An objective and rapid method for the determination of light dissemination in the lens

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Abstract. A method for the objective measurement of light dissemination in the lens was developed. There is an exponential relationship between the concentration of standard solutions and the intensity of light disseminated forwards. The light disseminated in non-pathological lenses from Sprague Dawley rats was registered as the equivalent standard concentration, C, and then transformed to $\log_{10}(C+1)$ and was found not to deviate from the normal distribution. The tolerance limit for light dissemination was derived by setting the probability to classify a non-pathological lens as pathological. An analysis of variance demonstrated that the inter-animal variation was the dominating source of imprecision. It is anticipated that the developed system will be useful in experimental toxicological risk assessment.

Key words: rat lens – cataract – light dissemination – toxicology – risk assessment.

The term 'cataract' implies lenticular opacification that results in impaired vision. This definition includes the imaging properties, usually measured as the visual acuity, and the light dissemination, commonly estimated in the slit-lamp microscope. In toxicological studies of cataractogenesis in animals, measurements of visual acuity is elaborate (Robbins & Zwick 1980) or impossible.

Various measures have been developed to assess the optical condition of the lens in humans and in experimental animals. These include grading of the colour of the lens nucleus (Pirie 1968; Marcantonio et al. 1980), registration of the image forming properties of the lens (Weale 1983), normalized anatomical description of cataract (Chylack 1978, 1984; Chylack et al. 1988a; Leske et al. 1988), standardized slit-lamp photography in vivo (Dragomirescu et al. 1978; Hockwin & Dragomirescu 1981; Hockwin et al. 1982, 1988), retro illumination photography (Kawara & Obazawa 1980), quasi elastic light scattering (Tanaka & Benedek 1975; Tanaka & Ishimoto 1977; Nishio et al. 1984; Libondi et al. 1986; Benedek et al. 1987), angular distribution of scatter (Bettelheim & Ali 1985; Bettelheim & Chylack 1985), and computer aided analysis of lenses imaged against a diffuse white background (Chylack et al. 1988b). These methods have been suitable for correlating the optical condition of the lens to toxic exposures to cataractogenic agents and to biochemical changes associated with cataract. However, these methods do not allow objective classification of a lens as pathological. Furthermore, it has been argued that the sensitivity of some of the cited classification systems varies with the anatomical distribution of a light disseminating zone within the lens (Datiles et al. 1987).

There are many difficulties involved in the evaluation of light dissemination in a pathological lens. Any lens that is illuminated gives rise to light dissemination, and therefore a well defined criterion for the condition of the lens must be made on an objective basis. Since the intensity and the pattern of light dissemination are determined by the illumination and the refractive properties in the ocular lens, the illumination and the mode of examination have to be standardized. Neither the intensity nor the pattern of light dissemination in the ocular media is a measure of the imaging properties of the lens and should not be directly related to biological variables.

In toxicological assessement of risk for cataract

development it seems necessary to restrict the definition of cataract development to occurrence of pathological light dissemination in the lens, as measured under specified conditions.

The aim of the present investigation was to develop a system that allows for a rapid and objective measurement of lenticular light dissemination by using a continuous scale. It was considered important that the measure is insensitive to the localization of the opacity. Such a system would permit a statistically defined classification of a lens as pathological or non-pathological.

Materials and Methods

Measurement device

The device developed for measurement of light dissemination was comprised of an illumination source and a photometry unit (Fig. 1). The illumination source was a Wild M3 Stereomikroskop Durchlichtstativ furnished with a 7023 Philips halogen lamp (3400°K colour temperature). The light from the illumination source run in dark-field mode struck the horizontal plane of the measurement cuvette at 45°. The phothometry unit consisted of a Canon AT-1 camera house fitted with a

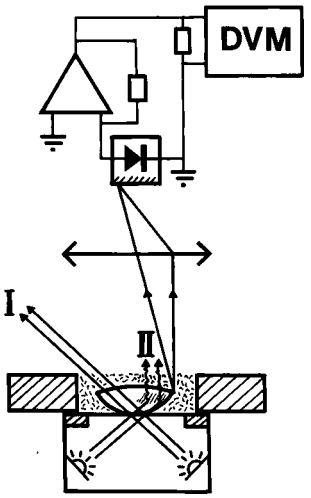

Fig. 1.
Schematic drawing of the light dissemination meter. Examined lens under dark-field illumination, I = ideally clear lens, II opacified lens. ⟷ = camera lens, —▶| = photo diode, △ = operational amplifier, DVM = digital voltmeter.

