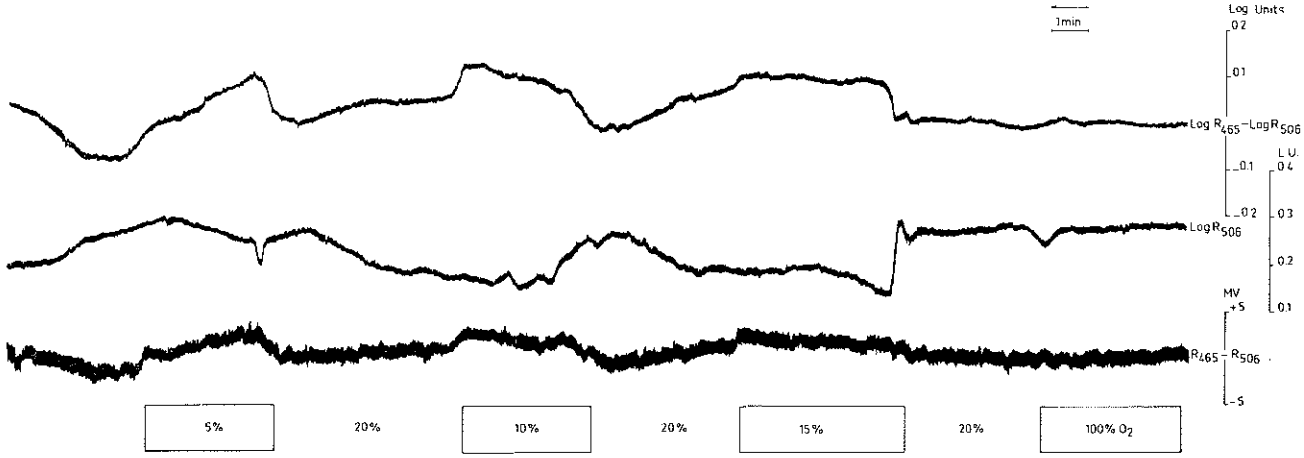


Fig. 17.

Energy returning from the eye of a pigmented rabbit, measured at wavelengths 465 nm and 506 nm, recorded respectively as log R_{506} and $R_{465} - R_{506}$, during ventilation with a varying mixture of O_2 and N_2O. The values of log R_{465} — log R_{506} have been calculated from the registrations of R_{465} and log R_{506}.

TABLE 3

Change in returning light energy, measured at 465 nm, expressed as percentage of the energy during ventilation with 20% oxygen.

percentage oxygen in gas mixture	energy change in albino rabbit	energy change in pigmented rabbit
15	+ 23	+ 9
10	+ 49	+ 13,5
5	+ 91	+ 15,5
100	— 9	— 3

over 2 kg bodyweight, they are a bit smaller then albino's, and so will be the size of their choroidal blood layer.

This however can only partially explain the extremely large difference. We take it for granted that both kinds of rabbits behave in the same way when exposed to hypoxia. The answer must lie, as discussed in another chapter, in the presence of the pigment in pigmented rabbits.

In both kinds of rabbits the log R_{506} curve showed an increase in density, during the hypoxic periods. These begin some time after the change in saturation, varying from 20 to 60 seconds. After changing the gasmixture back to 20% O_2 it took usually 1½ to 3 minutes before the density came to its original magnitude. In the registration in the albino rabbits in Figure 16, the fluctuation in density during the 5% period amounted to 0.128 log unit.

An increase in bloodlayer thickness of the order of 14 μ could account for this ($D = \varepsilon.c.d.$, where $\varepsilon = 5.1$ at 506 nm and the Hb was 8.9 mMol/L). The density difference points to a $d = 0.0028$ cm but this concerns a double passage.

In view of later findings on bloodlayer thickness (see chapter VIII) during periods of hypoxia we consider it unlikely that the whole of the density change should be attributed to an increase in thickness. A considerable part of the density change is to be ascribed to the stasis effect.

Although during ventilation with 100% oxygen a small change in $R_{detecting}$ is visible, changes in layer thickness or flow were not recognisable. Although the method can be used to visualize changes in blood oxygen saturation, no definite quantitative conclusions may be drawn.

1. It has been possible to register variations in SO_2 in the choroidal blood layer in rabbit eyes, without disturbing the physiology of the eye.

2. An influence of hypoxaemia on the bloodlayer thickness or the flow has been demonstrated.

3. A discrepancy was noticed between albino and pigmented rabbits in the magnitude of the change in the returning light during various states of hypoxia.

CHAPTER VII

THE INFLUENCE OF BLOOD FLOW ON TRANSMISSION

INTRODUCTION

In attempts to localize the position of isobestic wavelengths in vivo we noticed during transmission measurements in an arterial shunt in the common carotid artery of a rabbit that during hypoxaemia the transmission of blood decreased. For reasons to be clarified later, this phenomenon has been named stasis effect. In the literature it has been described (WEVER, 1954; MOOK, OSYPKA, STURM & WOOD, 1968; KLOSE et al., 1972; SCHMID-SCHÖNBEIN et al., 1972), but no satisfying explanation has been given. As much of the available information was inconsistent, we considered it opportune to investigate the stasis effect, especially as it might have some bearing on our experiments concerning oxygen saturation and bloodlayer thickness.

Experiment 1

It was necessary to establish that no qualitative or quantitative changes occurred in the flowing blood, which could explain a change in transmission.

Method

Four albino rabbits, between 2 and 3.5 kg, were induced, intubated and ventilated under positive pressure with a gas mixture of O_2 and N_2O. In the common carotid artery a shunt was prepared of about 50 cm length. In one place the polythene shunt was compressed between perspex sheets to form a cuvette of variable depth of 0.0–0.4 mm (Fig. 4). Transmission densitometry was performed with the apparatus described in detail in chapter II. The motor-driven mono-

chromator was used to scan a wavelength range from 600–500 nm (slit set at a half width of 1.2 nm). Transmitted light was brought to the photomultiplier by means of fiber optics. The diaphragmated lightbundle hit the cuvette at right angles. Registrations were made of the light transmitted through the shunt cuvette, at the end of a period of 3 minutes ventilation with alternately 100%, 5%, 100% and 5% O_2, complemented with N_2O.

Following each registration, a blood sample was taken out of the arterial blood in the shunt. Part of this sample was used to determine haematocrit and haemoglobin. The other part was oxygenated in a tonometer. While slowly rotating, the blood was flushed with 100% oxygen (1 liter/min.). After 6 minutes two haematocrit tubes were filled with this oxygenated blood. The remaining blood was mixed with powder of dithionite (10 mg on 1 ml blood). This chemical effects a complete reduction of the blood. Two haematocrit tubes were filled with this reduced blood. All haematocrit tubes were closed at the ends with plastic caps after introduction of a magnetic stirrer. The contents were haemolysed by immersion in methanol of —30° C. Freezing was repeated four times, each time for one minute duration. The haemolysed blood was thoroughly mixed and filled in a Neubauer leucocyte counting chamber. This chamber took the place of the shunt cuvette on the optical bench. Transmission measuring was performed immediately outside the ruled area of the counting chamber.

Results

The transmission curves made in the shunt during ventilation with 100% were nearly identical. So were both curves of the 10% O_2 period. When the 100% and 10% curves were projected over each other, there were no intersections at most of the usual wavelengths visible. The whole of the 10% O_2 curve showed a lower intensity than had been expected (Fig. 18). In contrast to this, the recordings of each oxygenated and reduced haemolysed sample, projected on each other, gave the usual intersections at the isobestic wavelengths 506, 522, 548, 569 and 585 nm. The results of the determination of Hb and Hc did not indicate any significant change.

Conclusion

A reversible and reproducible decrease in transmission of blood was registered in an arterial shunt during states of hypoxaemia. This was related to the lowered cardiac output and bloodpressure, which in turn resulted in a lower bloodflow in the shunt cuvette. It was demonstrated that no changes in the quality or quantity of the blood had occurred.

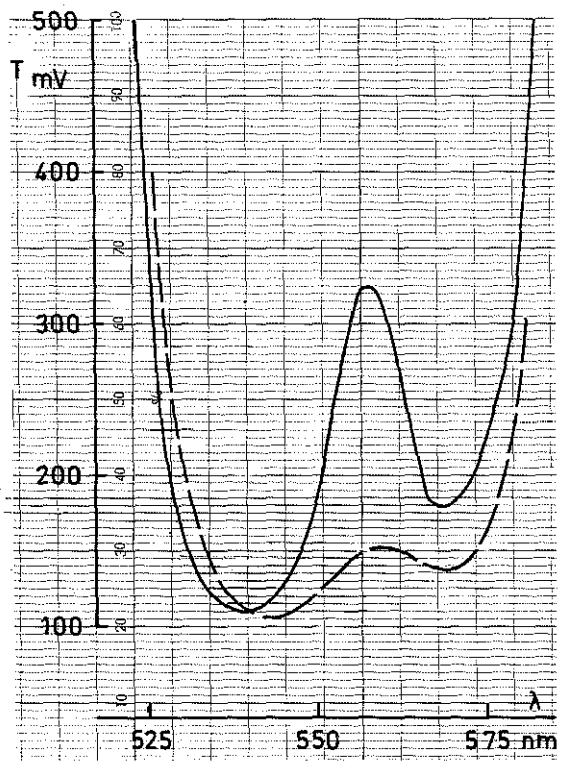


Fig. 18.
Energy of light transmitted through an arterial shunt cuvette over a wavelength range, recorded during ventilation with 100% O_2 (drawn line) and with 10% O_2 — 90% N_2O (interrupted line).

Experiment 2

It had to be demonstrated what the relation was between bloodflow and the stasis effect.

Method

In albino rabbits a shunt was prepared in the common carotid artery. Working in the direction of flow, there were mounted in this shunt, respectively an electromagnetic flowmeter (Transflow, type 600, Skalar, Delft), a shunt cuvette of variable depth, and a clamp enabling the shunt to be partially or totally closed. The arterial bloodpressure was measured in the femoral artery.

The clamp was fixed at the end in order to prevent any possible turbulent flow effects. Manipulating the clamp resulted in variations in flow. The depth of the cuvette was set at approximately 100 μ and not changed during the experiment. It was assumed that the total haemoglobin concentration remained constant. The intensity of the transmitted light of an isobestic wavelength was measured as a function of flow (Fig. 19).

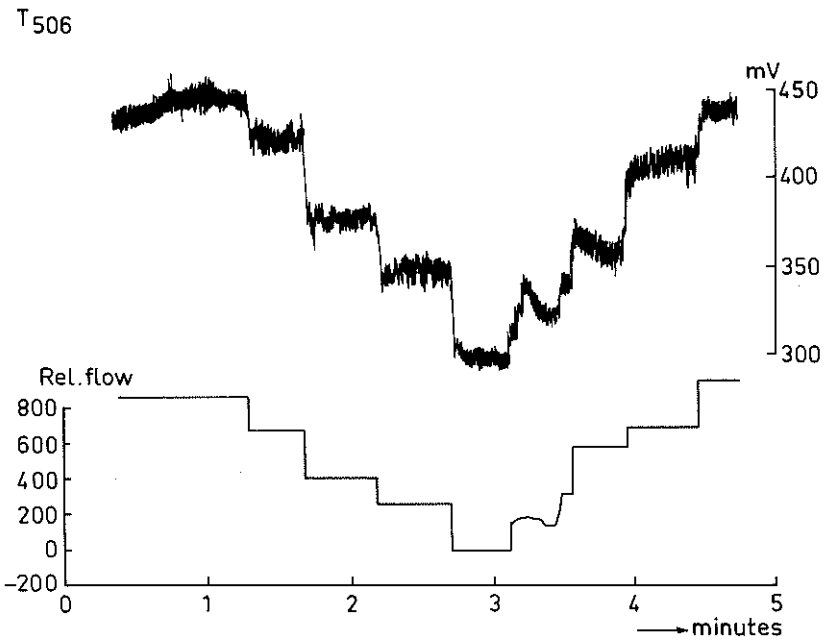


Fig. 19.
Registration of variations in relative flow and the effect thereof on the transmission through whole blood, measured at wavelength 506 nm in an arterial shunt in a rabbit.

The logarithms of the intensities of the transmitted light at an isobestic wavelength were plotted against the parallel registrations of relative flow measured with the electromagnetic flowmeter (Fig. 20).

In this experiment the light was guided from the cuvette to the photomultiplier by means of fiber optics, mounted immediately behind the cuvette. This means that diffused light could also be measured. When the experiment was repeated the magnitude of the stasis effect was different for a certain variation in flow, even if no change had occurred in the depth of the shunt cuvette.

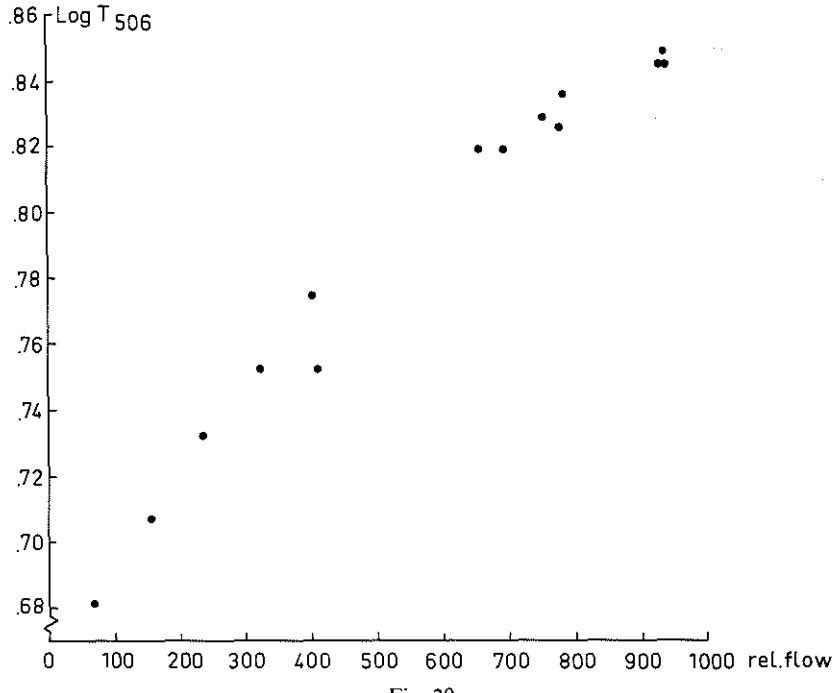


Fig. 20.

Relation between relative flow and the logarithmic recorded transmission through whole blood, measured at wavelength 506 nm in an arterial shunt in a rabbit.

Conclusion

An increase in bloodflow went parallel with an increase in transmission, or a lowered optical density. The density variations are larger for fluctuations in the low flow range.

Experiment 3

Dependence of wavelength in stasis effect on light transmission

In the same set-up as above, registrations were made of the light transmission during periods of high and low flow (Fig. 21). When for various wavelengths the relation between both light intensities was calculated, this proved to be a fairly constant ratio. The density of the blood at low flow and the density at high flow exhibit a constant difference at different wavelengths and therefore at varying ε (Fig. 22).

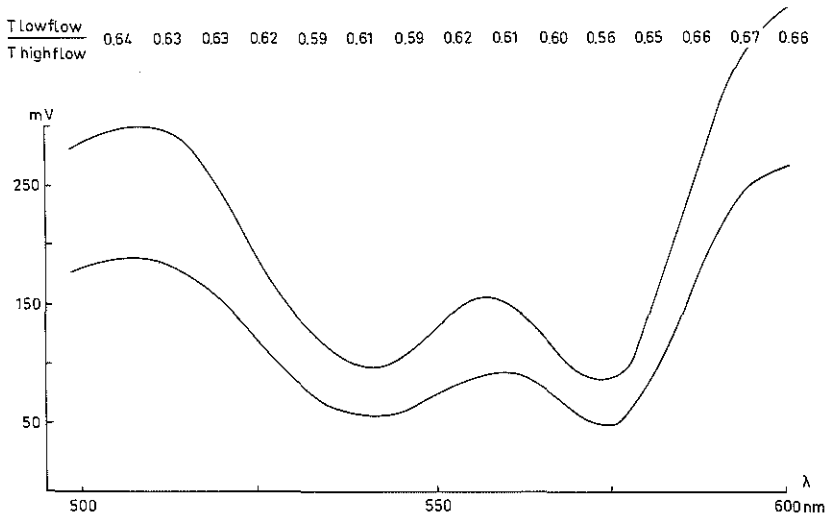


Fig. 21.

Registration of the energy of light transmitted through an arterial shunt during high (upper line) and low (lower line) bloodflow. The figures at the top indicate the relation between the energy at low and high flow measured at the wavelength at which each figure is written.

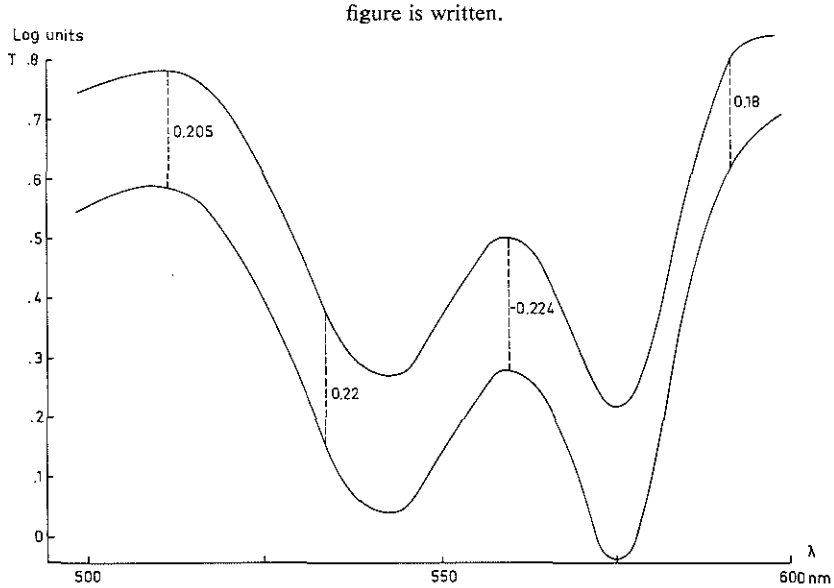


Fig. 22.

Logarithmic registration of the energy of light transmitted through an arterial shunt during high (upper line) and low (lower line) bloodflow. The figures indicate the difference in log units between both curves.

Conclusion

The stasis effect in a shunt cuvette is independent of the wavelength used to measure, and therefore independent from the ε-value.

Experiment 4

Dependence of oxygen saturation of the blood on the stasis effect

Method

In the same set-up as described above, registrations were made during ventilation with 100% and with 10% O_2, supplemented with N_2O, with high and low flow. As during hypoxic states the cardiac output was lower, the flow in the shunt cuvette was kept constant during the 100% and 10% period on information from the electromagnetic flowmeter by regulating the clamp. When for high flow as well as for low flow the 100% and 10% curves were projected on each other, the isobestic points showed up at the usual places, indicating that the isobestic ε-factors were independent of the state of oxygenation. Conversely when 100% and 10% curves were projected over each other, a similar decrease in density was revealed.

Conclusion

This experiment indicated that the stasis effect was independent of the state of oxygen saturation.

The conclusion of experiment 4 confirms that of experiment 3. A change in SO_2 implies only a change in the relation between the amount of Hb and HbO_2, while the sum of both components remains the same. When the stasis effect is ε-independent, no change could be possibly caused by SO_2 variations.

As mentioned before, the magnitude of the effects was not reproducible, probably due to minor changes in the set-up or the depth of the cuvette. Probably also the model of the cuvette was of influence on the results. No cuvette, whatever model or size, can ever be compared with a bloodvessel in the living eye. Therefore it was considered necessary to examine the influence of flow in the rabbit's eye.

We realized that this would be a difficult experiment. Any reduction in flow in the common carotid artery by clamping, even when the heterolateral carotis is ligated and the vertebral arteries as well, cannot give the certainty that the flow in the ophthalmic artery varies proportionately, to that in the carotis. Any variation in flow in the external and internal ophthalmic artery will influence

the flow in the choroid, which shows up in fundus reflectometry as an increase in bloodlayer thickness. The use of dyes as an indicator of layer thickness is impractical as within seconds the dye will begin to diffuse into the tissue of the choroid. It would seem impossible to combine in the same eye, reflectometry with flow measuring by means of labelled erythrocytes.

Therefore we used the following method for estimating stasis effect variations in the choroid of the albino rabbit's eye. It derives from an alternative method of calculating the stasis effect in a shunt cuvette, which therefore will be explained first.

Experiment 5

Alternative method to estimate variations in stasis effect measured in a shunt cuvette.

Method

In a shunt cuvette in an arterial shunt in a rabbit, ventilated with 100% O_2, the shunt was compressed completely. The registration, by a logarithmic amplifier, of the photocell response of the transmitted light served as bloodless value. The recording was made with the set-up as described in chapter II, using the motorised monochromator for the scanning of the range 600–500 nm, while the second monochromator, set at wavelength 506 nm, served as a control to monitor any unwanted variations in transmission during the short experiment. After obtaining a blank value, the cuvette was opened by degrees. At each step a registration was made of the logarithm of the transmitted light. (Fig. 23). The actual bloodlayer thickness could not be measured. To make figures 23 and 24 clearer, the layer thicknesses have been calculated as described in chapter VIII and serve only to identify the various lines and curves. For all isobestic wavelengths the densities of the bloodlayer were calculated by subtracting the log of the multiplier voltage from that of the bloodless value. The values found in this way were plotted against the ε-value at the particular wavelength, as these are known for haemolysed blood. Figure 24 demonstrates how there exists a linear relationship but not proportionality between the ε-value and the density.

Conclusion

From the above we thought it acceptable to take as a starting point that Lambert-Beer's law is valid, anyway for the thin layers in as far as it reflects the influence of layer thickness. The distance on the ordinate from the point of intersection

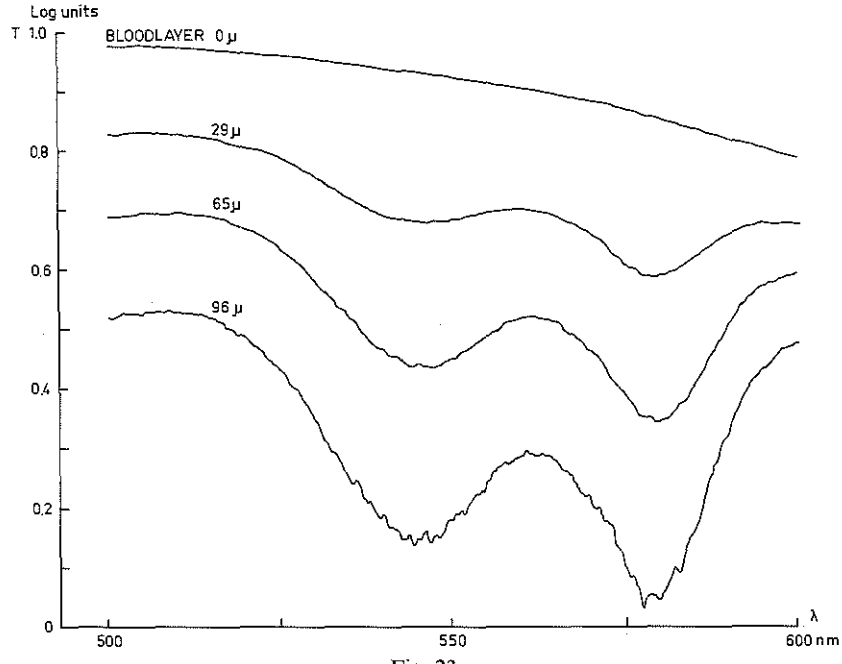


Fig. 23.

Logarithm of the energy of light transmitted through an arterial shunt (log T) registered over a wavelength range in a cuvette with variable depth.

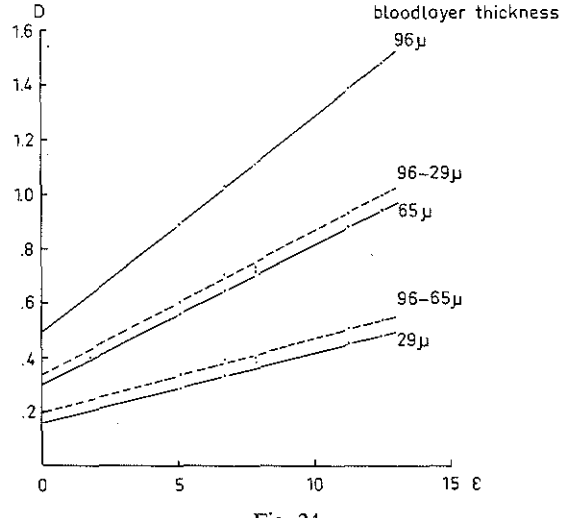


Fig. 24.

Relation between the optical density at various layer thicknesses (drawn line) or between the difference in density at various layer thicknesses (interrupted line) and the corresponding ε-value at some isobestic wavelengths, as measured in the registration presented in Figure 23.

to the centre represents the increase in density due to flow. When the cuvette is made wider, with a constant flow, the blood velocity decreases and the stasis effect becomes consequently larger.

Even when no bloodless values are available it is possible to have some information on the stasis effect. When the differences in optical density between the various layers are plotted against the ε-values of the wavelength where they were measured, the distance from where this line intersects with the ordinate to the zero point represents the difference in flow effect between the two situations in which the curves were registered.

Experiment 6
Registration of variations of the stasis effect in the albino rabbit eye

Method

Unless the choroid is pressed empty of blood by applying intraocular pressures of over 80 mm Hg, or by perfusing the eye at the end of an experiment, no bloodless values are available. Basically experiment 5 was repeated. One monochromator was set at 506 nm to check on unwanted variations during the recording, while the motorized monochromator scanned the range 600–500 nm. The arterial pressure was measured in a catheter in the femoral artery. The intraocular pressure was regulated by means of a needle in the anterior chamber connected with a saline reservoir, to which heparin was added. The height of the reservoir was adjustable. The actual pressure in the anterior chamber was measured by means of a second needle connected with a pressure transducer (Statham P23 Db)

Results

Figure 25 gives the combined recordings of two states of an albino rabbit eye under different intraocular pressures. In Figure 26 the difference between each pair of log values are plotted against the corresponding ε-values. It would not do to use other than isobestic wavelengths, even if the animal was ventilated with 100% O_2, because during periods of low flow the S_{O_2} certainly would become lower than 100%, as the oxygen consumption of the tissues would continue. Again an approximately straight line is produced, which intersects with the ordinate at 0.32. It indicates that when the intraocular pressure is decreased from 27 till 7 mm Hg, while the arterial pressure remains the same, the stasis effect increases, i.e. the light transmission of the blood decreases. The difference equals a density of 0.32 log units.

This change, however, points at an increase in optical density, when the intra-

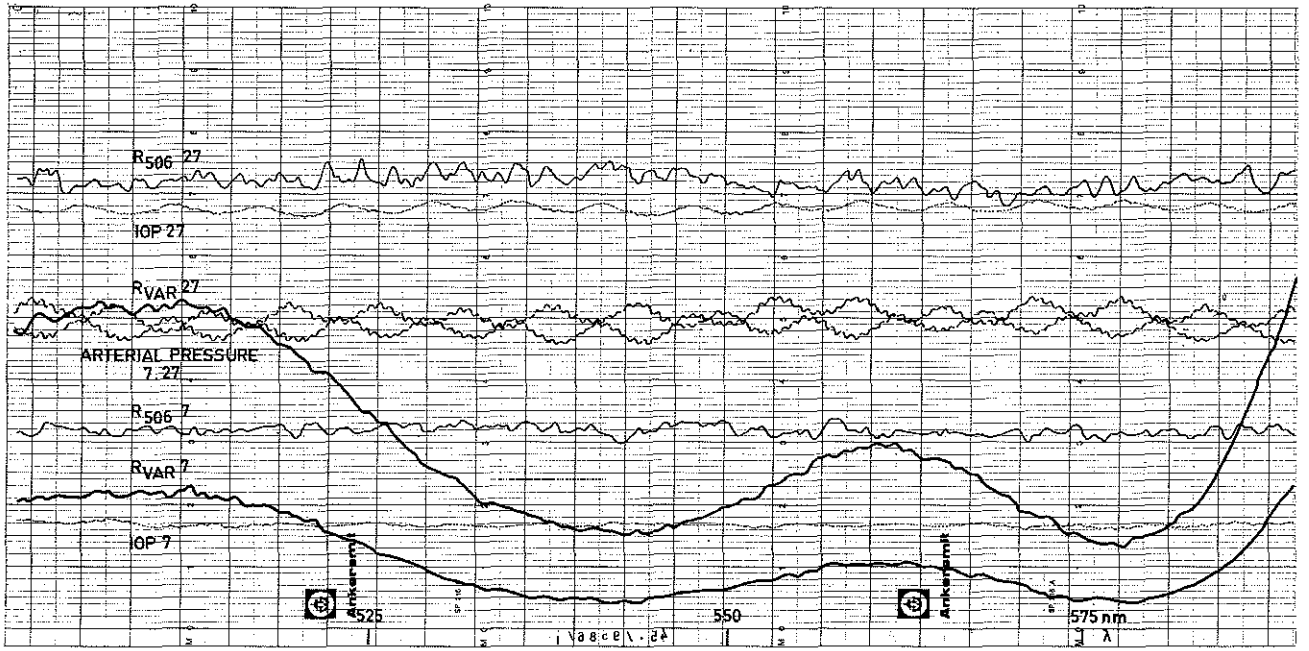


Fig. 25.