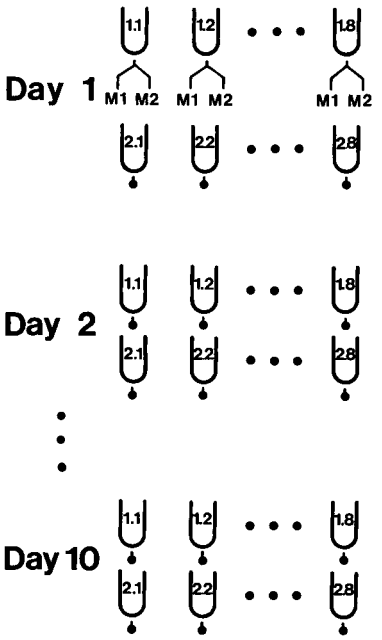

Fig. 2.
Design of the procedure for the establishment of the reference curve. The reference curve was obtained from ten pairs of dilution series. The two dilution series in each pair were independently prepared and measured in one day. Each dilution series consisted of 8 steps, and each step was measured twice (M1, M2).

12 mm extension tube and a Canon 35–70 mm zoom lens set to 35 mm focal length, 4.0 in focal ratio and infinite image distance. The camera lens projected an image of the object plane to a G1126 Hamamatshu (Japan) photo diode positioned in the film plane. The photo diode produced a current response which was linear to irradiance within the measured range.

The measurement cuvette consisted of a 3 mm thick black polyvinyl chloride sheet. A perforating hole, 7 mm in diameter, formed the sample holding cavity. The cavity was blocked with a cover glass at the lower end.

The principle for the measurements is outlined in Fig. 1. If the dark-field illumination hits a clear lens the light traverses the lens with little interaction. However, if the dark-field illumination strikes regions within the lens with a refractive index different from that of the surroundings, such regions will act as secondary light sources, disseminating the incident light diffusely. A fraction of the total amount of light disseminated in the lens was esti-

mated as the current response in the photo diode, evoked by the image of the equatorial plane of the ocular lens. The current response was amplified, voltage converted and finally presented on a digital voltmeter.

The lamp was fed with a fixed voltage in all measurements. Before each measurement the scale of the device was set to zero with the cuvette filled with 0.125 ml of balanced salt solution, BSS (Alcon, Sweden). Thereafter, the response of the photometer was calibrated substituting the cuvette with an opaque glass plate, used as a standard source of disseminated light.

Establishment of the standard

For comparison of data on light dissemination in ocular lenses a universal standard was developed by analyzing a variety of opaque solutions. A reference curve was established for the response of the light dissemination meter as a function of standard concentration. The reference curve was derived by adopting a plan of measurements outlined in Fig. 2. An aliquot of 0.125 ml of standard solution was put in the cuvette for each measurement.

The data from the standard solutions were analyzed assuming infinitely diluted light scattering solutions without absorption. The following equation was applied:

$$\Phi_S = \frac{1}{\alpha}(\Phi_i - \Phi_i e^{-klC}) \qquad 1)$$

The scattered power, detected by the photo diode, Φ_S, is a constant fraction, $\frac{1}{\alpha}$, of the total scattered power which equals the difference between the incident power, Φ_i, and the transmitted power, $\Phi_i e^{-klC}$. In this expression, e is the base of the natural logarithm, k is the scattering coefficient, l is the beam path length in the cuvette, and C is the concentration of light scattering particles. The expression was derived from basic equations for light scattering (Marshall 1978). The parameters in the model were estimated by fitting the experimental data to a non-linear regression (Snedecor & Cochran 1980a).

Measurement procedure

All the measured lenses that were defined as being non-pathological, were obtained from non-treated 6 weeks old Sprague Dawley male rats (150 g). Immediately after sacrifice, the eyes were enucleated, and the lenses were then extracted through a posterior incision in the bulb and transferred to a Petri dish with BSS (basal salt solution, Alcon, Sweden) at 20°C. Any reminiscents of the ciliary body were removed under a dissecting microscope. At this point the lens capsule was carefully inspected to assure its being intact.

The lens was then placed in the measurement cuvette and BSS was added until the cuvette was filled. The cuvette was positioned on the illumination source with the lens centered in the cuvette (Fig. 1). Since there is macroscopic similarity between the anterior and the posterior surface of the extracted non-pathological rat lens, it was not possible to standardize the antero-postero orientation of the lens in relation to the measurement device. However, the frontal plane of the lens was, by gravity, adjusted perpendicular to the optical axis of the probing optics. The lens was only illuminated during measurement.

The primary reading on the digital voltmeter was converted into equivalent standard concentration by adopting a polynomial function. The polynomial function was derived from the calibration data by regression analysis (Snedecor & Cochran 1980b). Each primary reading of light dissemination was converted to the equivalent standard concentration by solving the derived polynomial function numerically in accordance to the Newton-Raphsons method.

In order to obtain a preliminary estimate of the sources of variation, the light dissemination was measured twice for both lenses from 10 rats. An analysis of variance demonstrated that the inter-animal variation dominated. As a consequence, duplicate measurements were subsequently obtained and each measurement consisted of zero setting, then calibration against the standard source of light dissemination, and finally positioning the measurement cuvette on the source of illumination.