Combined recordings of the energy of light returning from the fundus of an albino rabbit eye at an intraocular pressure of respectively 27 and 7 mm Hg. Registrations were made over a wavelength range (R_{var} 27 and R_{var} 7; thick drawn lines) and of a fixed wavelength (R_{506} 27 and R_{506} 7; thin drawn lines) The pressure in the femoral artery was recorded (art. pres. 27 and art. pres. 7; interrupted lines) as well as the intra-ocular pressure (I.O.P.7 and I.O.P. 27; dotted lines).

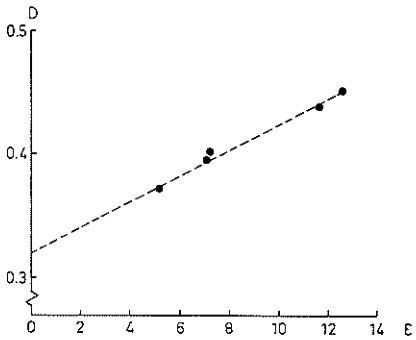


Fig. 26.

Relation between the difference in optical density of the choroidal bloodlayer during intraocular pressure of respectively 7 and 27 mm Hg, as registered in Figure 25, and the corresponding ε-values.

ocular pressure becomes lower. One may suppose on evidence presented by BILL (1962) and many others that an increased perfusion pressure results in an increased choroidal bloodflow. In this experiment a decrease in flow results in a decrease of optical density, i.e. the reverse of what had been found in the experiment in the shunt cuvette. In order to assess the stasis effect, expressed in log units, it is sufficient to compare the densities at two isobestic wavelengths only, for example at 506 and at 548 nm where a fair difference exists in ε-value. The change in optical density caused by a variation in stasis effect (named ΔF) is assumed to be the same for both wavelengths. Not so the change in transmission due to a variation in layer thickness which, as far as thin bloodlayers are concerned, obeys Beer's law.

The following equations can be deduced.

$$\Delta D_{506} = \Delta F + 5.11 \times \Delta d \times \text{conc.}$$

$$\Delta D_{548} = \Delta F + 12.51 \times \Delta d \times \text{conc.}$$

Δd, the variation in layer thickness in cm, ΔF, the variation in stasis effect in log units and ΔD, the variation in density in log units, are all positive when an increase in layer thickness or (apparent) density is involved.

Combining the two equations by subtraction results in:

$$\Delta D_{548} - \Delta D_{506} = (12.51 - 5.11)\, \Delta d \times \text{conc.}$$

The concentration, being the total haemoglobin, can be measured in the peripheral blood. Consequently Δd and ΔF can be solved.

$$\Delta d = \frac{\Delta D_{548} - \Delta D_{506}}{7.4 \times \text{conc.}}$$

$$\Delta F = 1.69\, \Delta D_{506} - 0.69\, \Delta D_{548}$$

Application

In albino rabbit eyes, where the intraocular pressure could be changed and measured as desired, the intensity of the returning light was registered in various situations.

Result

When the flow effect was calculated as described earlier, and plotted against the intraocular pressure, curves were obtained as shown in Figure 27. In other experiments with different rabbits the shape of this curve was nearly identical, but not the magnitude of the effect.

The shape of the curve in Figure 27 basically resembles the bloodflow/I.O.P. curve as presented by WEITER et al. (1973). Although the stasis effect probably has a relation to the choroidal bloodflow, it is by no means clear what this is. Equally a relation to blood velocity and vascular resistance could be expected. As the origin of the phenomenon is not known, no conclusion can be drawn.

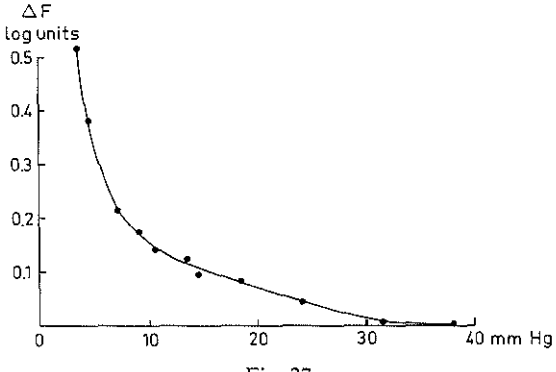


Fig. 27.
Relation between the stasis effect and intraocular pressure, measured in an albino rabbit eye.

Comment

The influence of bloodflow on the transmission was described as early as 1935 (KRAMER, 1935). WEVER (1954) explained the stasis effect on light transmission as a shift in the erythrocyte concentration, i.e. an increase in the axial stream and a decrease in the marginal zone. The blood velocity gradient does not only cause a considerable rotatory movement of the blood corpuscles, it also has an influence on static pressure in the vessels. Near the wall the blood velocity is zero. The

static pressure is here consequently maximal, and decreases towards the axial stream where the velocity is greatest. The stasis effect therefore is explained by WEVER as a deviation from Lambert-Beer's law. Although the erythrocytes certainly do concentrate in the midstream zone of the vessels and are nearly absent in the marginal zone, as is demonstrated in fluorescein angiography (WISE, DOLLERY & HENKIND, 1971) it is unlikely that the effect would be related therewith. The effect in that case would be dependent of the ε-value. One would expect the flow effect to approach zero, when the ε-value becomes very low as for blood at 600 nanometer wavelength. This, however, is certainly not the case.

This phenomenon has also been recognised in reflection photometry. During the development of a reflection cuvette oximeter (MOOK & ZIJLSTRA, 1957; ZIJLSTRA, 1958; BOSSINA, MOOK & ZIJLSTRA, 1960), it was observed that in vitro the light reflection of blood depends on the degree of rouleaux formation of the erythrocytes. The rate of rouleaux formation decreases with decreasing haemoglobin concentration and increases with the presence of macromolecular substances (e.g. Dextran) (JANSONIUS & ZIJLSTRA, 1965).

In vivo dextran 250 (mol. weight 250.000) proved to induce red cell aggregation in a rabbit ear chamber, while dextran 40 (Rheomacrodex^{R}) had no direct influence on the red cell aggregation. Rheomacrodex however had an apparent effect on the cell dispersion by increasing the speed of circulation. In dog and man Rheomacrodex appears to have a significant effect against the aggregating tendencies (ENGESET, STALKER & MATHESON, 1967). In vitro experiments (MOOK, OSYPKA, STURM & WOOD, 1968) not only demonstrated the effect of flow changes, but also the effect of a total cessation of the flow, which resulted in another decrease in transmission. This last effect, the measurement of which was described as syllectometry (BRINKMAN, ZIJLSTRA & JANSONIUS, 1963) was shown to be dependent on the fibrinogen content of the blood. Furthermore large variations were found for the blood of various species. It was explained by the formation of rouleaux, aggregations of erythrocytes. The process was reversible. It was dependent on erythrocyte concentration and on composition, viscosity, pH and temperature of the suspension medium.

Using a cone-plate chamber that was transilluminated under shear, KLOSE (KLOSE et al., 1972; SCHMID-SCHÖNBEIN et al., 1972) studied the photometric behaviour of whole blood under various shear rates. A biphasic pattern was seen. Starting from stasis or a low shear rate the light transmission first decreased as a function of shear, reached a minimum at a shear rate between 50 and 100 sec^{-1} and then increased with each increment of shear. The investigators had the opportunity to examine the content of the cone-plate chamber under a microscope.

Dispersion of aggregates corresponded with falling light transmission, while cell alignment corresponded with a rising transmission. They noted an absence of the effect when the shear experiments were repeated with human rigidified red cells. The stasis effect for normal erythrocytes also varies with the species. Whether registered at 400, 500, 600 and 700 nm wavelength, for a certain type of blood the same variation in shear rate resulted in the same variation in transmission. They remarked the inconsistency between their results and those of MOOK (MOOK et al., 1965) where the direction of the flow variation was just the opposite. It was evaluated as stasis effect giving more transmission and less reflection, and vice versa.

In short the existing literature on this subject is if not confusing certainly not consistent. All investigators demonstrated, whether with transmitted or reflected light, an influence of blood velocity or rate of shear on the light transmission. The reason why this effect however is of a different direction in different experiments is not clear, nor is the origin of the stasis effect itself.

Diffusion of the light certainly has influence on the stasis effect. In transmission experiments this could be demonstrated as no stasis effect was producible in haemolysed blood. When however the incident light consisted merely of diffused light as when a piece of thin, white paper was placed immediately in front of the cuvette, the effect was still present, but was considerably reduced. This is consistent with our experience that the opening of the photomultiplier is also of influence. When in transmission measurements in a cuvette the usual 4 mm diameter light guide, which was mounted immediately behind the cuvette, was replaced by a 1 mm opening in the photomultiplier window, placed 50 cm behind the cuvette, the stasis effect became somewhat smaller. As mentioned in the comment on experiment 6, the relation between bloodflow or blood velocity and the stasis effect compared with the shunt experiments, was found to be apparently reversed in the rabbit eye. The reason therefore cannot be explained.

The experiments of SCHMID-SCHÖNBEIN (1972) as well as of JANSONIUS & ZIJLSTRA (1965) made clear respectively that the stasis effect and the rouleaux formation are influenced by the same factors that are known to determine the viscosity.

The viscosity of blood, being a non-Newtonian fluid, is linear proportional to the negative logarithm of the rate of shear as well as to the negative logarithm of the temperature (BEGG, 1968).

The most important factors that determine the viscosity are the haematocrit value, the viscosity of plasma to which fibrinogen, albumin and the globulines contribute, the uniform dispersion of the red cells, the intrinsic viscosity of the

red cells (BEGG, 1968) and the ratio or eythrocyte diameter and vessel diameter (SKALAK, CHEN & CHIEN, 1972).

We investigated the impact of variations in the blood concentration on the stasis effect.

From blood from one and the same person three samples were prepared with Hb respectively 9.1, 5.6 and 2.6 mMol/L, by diluting with a varying amount of human plasma. The variable cuvette was clamped round the polythene tubing of an ordinary bloodgiving set and the cuvette depth set at approximately 0.3 mm. The cuvette was mounted at the end of the optical bench. The monochromator was set at 506 nm, the slits at 0.2 mm. The room temperature was kept constant at 26° C. In turn each of the three samples was perfused through the cuvette under pressures that were varied by changing the height of the reservoir. Registrations were made of the logarithm of the transmitted light. The more blood passed per second through the cuvette, the more light was transmitted. When the flow was made zero the density increased to a maximum. The changes in density were comparatively large between no flow and very low flow periods. As the amount of blood that flowed out of the lower end of the tube at a certain height of the reservoir was not the same for each blood

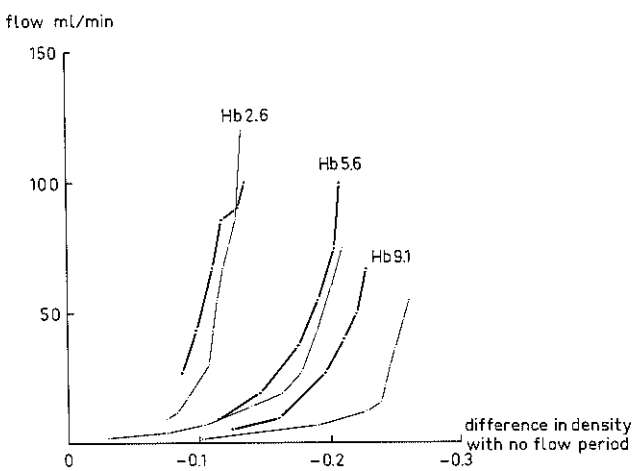


Fig. 28.

Relation between bloodflow and the difference in optical density of the bloodlayer, between periods of flow and stasis, measured in vitro with human blood in a shunt cuvette set at a depth of approx. 200 μ (thin lines) and 300 μ (fat lines) for blood of 2.6 mMol Hb/L, 5.6 mMol Hb/L and 9.1 mMol Hb/L.

sample, the outflow of blood was measured in ml/min for each blood sample at all pressures of the reservoir that were applied.

The experiment was repeated with the shunt cuvette set at approximately 0.2 mm depth. The results of the flow measurements and the density changes were reproducible within 5 %. In Figure 28 the various flows are plotted against the changes in density compared with no-flow periods. The results agree with the conclusions of SKALAK et al. (1972) who reported that the increase in Hc only results in a linear increase of viscosity for lower Hc. For higher Hc the increase in viscosity is less than expected. Furthermore we noticed that a narrower cuvette resulted in an increase of the stasis effect.

Although no concrete evidence can be produced, the foregoing nevertheless suggests that the blood viscosity is an important parameter for the stasis effect. In view of this suggestion, it is very questionable whether it is realistic to compare a shunt cuvette with a comparatively large lumen and smooth walls, with the complicated, microscopic meshwork of arterioles and capillaries in the choroid.

CHAPTER VIII

AN APPROXIMATIVE METHOD OF MEASURING THE LAYER THICKNESS AND OXYGEN SATURATION OF THE BLOOD IN THE VASCULAR LAYER OF UNPIGMENTED EYES.

INTRODUCTION

Oxygen saturation and bloodlayer thickness in the vascular layer of the eye might be valuable parameters in the study of pathogenesis and treatment of vascular pathology. So far reports have been published about the arterio-venous oxygen difference in human retinal vessels (HICKAM & FRAZER, 1966) and in the uveal tract in dogs (ELGIN, 1964). TROKEL (1965) described a method for measuring the thickness of the bloodlayer in the choriod in albino rabbits. A more precise system for bloodlayer measurements was developed by PEREGRIN & DODT (1969). In both methods the destruction of the eye was a necessary part of the measurement. SVERAK & MALESSA (1970) published an elegant method, which however was dependent on a standard bloodless eye.

This paper presents a method which enables measurement of the bloodlayer thickness, combined with that of the blood oxygen saturation, both in the vascular layer of the unpigmented eye, without the use of a separate blank value, and without disturbing the physiology.

METHOD

In fundus densitometry much of the incident light gets lost. For several reasons (for instance the amount of reflection against cornea and lens etc., scatter, absorption by the pigment and blood in the fundus, and stray light) it is virtually impossible to predict in an experiment which part of the incident light energy will come back through the pupil. More information can be obtained by com-

paring the spectral characteristics of the incident and out-going light. By 'characteristic' we mean the interrelationship of the energy of the light at each wavelength over a certain range. When the logarithms of the energy of the spectral components of a certain light are plotted against wavelength, a decrease or increase of the total intensity of the light shows up in the curve as a shift downwards or upwards of the whole curve, without the form being changed. The light of a Xenon arc has a certain spectral characteristic, the recorded registration of which depends on the type of photomultiplier used. Any loss for reasons mentioned above will only result in a lowering of this curve, as in Figure 29, towards the position of curve Xe_2.

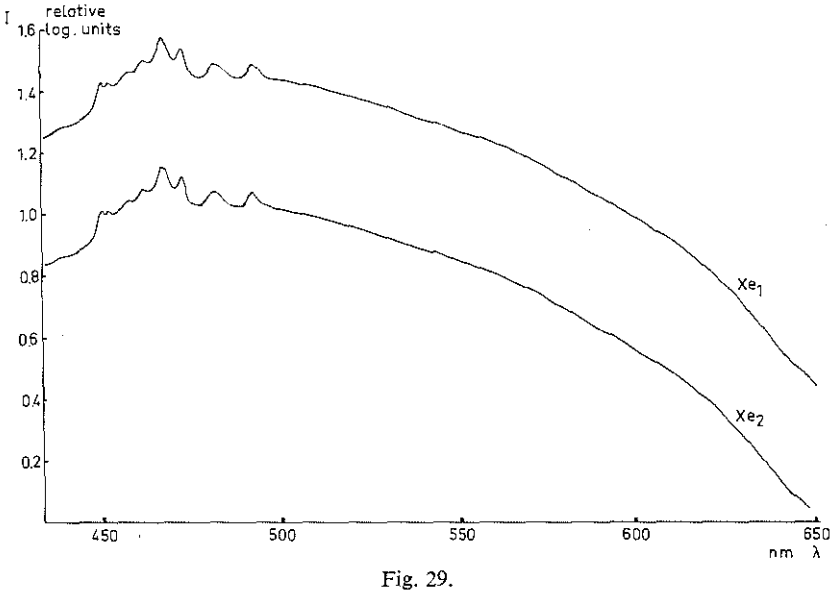


Fig. 29.
Spectral characteristics of a Xenon arc, between 450 and 650 nm, registered with a half intensity band width of 1.2 nm, with two different intensities.

Part of this loss may be accounted for by light transmitted through the sclera and consequently lost for measurement. The media of the eye can be considered as filters. It is irrelevant in what sequence the light passes through them.

Theoretically the interchangeability of filters is limited to non-diffusing filters. Although lens and cornea certainly do diffuse, the amount of diffused light is not important. Moreover most of the diffused light will not reach the photomultiplier. Therefore one may use a model, where the light traverses first all

the non-blood media. Keeping in mind the supposition that light traverses the media, is reflected against the shining white surface of the sclera, anyway partly, and passes the media a second time (OOSTERHUIS, BAKKER & VAN DEN BERGE, 1970), then Figure 30 gives a schematic drawing of the situation. As all the non-blood containing media possess an unvariable density, the spectral composition of the light will now be different, but still independent of blood. We named the light with this quality 'B' (Fig. 31).

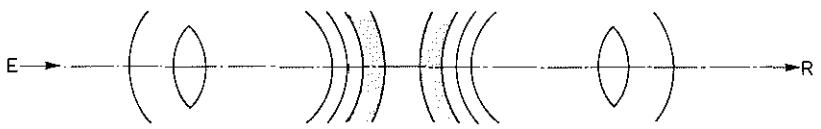


Fig. 30.
Scheme of the passage of light through the media of the eye.

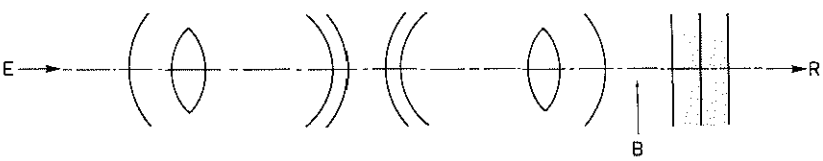


Fig. 31.
Scheme of the passage of light through the eye when the non-blood media are traversed first.

In principle this should have the same spectral composition as the light that is reflected by a bloodless eye. In practice however, a bled animal usually has some blood left in the vessels of the choroid. In order to assess the impact of the various ocular media on the characteristics of the emission spectre of the Xenon arc, experiments were done to measure the optical density of various separate media. A detailed account of this will be given in a separate chapter.

Summarizing these investigations we may say that for lens, cornea and vitreous, as well as for retina without pigment epithelium, and choroid of unpigmented animals, the density spectrum is represented by a nearly horizontal, more or less straight line (see for details chapter IX).

Pigmented choroid and retinal pigment epithelium have a higher density for shorter wavelength light. Still the density spectrum is a nearly straight line although its slope depended on the degree of pigmentation.

Figure 32 schematizes how the incident light (log E) after passage through the non-blood media is reduced to log B. When this light in turn passes the blood-layer only light with spectral composition log R is left, which is what can be recorded. As the degree of pigmentation is not to be assessed in vivo, and neither therefore the spectral composition of light B, this analysis does not bring us much further in resolving the problem of measuring bloodlayer thickness and oxygen saturation. However, the passage of the light through the blood layer in the eye depends on the varying light absorption at the various wavelengths,

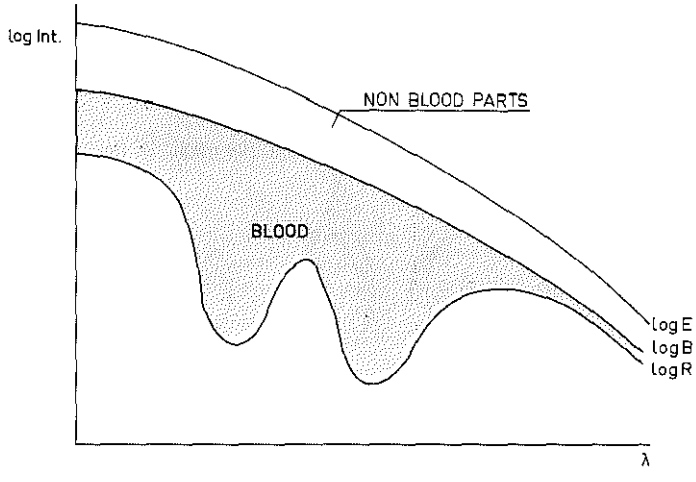


Fig. 32.
Scheme indicating that the energy of light returning from the eye is what is left from the energy of the incident light (log E) after passage through successively the non-blood media (remains log B) and the bloodlayer (remains log R).

which in its turn, is related to the molecular extinction coefficient ε of the constituents of the blood. The main constituents are haemoglobin (Hb), oxyhaemoglobin (HbO_2), carboxyhaemoglobin (HbCO), hemiglobin (Hi) and other haemoglobin derivatives, furthermore haemoglobin degeneration products, other compounds and traces of free iron (VAN KAMPEN & ZIJLSTRA, 1968). For our purpose we will consider Hb and HbO_2, as the other factors play a minor role. The extinction coefficient spectra of both are known (Fig. 33) (for details see separate chapter). In the red-green range wavelengths 522, 548, 569 and 585[5] were found to be isobestic, while 537[5], 560 and 577 showed maximal difference in extinction coefficient for Hb and HbO_2.

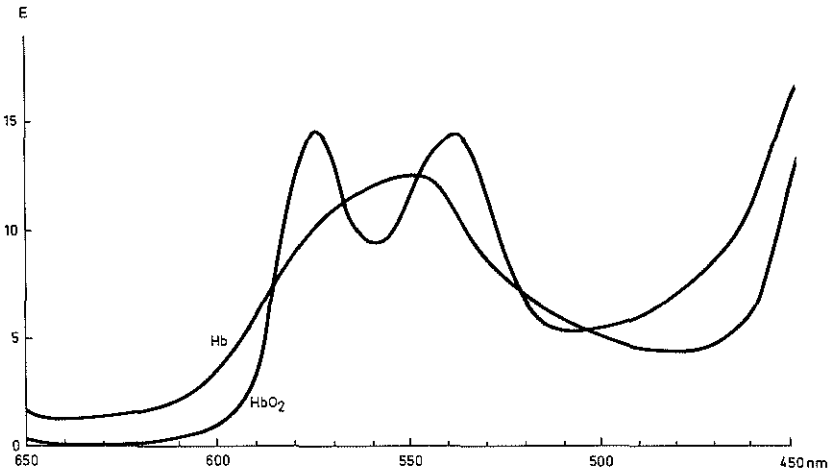


Fig. 33.
ε/λ curve for Hb and HbO_2.

In Figure 34 hypothetic log B curve, in which all proportional factors implied in the optics of the system are accounted for, is drawn together with a log R curve as can be measured in the intact eye. In view of the density spectra for the various eye media, we assumed the log B curve to be straight between 522 and

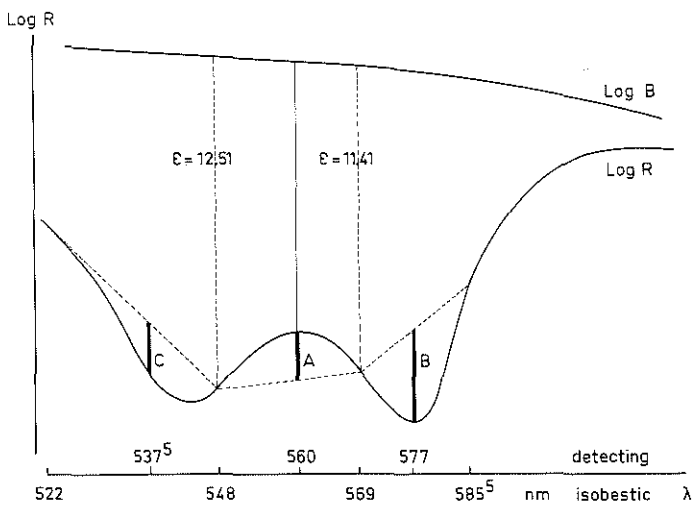


Fig. 34.
Scheme, indicating the calculation of bloodlayer thickness and oxygen saturation.

548 nm, between 548 and 569 nm and between 569 and 585[5] nm, all of them isobestic wavelengths. At these wavelengths the distances between the log B and log R curve represent the respective densities, which can be expressed as a function of layer thickness (d), concentration (c) and extinction coefficient (ε). The problem of deviations from the haemolysed blood values will be neglected. As an example we will do the calculation at 560 nm. When a line is drawn through the log R values for both isobestic wavelengths, 548 and 569 nm, then on this line at 560 nm which is a wavelength of maximal difference between the extinction coefficient for reduced and oxygenated blood, the distance to the log B curve at 560 nm can be expressed

$$\begin{aligned} \text{line}_{560} \text{ to log } B_{560} &= \text{d.c. } (\varepsilon_{548} - \frac{12}{21} \times \varepsilon_{548} - \varepsilon_{569}) \\ &= \text{d.c. } 11{,}87 \end{aligned} \tag{1}$$

As the saturation is unknown, the blood density at 560 nm can be given when the ε is expressed as the ε for reduced blood, decreased with part of the difference $\varepsilon_{\substack{560 \\ \text{red.}}} - \varepsilon_{\substack{560 \\ \text{oxyg.}}}$ depending on the saturation (S). S has to be expressed as a fraction.

$$\begin{aligned} \text{blood } D_{560} &= \text{d.c} \left[\varepsilon_{\substack{560 \\ \text{red.}}} - S \left(\varepsilon_{\substack{560 \\ \text{red.}}} - \varepsilon_{\substack{560 \\ \text{oxyg.}}} \right) \right] \\ &= \text{d.c } [12.76 - S (12.76 - 8.91)] \\ &= \text{d.c } (12.76 - 3.85 \, . \, S) \end{aligned} \tag{2}$$

The difference (A) between the theoretical blood density on the line and the real blood density, both at 560 nm, can be measured. Subtracting (2) from (1) gives

$$\begin{aligned} A &= \text{d.c } [11.87 - (12.76 - 3.85 \, . \, S)] \\ &= \text{d.c } (3.85 \, . \, S - 0.89) \end{aligned} \tag{3}$$

The same procedure can be repeated for wavelength 577 and 537.5 nm, when the measurable differences between the real blood density and the theoretical density on the line connecting the neighbouring isobestic points are named respectively B and C.

$$\begin{aligned} B &= \text{d.c } (9.47 + 5.31 \, S - 9.73) \\ &= \text{d.c } (5.31 \, S - 0.26) \end{aligned} \tag{4}$$

$$\begin{aligned} C &= \text{d.c } (9.75 + 4.01 \, S - 10.23) \\ &= \text{d.c } (4.01 \, . \, S - 0.48) \end{aligned} \tag{5}$$

By solving from respectively (3) and (4), (4) and (5) and (3) and (5), either S, or d.c, for each 3 equations can be obtained.

$$S_{AB} = \frac{0.89\,B - 0.26\,A}{3.85\,B - 5.31\,A}$$

$$S_{AC} = \frac{0.89\,C - 0.48\,A}{3.85\,C - 4.01\,A}$$

$$S_{BC} = \frac{0.48\,B - 0.26\,C}{4.01\,B - 5.31\,C}$$

$$cd_{AB} = 1.03\,B - 1.43\,A$$

$$cd_{AC} = 2.22\,C - 2.31\,A$$

$$cd_{BC} = 2.66\,B - 3.51\,C$$

RESULTS

To see whether the method can be used, first a check was done in vitro. 5 blood-samples were oxygenated by flushing with 100% oxygen. Transmission measurement was done in a leucocyte counting chamber of 0.01 cm depth. The results are given in Table 4.

The saturation calculation from A and B is the only one that gives a more or less acceptable result. Therefore no further use was made of calculations from A–C and B–C. To control the reliability of the formulas in vivo, a 3½ kg albino

TABLE 4

Results of the calculation of SO_2 and bloodlayer thickness in a cuvette of 100 μ thickness from the measurements of A, B and C, in 4 bloodsamples with known Hb concentration.

sample no	known Hb mMol/L.	known SO_2	S_{AB}	S_{AC}	S_{BC}	mean calc. SO_2	$d_{AB}(\mu)$	$d_{AC}(\mu)$	$d_{BC}(\mu)$	mean (μ)
1	9.2	1.0	1.07	1.22	0.87	1.05	82	72	109	87
		0	0.04	–0.02	0.04	0.02	100	76	138	104
2	8.5	1.0	0.84	–	0.34	–	91	–	293	–
		0	0.04	0.01	0.04	0.03	92	79	108	93
3	8.7	1.0	1.02	1.47	0.65	1.05	55	36	94	62
		0	0.03	0.01	0.03	0.02	116	105	131	117
4	9.1	1.0	1.06	31.89	0.44	–	66	2	177	81
		0	0.03	–0.01	0.03	0.02	103	88	125	105

rabbit was anaesthetized with Hypnorm on 6 consecutive days. The animal was given a retrobulbar injection with procain 4% 2 ml in order to obtain akinesia. The pupil was dilated with Phenylephrine 10% and Mydriaticum Roche. Each day three registrations were made with fundus reflectometry, whereby the intensity of the returning light was registered.

On day 2, 4, 5 and 6 100% oxygen was given by means of a rather badly fitting rubber mask.

The results of the SO_2 calculations are given in Table 5.

TABLE 5

The SO_2 in an albino rabbit's choroidal bloodlayer, calculated from the mean values for A and B, as measured in three registrations on six consecutive days. On day 2, 4, 5 and 6, the animal was administered 100% O_2.

day	1	2	3	4	5	6
S_{AB}	1.02	0.61	0.92	1.23	1.21	2.14

It was useless to calculate the bloodlayer thickness as no usable figures were obtained.

Any small error in A, B or C has a large influence on the outcome of the SO_2. This is understandable especially for S_{AC}, where when C was measured slightly smaller than usual, for whatever reason, the denominator 3.85 C — 4.01 A became very small, if not negative. The result consequently also became extremely large or negative. For S_{BC} the problem was the same. For S_{AB} the larger difference between A and B allowed some fluctuation without resulting in absolute nonsense.

In view of the very unsatisfactory results it was preferable to take the risk of increasing the error by widening the span of the connecting line, in order to obtain a more reliable outcome. Therefore we connected the log R values at the isobestic wavelengths of 522 and 569 nm with a straight line (see Fig. 35). On this line at 548 nm the D = c.d. 9.38. By subtracting this from the real blood density at 548 nm i.e. 12.51 × c.d the measurable piece D (see Fig. 35) can be expressed as

$$D = \text{c.d. } 3.13. \quad (6)$$

Out of the now known layer thickness the SO_2 can be calculated at any detecting wavelength, using A, B or C.

For example

$$B = \text{c.d. } (5.31 \cdot s - 0.26) \quad (4)$$

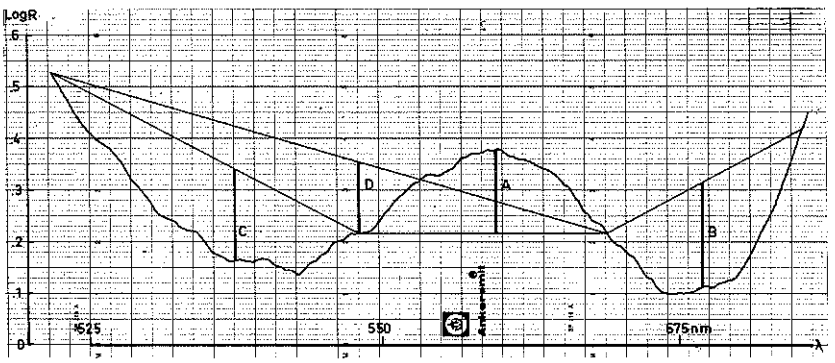


Fig. 35.
Example of a logarithmic registration of the energy of the returning light from an albino rabbit eye, over the range 522 to 586 nm, in which the auxillary lines, necessary to calculate d and SO_2 are indicated.

From (6) and (4) we can arrive at the saturation:

$$S = \frac{B}{D} \times 0.61 + 0.05$$

In the same way the saturation can be calculated from A and D, and C and D.

$$S_{AD} = 0.813 \times \frac{A}{D} + 0.23$$

$$S_{CD} = 0.781 \times \frac{C}{D} + 0.12$$

Applied on the same bloodsamples as treated in Table 4 this gave a better result (see Table 6).

When we reconsidered the registrations made in one albino rabbit on 6 consecutive days, the results were more consistent than before. Also the results of the calculation of the bloodlayer thickness proved to be more reliable and came to rather plausible values. These values lie consistently below those found in the literature (PEREGRIN & DODT, 1969; SVERAK & MALESSA, 1972). which may be due to the elimination of the stasis effect. The results are tabulated in Table 7.

The differences between the saturations as calculated in the transmission measurements in a cuvette and in the rabbits' eyes can be explained by the fact that the formulas are based on rather theoretical suppositions, the application of which does not take into account minor changes in diffusion and reflection due to the different geometry of the set-up.

TABLE 6

Calculation of the SO_2 and bloodlayer thickness from the measurements of A, B, C and D in 4 bloodsamples with known Hb concentration and a layer thickness of 100 μ.

sample no	known SO_2	Hb mMol/L	S_{AD}	S_{BD}	S_{CD}	mean calculated SO_2	calculated layer thickness d_D (μ)
1	1.0	9.2	0.89	0.92	0.92	0.91	102
	0		–0.00	0.04	0.01	0.01	88
2	1.0	8.5	0.83	1.02	0.95	0.93	90
	0		0.00	0.04	0.03	0.02	76
3	1.0	8.7	0.84	1.03	0.89	0.92	68
	0		–0.01	0.03	–0.03	–0.01	88
4	1.0	9.1	0.80	0.90	0.90	0.87	85
	0		–0.01	0.03	–0.01	0.00	86

TABLE 7

Oxygen saturation in the choroidal bloodlayer, measured on 6 consecutive days, calculated from the mean values for A, B, C and D, as measured in three registrations. On day 2, 4, 5 and 6 the animal was administered 100% O_2.

day	S_{AD}	S_{BD}	S_{CD}	mean SO_2	bloodlayer thickness (μ)
1	0.89	0.89	0.98	0.92	25.4
2	1.08	1.08	1.11	1.09	26.8
3	0.93	0.95	0.98	0.95	28.5
4	1.23	1.06	1.16	1.15	26.5
5	1.21	1.06	1.11	1.13	27.3
6	1.12	1.06	1.08	1.09	29.8

In two albino rabbits the bloodlayer thickness and oxygen saturation were calculated for periods of ventilation with 100%, 25%, 15% and 10% oxygen supplemented with N_2O. We used three registrations for each period and the mean values for A, B, C and D provided three values for the saturation, the mean of which is presented in Table 8.

Variations in saturation are not necessarily errors in the method of calculation. Part of the differences will be due to inaccuracies in the gas-flow meters.

TABLE 8

Blood oxygen saturation (SO_2) and bloodlayer thickness (d) in the choroid of 2 albino rabbits ventilated with respectively 100%, 25%, 15% and 10% oxygen. The mean values of A, B, C and D in three registrations were used to perform the calculation. In a third albino rabbit the calculation was performed for ventilation with 100% O_2 only.

ventilation with oxygen	albino rabbit I		albino rabbit II		albino rabbit III	
	d	SO_2	d	SO_2	d	SO_2
100%	27.6	1.08	28.0	1.01	26.2	1.13
25%	30.9	0.98	29.2	1.00		
15%	29.8	0.85	32.3	0.75		
10%	28.3	0.39	31.0	0.51		

In rabbits variations of several percents in saturation are possibly due to the phenomenon of 'shunting', whereby in the lung arterial blood is mixed with venous blood. Therefore a lower oxygen saturarion might be reached than expected (NUNN, 1969). Especially during ventilation with positive pressure as was used by us, shunting is increased.

SOURCES OF ERROR

The stasis effect due to the presence of erythrocytes causes the optical density of whole blood to be apparently increased compared with haemolysed blood. Whether this increase is similar for light of different wavelength depends partly on the geometry of the set-up. Diffusion depends on many factors and it would be very difficult if not impossible, to prove anything concerning the size or the wavelength dependency of the density increase.

In experiments in vitro, i.e. the transmission measurements of blood in a leucocyte counting chamber, identical curves were recorded logarithmically for haemolysed and non-haemolysed blood samples. For example at wavelength 548 nm the log T value for the whole blood indicated a density of 0.82 higher than that for haemolysed blood. Where it concerned an experiment with blood of Hb 9.1 mMol/L, this would suggest an increase in ε-factor from 12,51 to 21.52. Measured at various isobestic wavelengths, however, the discrepancies between the two curves when projected over each other, were well below 1% of the density for haemolysed blood. For flowing blood the effect of measuring whole blood versus haemolysed blood is less outspoken if measured in a shunt. For the situation in the rabbit eye, we have no evidence for our supposition that the

stasis effect is of the same magnitude over the wavelength range. In practice however the method which is based on this assumption works passably well.

The respiration of the rabbit, which has a frequency of approximately 30 cycles per minute, results in a kind of oscillatory curve when the animal is subjected to fundus reflectometry.

When measured at various fixed wavelengths, the excursions could amount to 0.0215 log unit (mean value of 3 respiration excursions at wavelengths where blood has extinction values from 0.9 to 14.78; lowest change in density 0.018, highest 0.026).

As the lengths of the lines A, B, C and D in the rabbit eye registrations are in the order of 0.0150 log units, it will be clear that the respiration excursions can be a serious hindrance. The respiration excursions showed to be of the same order whether registered at 600 nm where blood of a saturation of 1.0 has an extinction of 0.9 or at 577 nm where the extinction is 14.78 (see Fig. 36).

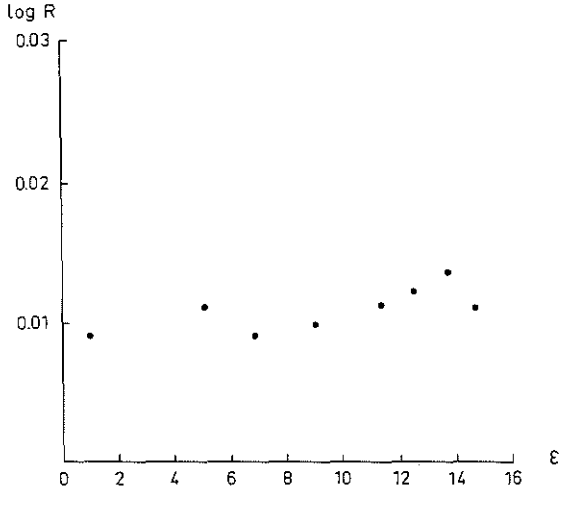


Fig. 36.
Variation in the optical density of the choroidal bloodlayer in albino rabbits, under influence of respiration, plotted against the corresponding ε-value.

Superimposed on the excursions due to respirations, are small pulse-synchronous waves of approximately 0.005 log unit (see Fig. 37). When considering both the respiration and the pulse effect (PEREGRIN, MALESSA & SVERÁK, 1970), the question arises whether we deal with an increase in bloodlayer thickness or with the effect of temporarily increased blood velocity due to pulsatile flow. There is some confirmation for the latter assumption in the following.

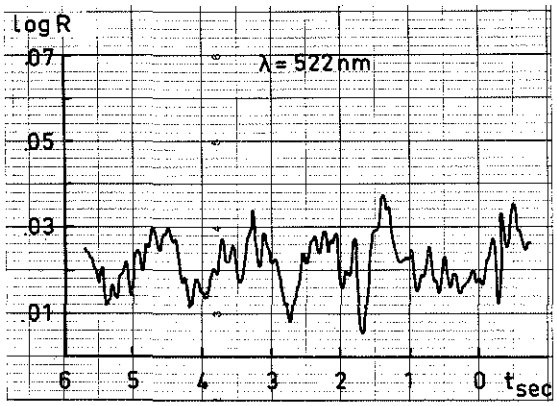


Fig. 37.
Example of respiration and pulse waves in a recording of the logarithm of the returning light energy measured at a fixed wavelength (522 nm).

Even when recorded at a higher sensitivity the magnitude of the excursions due to the pulsewave in the logarithmic recording of the returning light could not reliably be measured. Moreover our recorder was not fast enough to follow the changes in detail. Therefore the impulses as received from the photomultiplier were fed into an averager (CAT 400 C, TMC) after amplification and logarithmisation. The averager was synchronized with the E.C.G. At a range of wavelengths where the blood has ε-values from 1.0 to 14.78, 400 signals were summated and averaged. The E.C.G. was treated in the same way. The result was not very consistent, probably due to the fact that both density changes, one due to the stasis effect, the other to a layer change, merged together. No ε-dependency could, however, be recognised. Any change in the geometry of the set-up resulted in a change in magnitude of the apparent density increase, due to the pulse wave. Consequently both the respiration and the pulse wave in the recording can be considered to be the result of the stasis effect due to fluctuations in blood flow.

Lack of a second logarithmic converter made it impossible to register the differential recording of the logarithmic curve over a wavelength range and a logarithmic registration at a fixed isobestic wavelength with the second light-bundle. This would have yielded a registration of the returning light energy, free of disturbances.

Up to now the log B curve was taken to be a straight line. In order to control any deviations from the straight line the values for A, B, C and D were calculat-

ed in recordings of eyes of three albino and three pigmented rabbits, perfused with Macrodex. Table 9 gives the results.

This indicates that the values used in our experiment for A, B and C should be corrected for 1% at the most, whereas for D, usually in the order of 0.150 log units, the correction could amount to 6%.

For pigmented rabbits the error for A, B and C would be about the same as for albino rabbits.

TABLE 9

Deviation of the log B curve from a straight line, expressed as values for A, B, C and D in logarithmic units (mean of three registrations).

	A	B	C	D
albino rabbit	+0.001	0	0.002	0.008
pigmented rabbit	+0.001	—0.002	+0.003	+0.014

The deviation in D is +10%, which means that if the D would be used for estimating the layer thickness a correction of 0.014 log units should be applied. As is discussed, the use of this method in pigmented eyes does not yield reliable results. Therefore this point is not further elaborated.

No consideration was given to the existence of macular and visual pigment. The density of the first pigment is relatively small (Fig. 38). The visual pigment will be exposed to a constant intensity of light. Therefore it was supposed that

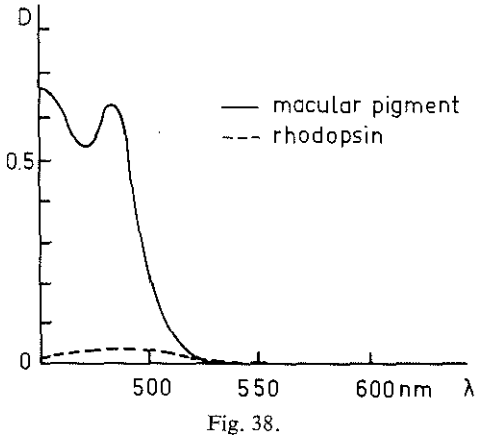


Fig. 38.
Optical densities of human macular pigment and rhodopsin between 450 and 600 nm. (After DARTNALL and after WALD).

the density of rhodopsin would not change as bleaching and regeneration would be in balance. Consequently these pigments can be arranged under the non variable layers.

As mentioned earlier part of the light from the Xenon arc never reaches the vascular layer of the eye, but is reflected against cornea, lens, opacities in the vitreous, anterior limiting membrane of the retina and whatever planes of transition between media with a different refractive index there exist in the eye. Part of these reflections are received by the photomultiplier, and 'dilute' the information received from the vascular layer. The amount of these false reflexes is dependent of the angle of incidence of the light, clearness of the lens and many other unpredictable factors. The value of quantitative measurements depends completely on the possibility of assessing the amount of these false reflections. The physical layman would suggest that it must be possible to calculate the size of each component from information over a certain range, built up out of a constant part, (the false reflections) and a part dependent on the properties of the blood. We were informed that this was however very difficult if not impossible (VAN DER VEN, VAN DER WILDT & MEYER, personal communication, 1974). A solution was found in the existence of the Soret bands. At the isobestic wavelength 412.5 nm the ε-factor, according to VAN KAMPEN & ZIJLSTRA (1968) is 110. Although we found considerable lower values (32–35), still a bloodlayer of 30 μ of an Hb of 10 mMol/L is sufficient to reduce the transmission to less than 0.01. This is in a recording a negligible amount. All the light that was measured at 412.5 nm was considered to be due to 'false' reflections. When false light was present, it was normalised with respect to the spectral composition of the Xenon arc light as seen by the photocell. This percentage could be used as a correction for any other wavelength. Blue light has a higher diffusion than light of longer wavelength. Within the scope of our experiment however we can accept the reflection as independent of wavelength since diffused light is hardly measured. Therefore considering the small contribution of 'false' light in the blue, we can neglect the contribution at other wavelengths except that to be described in pigmented animals. This result was duly confirmed by the following experiment. Evans Blue 5% solution was injected intravitreal in two perfused albino rabbit eyes. In one eye no reflection was measurable any more. In the second eye only 0.2% was left from the original returning light.

CHAPTER IX

THE OPTICAL DENSITIES OF SOME MEDIA IN THE RABBIT AND HUMAN EYE MEASURED BETWEEN 650 AND 450 NM.

INTRODUCTION

As part of the work on fundus reflectometry, many experiments were done on isolated media of the rabbit and human eye. As only a little information concerning this matter could be found in the current literature (GEERAETS, 1960; PRINCE, 1964; DUKE ELDER, 1968 and HUNOLD & MALESSA, in press), it might be of interest to others to report our results.

MATERIAL AND METHODS

Human eyes were obtained from an eyebank. Enucleation took place within 8 hours p.m. The examination took place within 24 hours, up to which time the eyes were stored in a closed container at 4°C with a wet gauze at the bottom.

Rabbit eyes were used from bled animals. Some of the rabbits were perfused with Macrodex as bleeding proved to be not always efficient. The experiments were done within one hour after death.

The apparatus used has been described in chapter II. Only the motor-driven monochromator was used. The incident light was diaphragmated to a 1.0 mm diameter parallel bundle. The photomultiplier was placed 20 mm beyond the tissue whose density had to be measured. The opening in the photomultiplier window was either 10 mm in order to measure diffused light as well or the collimating unit was mounted on top of the photomultiplier, in such a way that only collimated light could be registered. Recordings were darkstream and background illumination compensated by means of differential registration of the chopped

light. Slitwidth was usually 0.01 mm, resulting in monochromatic light with a half width of 0.07 nm. Registrations were made of the intensity of the transmitted light, recorded exponentially and compared with a suitable blank. The densities were calculated by subtracting the measured logarithmic value from the blank logarithmic value. These subtractions were performed every 5 nm. Where rapid changes in density took place the subtractions were done more frequently. The densities were plotted in a graph against the wavelength.

CORNEA

The corneal radius is usually between 7.0 and 7.5 mm (PRINCE, 1964). Corneal densities were measured after the cornea had been excised and placed in a perspex block with a spherical excavation with a radius of 8 mm. The thickness of the perspex layer under the centre of the excavation, where the measurements took place, was 0.5 mm. In order to measure also any light that was scattered or diverted from its original direction, the 10 mm opening in the photomultiplier window was used and placed at a distance of 20 mm under the perspex block. The perspex block was filled with saline and covered with a microscope slide. The object of this was to have the same percentage reflections from the surface at each measurement. Blanks were made with the perspex block filled with saline only and covered with a slide. The results of the measurements are given in Figure 39 and 40. No distinction was made between corneas of albino and pigmented rabbits. The higher density in human corneas is probably due to edema of the endo- and epithelium.

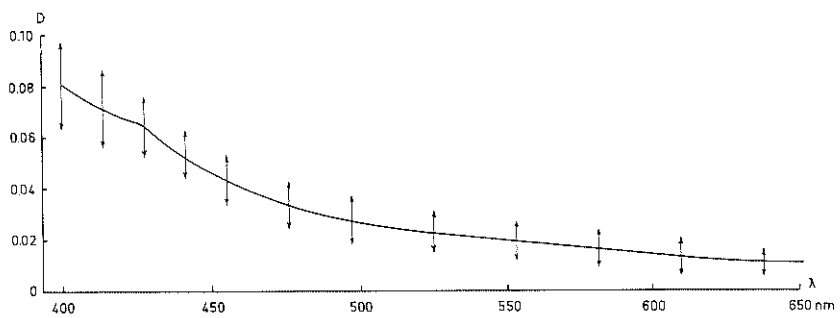


Fig. 39.
Optical density of rabbit cornea. Mean values of measurements in 6 corneae with standard deviations.

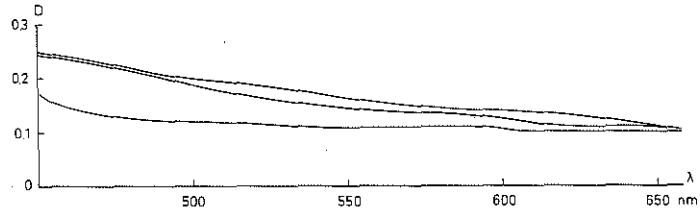


Fig. 40.
Optical density of three human corneae.

LENS

Lenses were freed from zonulae and vitreous with blunt scissors. Only lenses with intact capsules were used. As the measuring exactly through the centre of the lens was difficult for technical reasons and any slight deviation from the centre resulted in deviation of the light, a special cuvette was prepared. This consisted of two microscope slides fitted at a distance of 3.5 mm for human lenses, at 6.6 mm for rabbit lenses. When a lens was placed in the cuvette the anterior and posterior side became slightly flattened. This made measurements in the centre more reliable and had furthermore the advantage that the layer thickness was known. To obtain a blank value two microscope slides with a drop of water in between were placed in the light path. The results are shown in Figure 41 and 42. The higher density for human lenses is probably due to the presence of cataract.

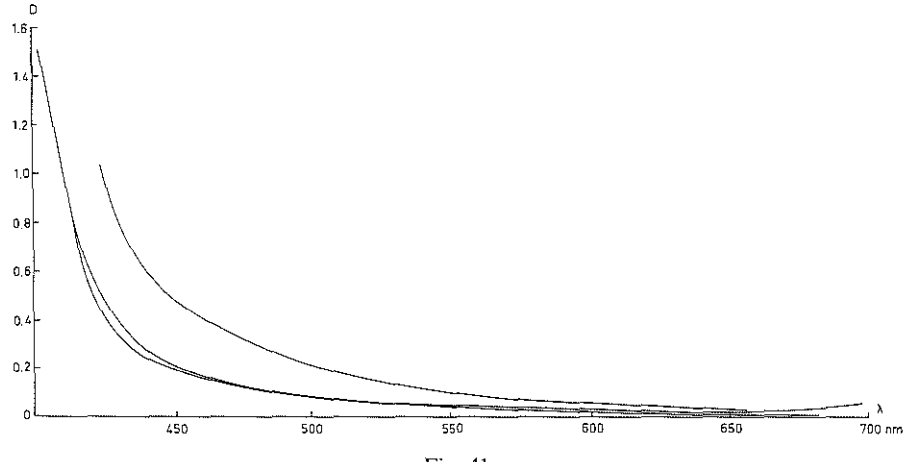


Fig. 41.
Optical density of three human lenses. Thickness of lenses 3.5 mm.

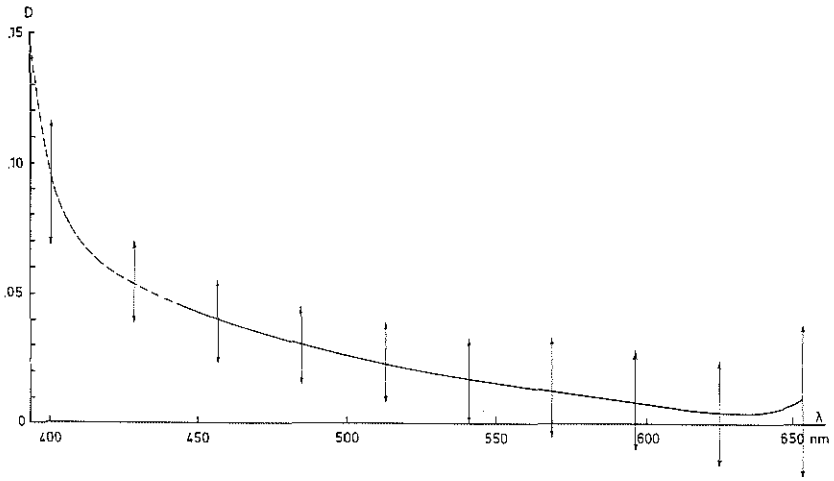


Fig. 42.
Optical density of albino rabbit lenses. Mean value of 8 lenses (drawn line) and of 3 lenses (interrupted line) with the standard deviations. Thickness of lenses 6.6 mm.

VITREOUS

Vitreous was removed from the opened eye with a forceps and scissors. The material was put into the measuring chamber, a 5 mm high and wide perspex cylinder closed on top and bottom side with microscope slides. Blank values were obtained as for the lens. The results are given in Figure 43.

RETINA WITHOUT PIGMENT EPITHELIUM

Retinal tissue became edematous very soon after death, probably due to anoxia. When separated from the choroid and sclera the elasticity of the retina made it shrink, which certainly would result in an apparent increase in optical density. For that reason it was thought more reliable to measure first choroid and retina together. Then the retina was to be removed. By subtraction of the density values for choroid without retina from those for choroid with retina, the density for retinal tissue without pigment epithelium was to be obtained. As it proved to be very difficult to measure exactly at the same spot of choroid, before and after removing the retina, this idea had to be abandoned. For lack of a better

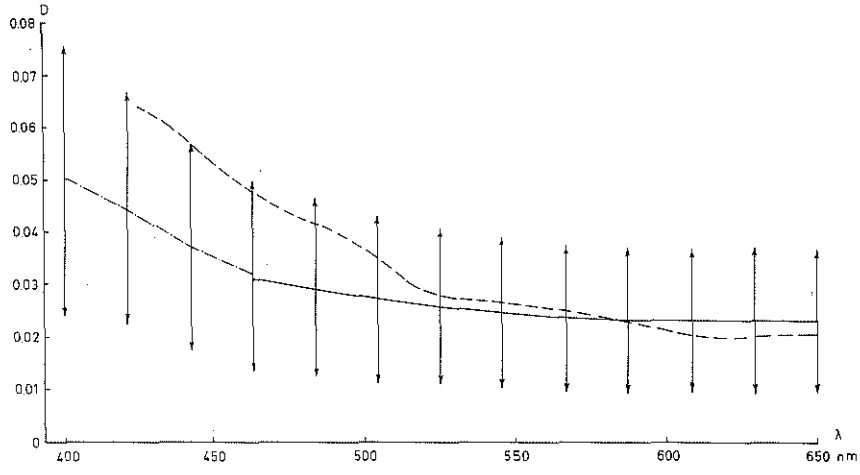


Fig. 43.
Optical density of vitreous, measured at a layer thickness of 5 mm. Mean values of 3 samples rabbit vitreous (–·–·–), 5 samples rabbit vitreous (———), 1 sample human vitreous (– – –) with the standard deviations.

technique, we had to rely on mounting a piece of retina between two microscope slides and just hoping that the slides would not flatten the retinal tissue too much. Therefore the results do not indicate absolute values.

Only the spectral characteristics can be considered to be reliable. The results are given in Figure 44 and 45. No distinction was made between the retina of albino and pigmented rabbits.

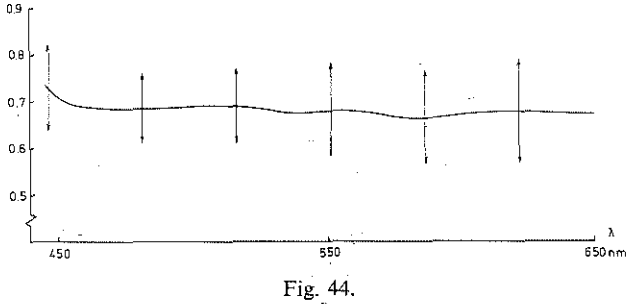


Fig. 44.
Optical density of albino rabbit retina without retinal pigment epithelium. The mean values and standard deviations of three samples.

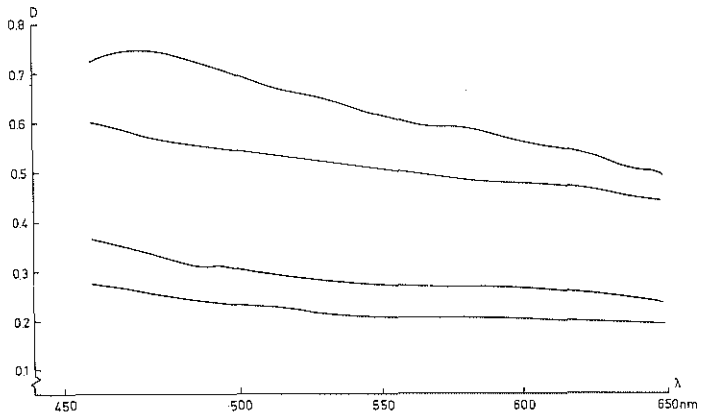


Fig. 45.
Optical density of human retina without retinal pigment epithelium measured in four samples.

CHOROID WITH RETINAL PIGMENT EPITHELIUM

A window was prepared in the sclera leaving bare choroid exposed. The edges of the window were glued with Cyano acrylate on a microscope slide. Then the bulbus was perforated. All parts were removed with exception of the posterior pole. After the retina and vitreous had been removed, the choroid was covered with a microscope slide. Any airbubbles were removed and replaced by saline. In this way the choroid was prevented from shrinking. Measurements were done with a small and large photomultiplier opening. When the eye used for measuring the choroid had been bled, frequently residual blood was visible in the spectral characteristic of the tissue. The presence of blood can be recognized by small density increases at approximately 575 and 540 nm. The results are given in Figure 46.

ALBINOTIC CHOROID

The measurement was performed as for pigmented choroid. The results are presented in Figure 47.

SCLERA

A well hydrated piece of sclera was compressed with a drop of saline in between two microscope slides. Blank values were obtained by measuring the density of two microscope slides with a drop of saline in between. The results are shown in Figure 48.

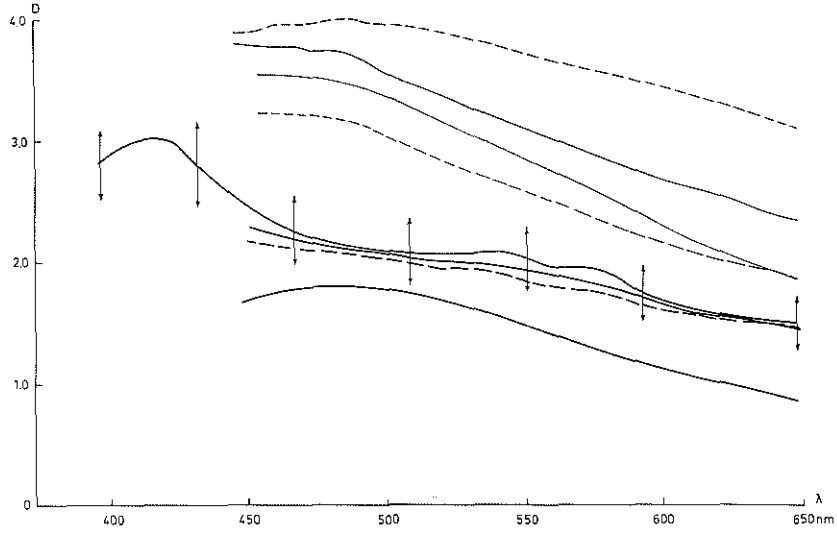


Fig. 46.
Optical densities of the choroid with retinal pigment epithelium of pigmented rabbit's eyes (drawn lines) and human eyes (interrupted lines). Measured with collimated light (thin lines) and diffused light (thick lines).

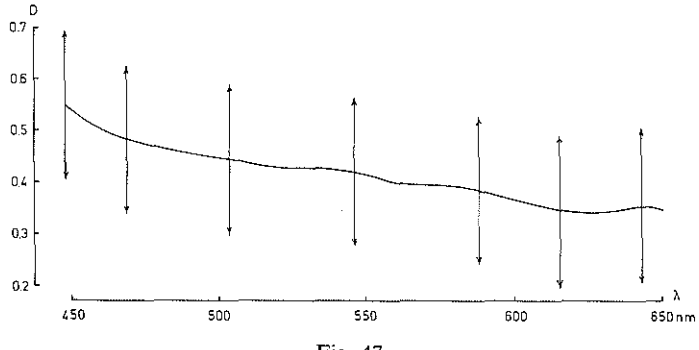


Fig. 47.
Optical density of choroid with retinal pigment epithelium of albino rabbits. Mean of 3 samples with the standard deviations.

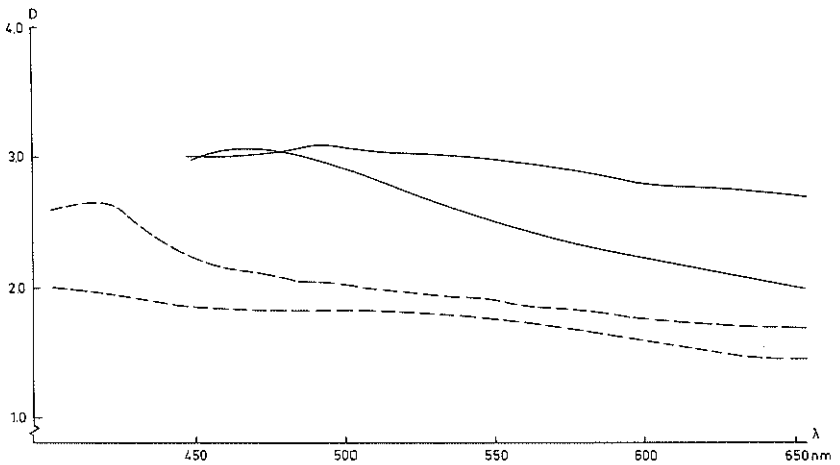


Fig. 48.
Optical densities of human (drawn lines) and albino rabbit sclera (interrupted lines).

DISCUSSION

For tissues with low densities like retina, cornea etc., it did not make much difference whether the measurement took place with wide or narrow opening of the photomultiplier, in other words, it made hardly any difference whether the light was diffused or not.

For tissues with a high density at the other hand, like pigmented choroid with the adjoining retinal pigment epithelium, especially in the lower wavelength range, considerable differences were noticed. This is illustrated in Figure 46. For those tissues where the density curves were rather similar, a mean value was calculated together with the standard deviation. As the standard deviation should ideally have been calculated over the transmission, and then logarithmized, the values indicate somewhat too small or too large deviations in the respectively negative or positive direction.

Our figures for pigmented choroid are much higher than reflectometrically found values as reported by PEREGRIN & MALESSA (in press). As mentioned before this may be related to the fact that in reflectometry probably only the density of the pigment epithelium is registered.

CHAPTER X

CALCULATION OF THE MOLECULAR EXTINCTION COEFFICIENT FOR Hb AND HbO_2 BETWEEN 650 AND 450 NM.

INTRODUCTION

Although there are no reasons to believe the contrary, we had to make sure that the ε-values for Hb and HbO_2 in rabbit blood were the same as for human. Indeed no differences were found. Further investigations were all performed on human blood as this was easily available. The conclusions hold therefore for rabbit blood as well.

Several authors produced ε-values over about the same wavelength range (VAN KAMPEN & ZIJLSTRA, 1965; SIGGAARD ANDERSON et al., 1972). Nevertheless it was worthwhile to repeat these calculations for several reasons. First there is some confusion on the exact wavelengths that are isobestic, i.e. where the ε-values for Hb and for HbO_2 are equal. For the present study the position of these wavelengths was of paramount importance. In the second place the ε-values given by VAN KAMPEN & ZIJLSTRA are calculated with very narrow half-intensity bandwidth. This is of course the correct method to use, but as in fundus reflectometry shortage of light was frequently experienced, slits of 0.2 mm were used. This resulted in a half-intensity bandwidth of 1.4 nm. This certainly will have some influence on the ε-values for wavelengths, where the ε-curve is not flat.

METHOD

Venous blood was taken from non-smoking healthy individuals. The blood was allowed to stand for 1 hour, so that methaemoglobin would convert into Hb and HbO_2. Two ml. undiluted blood was during 6 minutes flushed with 100%

oxygen in a tonometer, a ten ml pipette, circulating with 32 rev/min. The oxygen was humidified. From the now presumably 100% saturated blood, two haematocrit tubes were filled. The remaining 1.5 ml blood was poured into a testtube containing 15 mg Sodium dithionite, 30 mg Natricus biboras, both as dry powder and some glassbeads. The testtube was shaken carefully. The blood was allowed three minutes to become completely reduced. Then two haematocrit tubes were filled with this reduced blood and the remainder used to calculate the total haemoglobin content. All haematocrit tubes were sealed with plasticine and closely fitting plastic caps after a stirring magnet had been introduced. The blood was haemolysed by immersion three times one minute in methanol of —30°C. In a Bürker-Türk leucocyte counting chamber of a depth of 0.1 mm, immediately outside the ruled area, the intensity of the transmitted light was measured (SIGGAARD ANDERSON et al., 1962). The set-up as described in Chapter II was used. Blank values were found by measuring in the same counting chamber, filled with water. The blood densities were calculated by subtracting the logarithms of the intensities of transmitted light in blood measurements from that of the water measurements. Out of the obtained values for the optical density the ε-factor could be calculated with help of Lambert-Beer's law, $D = c.d.\varepsilon$. c stands for concentration (mMol/L) and d for layer thickness (cm), both known factors. The haemoglobin was determined with the cyanide method. Due to several factors, the standard error of this method is about 1%. Three measurements were taken and the mean value accepted. The error in the Hb determination returns in the ε-value.

RESULTS

A representative specimen of a ε/λ curve for Hb and HbO_2 is presented in Figure 49. Table 10 gives the mean wavelengths that were found to be isobestic. Table 11 gives the outcome over 7 measurements of the ε-values of the isobestic wavelength and of those wavelengths where a maximal difference exists between ε_{Hb} and ε_{HbO_2}.

COMMENT

The optical density for plasma was compared with that of water. No measurable difference could be measured. We noticed the influence of the kind of light used. When the opening of the photomultiplier was 1 cm in diameter, diffused light was measured as well. Especially for the lower wavelength range the ε-values were higher than when measured with collimated light. For 506 nm we noticed

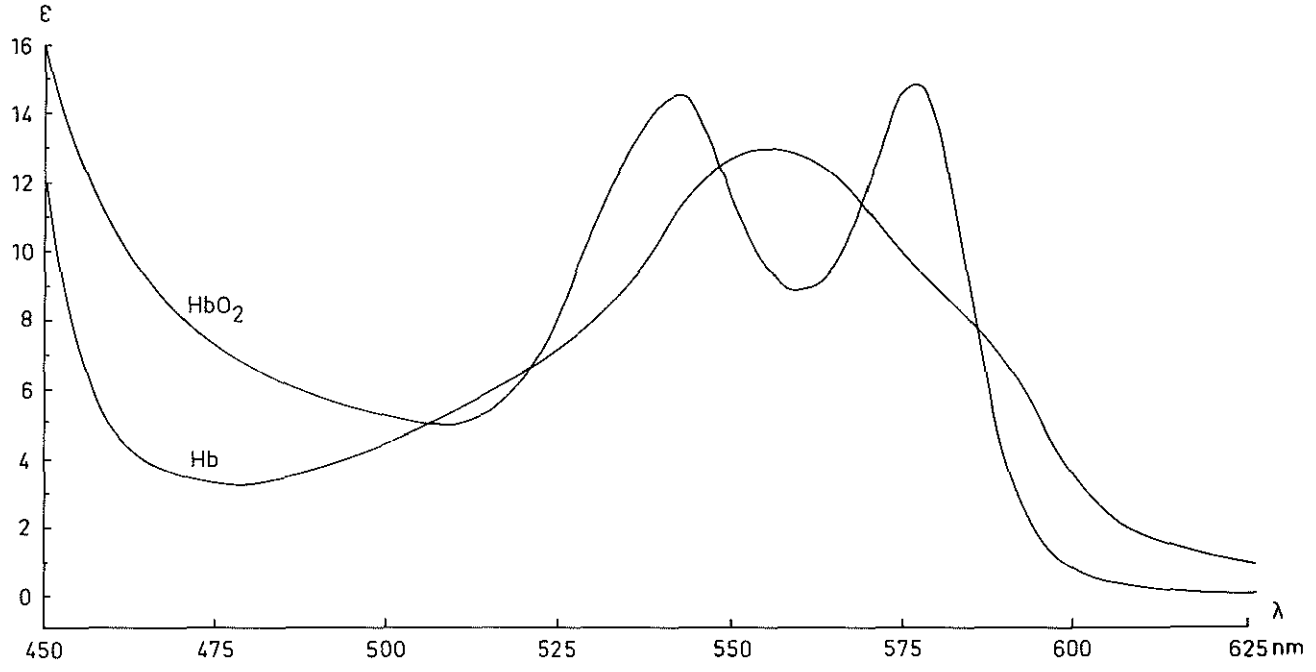


Fig. 49.
ε/λ curve for Hb and HbO_2, calculated in a sample of human blood.

TABLE 10

Mean values and standard deviations calculated over n measurements of those wavelengths, that are isobestic for Hb and HbO_2.

approx. wavelength (nm)	n	mean (nm)	S.D.
506 nm	6	505.97	0.03
522 nm	6	521.77	0.35
548 nm	8	548.02	0.07
569 nm	8	569.15	0.18
586 nm	8	585.58	0.04

TABLE 11

Mean values of the extinction coefficients of HbO_2 and Hb and their standard deviations, at some isobestic wavelengths and at some wavelengths with maximal difference between ε_{Hb} and ε_{HbO_2} in n measurements.

isobestic wavelength	n	mean ε	S.D.	highest value	lowest value
506 nm	7	5.11	0.09	5.20	4.93
522 nm	7	6.87	0.09	7.00	6.70
548 nm	7	12.51	0.09	12.61	12.39
569 nm	7	11.40	0.07	11.53	11.29
585.5 nm	7	7.86	0.09	7.99	7.78
detecting wavelength					
537.5 nm HbO_2	8	13.76	0.21	13.99	13.48
Hb	7	9.75	0.05	9.84	9.68
560 nm HbO_2	8	9.02	0.11	9.11	8.83
Hb	7	12.76	0.08	12.87	12.66
577 nm HbO_2	8	14.78	0.26	15.25	14.49
Hb	7	9.47	0.07	9.57	9.39

an ε-value of 5.46 (n = 6; SD = 0.25), for 522 nm 7.03 (n = 6; SD = 0.20).

Our results confirm largely the findings of VAN KAMPEN & ZIJLSTRA in respect of the localization of the isobestic wavelength. The exact extinction values do not all agree but it is not unlikely that these differences are a consequence of the different geometry of the respective set-ups.

CHAPTER XI

CONCLUSIONS AND SUMMARY

Notwithstanding the development of new techniques like the use of radioactive isotopes, not much has been added to the knowledge on choroidal circulation, since the experiments of Bill (1962a; 1962b).

Moreover most experimental methods are limited to registering at one moment only, or are traumatising to the eye.

The object of this study was to evaluate the possibilities of fundus reflectometry as a tool to investigate the choroidal blood circulation. The primary aim was to measure blood oxygen saturation and the bloodlayer thickness.

The obvious advantages of the method are that it enables continuous measurements without disturbing the physiology of the eye at all, except that mydriasis is essential.

In the present experiments it has been demonstrated that a large difference exists in the way light in fundus reflectometry returns from an albinotic and a pigmented fundus. While in experiments with albino rabbit eyes perfused with Evans blue, the returning light was shown to consist more than 99% of transmitted light reflected against the sclera, in pigmented rabbits the removal of the sclera had no influence on the light transmission. Furthermore the optical density of the pigmented layers makes it very unlikely that any light could be transmitted. Reflections from the erythrocytes can be excluded as source of the returning light and so can direct scattering against the cornea or lens.

Although in pigmented eyes the amount of reflected light is much less than in albinotic eyes, when bloodless, the spectral composition of the returning light was shown to be rather similar in both kinds of eyes. It is difficult to ascertain

in how far this agrees with the spectral composition of the pigment, but as we have measured in the considered range from 500 to 600 nm, no considerable deviations can be expected. The most likely explanation is based on the reflecting properties of both pigmented layers in the eye, notwithstanding their high density. This is supported by histologic examinations. Part of the light is reflected against the uveal pigment and returns after having traversed the choriocapillaris and possibly some of the uveal blood layer. Another part of the incident light is reflected by the retinal pigment epithelium and passes no blood at all, but only causes dilution of the information about the blood which could be obtained from the first part. As the effective bloodlayer thickness as well as the share of the reflections in the total returning light probably depends on the amount of pigment present in the layers concerned, at the present no quantitative conclusions can be drawn from fundus reflectometric registrations in pigmented eyes.

As in albinotic eyes we deal with transmission, it had to be demonstrated that although blood is a non-homogeneous solution, Lambert-Beer's law is valid. For blood of 10 mMol/L Hb, this was shown in vitro to be the case for bloodlayers up to 150 μ thickness, which is more than will be encountered in rabbits. The results in the living eye corroborated these findings.

The method of reflectometry as performed by us, uses a narrow, collimated light-bundle, entering the dilated pupil of the eye. Light was only allowed to be measured by the photomultiplier when its returning course was parallel with that of the incident light. The purpose of this was to prevent the registration of light energy from reflections against cornea and lens. The percentage of these reflections was limited indeed to less than 0.2% of the total returning light in albino rabbits, as was shown with experiments with Evans blue, injected in the vitreous. The image of the incident light bundle formed a spot of $1 \times 1\frac{1}{2}$ mm on the fundus, within which area the light was supposed to traverse an intermingled structure of venous and arterial blood. Based on reports on histologic examinations of the choroid and clinical evidence, it was assumed that no large variations in bloodlayer thickness exist.

The factors influencing the optical density of this bloodlayer are not oxygen saturation and layer thickness only. In attempts to localize isobestic wavelengths in vivo, it became apparent that bloodflow had influence on the light transmission. The magnitude of this effect, named stasis effect, proved to be highly influenced by the diffusion of light, a factor always influencing the quantitative results of measuring whole blood. The effect of apparent diminished density if flow increased was present, but was of different magnitude in different experiments as it depended partly on the geometry of the set-up. It was possible to demonstrate in a shunt cuvette that the stasis effect was:

1. related with the magnitude of the flow
2. rather independent of the ε-value of the blood
3. dependent on the Hb concentration in the blood.

The first two of these conclusions were proved to be valid in the albino rabbit eye, if the geometry was kept unchanged. It was established that haemolysed blood was not subject to this effect.

Variations in blood oxygen saturation in the choroidal bloodlayer were measured in albino and pigmented rabbits with a differential method. The measurement was continuous using a two-colour method with fixed wavelengths. The expected effects were demonstrated. Differential recording will indicate changes in saturation but these cannot be quantitatively measured. The registration of the logarithm of the returning light of an isobestic wavelength made it possible to follow changes in the optical density of the bloodlayer, whether caused by fluctuations in layer thickness or flow. In albino rabbits these changes can be interpreted quantitatively. The logarithmic recording of returning light proved to depend on layer thickness and oxygen saturation, but its spectral characteristic is independent of the flow effect. For the various isobestic wavelengths, there will be an interdependency of the respective blood densities. This served as a base for calculation of the bloodlayer thickness and oxygen saturation, from a fast logarithmic registration of the returning light over the wavelength range 586 to 522 nm. With respect to this it was indispensible to know the exact ε-value for Hb and HbO_2. Therefore ε/λ curves were prepared for both kinds of blood with the same half-intensity bandwidth as used in fundus reflectometry. The method of calculation depends on the fact that the density spectra for the various media in the eye are straight or nearly straight lines over short wavelength ranges. To verify this D/λ curves were made of the media involved. There are several variations of the calculation method. Based on a range from experiments the best solution was chosen. This had a reliability of about 10% for the oxygen saturation and layer thickness calculation. Part of the error is due to respiration and pulse waves. Some preliminary experiments about these effects were performed.

GLOSSARY

Optical density = $D^{\lambda} = \varepsilon_1^{\lambda} \cdot c_1 \cdot d + \varepsilon_2^{\lambda} \cdot c_2 \cdot d \ldots . + \varepsilon_n^{\lambda} \cdot c_n \cdot d$ (Lambert-Beer's law).

ε_n^{λ} = molecular extinction coefficient of component n at wavelength λ.

c_n = concentration of component n (mMol/L).

d = layer thickness in cm.

Isobestic wavelength = λ isobestic, e.g. for Hb and HbO_2: wavelength where the ε-factors for Hb and HbO_2 are equal.

$\text{Transmission}_{\lambda}$ = percentage of light of a wavelength λ, passing through a solution or substance.

$$= \frac{\text{transmitted light}}{\text{incident light}} \times 100.$$

Stasis effect = the phenomenon of changing light transmission under influence of variations of blood flow or blood velocity, expressed in log units. This special term was chosen for use in this study because, compared with the density of haemolysed blood of the same concentration and layer thickness, the deviation of the density of whole blood is maximal in complete stasis.

nm = nanometer = cm $\times 10^{-7}$.

T = symbol used for the photomultiplier output of transmitted light (and not in the usual sense of transmission).

R = symbol used for the photomultiplier output of light returning from the eye, irrespective its origine or nature.

S_{O_2} = oxygen saturation, expressed as fraction.

SAMENVATTING

Ondanks het ontwikkelen van geavanceerde onderzoektechnieken, zoals het gebruik van radioactieve isotopen, werd tot nu toe weinig vooruitgang geboekt in het onderzoek naar de haemodynamica van de chorioidea. De meeste methoden registreren slechts de toestand op een enkel moment; andere, waarmee over langere perioden gemeten kan worden, zijn traumatiserend voor het oog. Fundus reflectometrie is een methode waarbij continue metingen verricht kunnen worden zonder dat de fysiologie van het oog verstoord wordt anders dan door het wijd maken van de pupil. Deze onderzoektechniek is vooral ontwikkeld door RUSHTON, die de methode toepaste bij het onderzoek naar het gezichtspurper. Er wordt gebruik gemaakt van een lichtbundel die de verwijde pupil van het oog binnentreedt. Een deel van dit licht treedt via de pupil weer uit en de energie hiervan kan gemeten worden. Verscheidene groepen onderzoekers hebben de methode in de loop der jaren verfijnd. (BROADFOOT, GLOSTER & GREAVES; TROKEL). Wij meenden dat het wenselijk was na te gaan welke de mogelijkheden zijn die fundus reflectometrie kan bieden als middel tot onderzoek. Gebruik werd gemaakt van apparatuur, die grotendeels was ontworpen door IR. C. A. DE COCK. Daarbij werden twee monochromatische lichtbundels van verschillende golflengten alternerend in het oog geworpen. De gemeten energie van het terugkerend licht werd voor elke kleur gecorrigeerd voor stoornissen van donkerstroom en achtergrond verlichting. Op vele manieren werd geprobeerd een enerzijds efficiënt gebruik van het beschikbare licht te maken, zonder dat dit aan de andere kant zou leiden tot het meemeten van reflecties tegen de media in het voorste oogsegment.

Het door ons verrichtte onderzoek had als voornaamste doel het ontwikkelen

van een methode om de zuurstof saturatie en de laagdikte van het bloed in de chorioidea te meten.

Als proefdieren werden konijnen gebruikt omdat bij deze dieren het retinale vaatstelsel beperkt is tot een klein gebied vlak bij de papil. De metingen betreffen derhalve uitsluitend de choroidale bloedlaag. In de door ons uitgevoerde experimenten werd aangetoond dat er een groot verschil bestaat tussen de manier waarop licht bij fundus reflectometrie terugkomt uit een albinotische en uit een gepigmenteerde fundus.

In de experimenten met albino konijnen waarbij het oog geperfundeerd werd met Evans blue bleek het terugkomende licht voor meer dan 99% te bestaan uit licht, dat na door de weefsels te zijn gegaan tegen de sclera was gereflecteerd en de weg terug had afgelegd.

In gepigmenteerde konijnen daarentegen werd gezien dat het verwijderen van de sclera geen enkele invloed had op de hoeveelheid terugkomend licht. Ook de optische dichtheid van de gepigmenteerde weefsellagen maken het erg onwaarschijnlijk dat een meetbare hoeveelheid licht getransmitteerd zou kunnen worden.

Er naar zoekend hoe dan wel licht uit de gepigmenteerde fundus terug keerde, kon worden vastgesteld dat de reflecties van de erythrocyten geen belangrijke rol spelen, evenmin als strooilicht tegen de cornea en de lens. Hoewel in gepigmenteerde ogen de hoeveelheid terugkerend licht veel kleiner is dan in albinotische ogen, bleek dat wanneer de ogen bloedloos waren, de spectrale compositie van het terugkerend licht in beide gevallen ongeveer gelijk was. Het is moeilijk met zekerheid te zeggen in hoeverre dit overeenkomt met de spectrale compositie van pigment, maar, zoals wij in pigmenthoudende weefsels gemeten hebben in het betreffende gebied van 500–600 nanometer, zijn aanzienlijke afwijkingen niet te verwachten. De meest waarschijnlijke verklaring voor de terugkeer van licht uit de gepigmenteerde ogen is dat pigment reflecteert, niettegenstaande de hoge optische dichtheid. Deze veronderstelling wordt ondersteund door histologisch onderzoek. Een deel van het licht wordt gereflecteerd door het uveale pigment en keert terug nadat de choriocapillaris en mogelijk een deel van de uveale bloedlaag doorlopen is. Een ander deel van het invallende licht wordt gereflecteerd door het retinale pigment epitheel, zonder bloed te hebben gepasseerd. Licht van deze laatste categorie veroorzaakt verdunning van informatie verkregen uit het licht dat wel door het bloed is gegaan. Aangezien de gemeten bloedlaag dikte evenals het aandeel van reflecties in het totaal terugkerende licht waarschijnlijk afhangt van de hoeveelheid pigment aanwezig in de betreffende lagen, is het op dit moment niet mogelijk quantitatieve conclusies te trekken uit fundusreflectometrische waarnemingen in gepigmenteerde ogen.

In albinotische ogen hebben wij te doen met transmissie. Daarom moest aangetoond worden dat hoewel bloed een niet-homogene vloeistof is, de wet van Lambert-Beer toch geldt. Voor bloed van een Hb van 10 mMol/L werd aangetoond in vitro dat dit het geval was voor laagdikten tot 150 μ, wat meer is dan de verwachte laagdikte in konijnen. Dat de wet van Lambert-Beer in het oog mag worden toegepast kon niet bewezen worden, maar is op grond van uitgevoerde experimenten aannemelijk.

De methode van reflectometrie, zoals door ons uitgevoerd, maakt gebruik van een smalle gecollimeerde lichtbundel, die binnentreedt in het oog door de verwijde pupil. Alleen dat licht wordt gemeten door de photomultiplier, dat bij teruggang uit de fundus parallel loopt aan het invallende licht. De bedoeling hiervan was te voorkomen dat lichtenergie van verstrooiing en reflecties door en tegen lens en cornea, zouden worden meegemeten. Op deze manier bleek het mogelijk het aandeel van reflecties in de totale hoeveelheid terugkerend licht te beperken tot minder dan 0.2 % bij albino konijnen. Dit kon worden aangetoond in experimenten, waarbij Evans blue geinjiceerd werd in het glasvocht. Het binnenvallende lichtbundeltje vormde een beeld van 1 $\times$ 1½ mm op de fundus binnen welk gebied het licht geacht wordt een constante verhouding van veneus en arterieel bloed te passeren. Op grond van histologische beschrijvingen van de chorioidea en klinische ervaringen, werd aangenomen dat geen grote variaties in bloedlaag dikte bestaan.

De factoren die de optische dichtheid van de bloedlaag in de chorioidea beïnvloeden zijn niet alleen zuurstof saturatie en bloedlaag dikte. Bij pogingen om de isobestische golflengten in vivo te bepalen bleek dat de bloed stroomsnelheid invloed heeft op de lichttransmissie. De grootte van dit effect, stasis effect genoemd, bleek sterk te worden beïnvloed door diffusie van licht, een factor die steeds de quantitatieve resultaten van metingen in niet gehaemoliseerd bloed beïnvloedt. Het effect van de schijnbaar verminderde optische dichtheid wanneer bij metingen in een shunt cuvette de stroomsnelheid toeneemt is steeds aantoonbaar maar van een verschillende grootte in verschillende experimenten. Een deel van deze verschillen kunnen worden geduid als verschillen in de geometrie van de opstelling. Het bleek mogelijk aan te tonen in een shunt cuvette dat het stasis effect:

1. afhankelijk is van de bloedstroom
2. onafhankelijk is van de ε-waarde van het bloed
3. afhankelijk is van de concentratie van het bloed.

De eerste twee van deze conclusies konden bewezen worden in het albino konijnen oog, wanneer de geometrie van de opstelling onveranderd werd gehouden. Er werd vastgesteld dat gehaemolyseerd bloed dit effect niet vertoonde.

Veranderingen in de zuurstof saturatie van het bloed in de chorioidea werden gemeten in albino en gepigmenteerde konijnen door gebruik te maken van een differentiaal methode. De meting was continue en werkt met twee lichtbundels met vaste golflengte, waarvan de één gevoelig is voor kleurveranderingen van het bloed, de ander juist niet. De te verwachten effecten werden aangetoond. Verschilschrijving geeft weliswaar veranderingen in de saturatie aan, maar deze kunnen niet quantitatief worden beoordeeld. De registratie van de logarithme van het terugvallend licht van een isobestische golflengte, maakte het mogelijk om veranderingen te volgen in de optische dichtheid van de bloedlaag, veroorzaakt door fluctuaties in hetzij de laagdikte of de flow. In albino konijnen kunnen deze veranderingen quantitatief bepaald worden.

De logarithmische registratie van terugkerend licht bleek afhankelijk te zijn van laagdikte en zuurstof saturatie, maar de spectrale karakteristiek is niet beïnvloed door het stasis effect. Metend bij diverse isobestische golflengten bestaat er een onderlinge afhankelijkheid van de optische dichtheid van de bloedlaag, afhankelijk van de extinctie coefficient van het bloed bij die golflengte. Deze waarneming fungeerde als grondslag voor de berekening van de bloedlaag dikte en de zuurstof saturatie. Hiervoor werd een snelle logarithmische registratie gedaan van het terugkerend licht over een golflengte gebied van 568 tot 522 nm. Om de berekeningen te kunnen uitvoeren was het noodzakelijk om precies de ε-waarde te weten voor Hb en HbO_2. Van uitsluitend Hb of HbO_2 bevattend bloed werden ε/λ curves gemaakt met dezelfde bandbreedte van het licht, als werd gebruikt bij fundus reflectometrie. De methode is afhankelijk van het feit dat de optische dichtheid van de diverse media in het oog, gemeten over korte golflengtegebieden, rechte of bijna rechte lijnen zijn. Om dit te controleren werden optische dichtheid spectra gemaakt van de betreffende media. De berekening bleek op verschillende wijze mogelijk te zijn. Op grond van experimenten werd de beste oplossing gekozen. Deze had een betrouwbaarheid van ongeveer 10% voor de zuurstof saturatie en de laagdikte berekening. Deze fout is gedeeltelijk het gevolg van de ademhaling en pols fluctuaties. De hierdoor veroorzaakte variaties in optische dichtheid van de bloedlaag werden in enkele experimenten nader onderzocht. Hierbij bleek dat deze fluctuaties voor het merendeel berusten op veranderingen in de stroomsnelheid of flow van het bloed.

CURRICULUM VITAE

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