Statistical characterization of the non-pathological sample

To investigate the frequency distribution for light dissemination in a reference population, the lenses from another two groups of 25 and 26 rats, respectively, were examined on different days. These measurements were statistically analyzed with a *t*-test (Snedecor & Cochran 1980c) to determine if there was no difference between groups of lenses. Left and right eye differences was examined with a

paired *t*-test for the lenses from all the 61 rats (Snedecor & Cochran 1980c).

The lenses from all the rats were randomized by including the right lens from 31 rats and the left lens from the remaining rats in one group, and the contralateral lenses in the other group. The frequency distribution was estimated as a frequency histogram for each group and in order to facilitate the evaluation of the frequency distribution, the distribution function was also estimated (Draper & Smith 1980). The estimated distribution function was judged as its 'normal equivalent deviate' (NED) transform (Finney 1971). An NED transformation of an estimated distribution function for a normal distribution results in a straight line around the mean. The straight line is symmetrical around 0 at the ordinate. The extremes usually deviate from the straight line because of the low precision in the determination of rare events. Deviation of the estimated frequency histogram from a normal distribution was tested for with a χ^2-analysis (Snedecor & Cochran 1980d). This test was based on the data of the group that gave rise to the highest estimated mean.

Estimation of the tolerance limit for non-pathological light dissemination

The tolerance limit was set based on the condition that 5% of examined lenses are allowed to be classified as pathological although they are non-pathological. The tolerance limit was estimated (Beyer 1966) from the same data as those that were used for the distribution test.

Analysis of the sources of variation

In order to estimate the components of variation in the measurements of the entire sample of lenses, the experimental data were planned to be analyzed according to the following model (Snedecor & Cochran 1980e):

$$x_{ijk} = \mu + A_i + B_{j(i)} + \varepsilon_{k(ij)} \qquad 2)$$

The light dissemination in an individual lens, x_{ijk}, equals the sum of the expected total mean, μ, a factor for the variation among rats, A_i ($i = 1,..,61$), a factor for the random variation among eyes within rat, $B_{j(i)}$ ($j = 1,2$), and a factor for the experimental error, $\varepsilon_{k(ij)}$ ($k = 1,2$).

Selected probabilities in the statistical analyses

Significance levels and confidence coefficients were set to 5 and 95%, respectively.

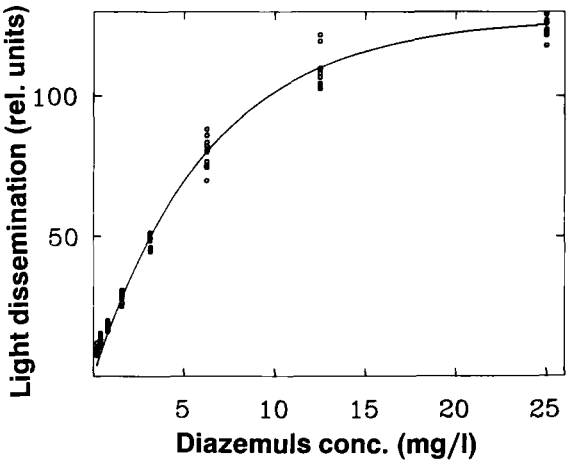

Fig. 3.
The response of the light dissemination meter expressed as a function of Diazemuls concentration. O = the mean response at a certain concentration of Diazemuls within the pair of dilutions prepared in one day. — = the best fitting non-linear regression according to an exponential model.

Results

Characteristics of the standard

Comparative measurements of light dissemination in ocular lenses necessitate a defined universal standard for calibration. To meet with this demand a variety of established standards were examined. An accepted standard for measurement of turbidity in water (Swedish Commission for Standardization 1974) was tested, but abandoned because it produced light dissemination of much lower intensity than that occurring in opacified lenses. Finally, a commercially available emulsion, Diazemuls (Kabi Vitrum, Sweden) was adopted for calibration. Diazemuls is a licensed preparation of oil in water emulsion with diazepam dissolved in the oil phase.

The fitting of the calibration data (Fig. 3) with non-linear regression, in accordance with the assumed model (Eq. 1), resulted in the following expression:

$$\Phi_S = 128 - 128e^{-0.157C} \qquad 2)$$

Here, Φ_S (rel. units) is the response of the light dissemination meter and C (mg/l) is the concentration of Diazemuls. At very high concentrations of Diazemuls the intensity of forward light dissemination decreased.

The light dissemination in non-pathological

lenses corresponded to the lowest concentrations of Diazemuls in the calibration solutions. The exponential expression obtained by non-linear regression (Eq. 2) deviated slightly from the experimental data at low intensities of light dissemination (Fig. 3). For conversion of recordings of light dissemination in lenses from relative units to equivalent Diazemuls concentration (mg/l), EDC, it was therefore decided to fit a polynomial function. The expression

$$R = 5.84 + 15.69C - 0.65C^2 + 3.39 \times 10^{-4}C^4 \quad 3)$$

proved to be an adequate approximation of the response, R, of the digital voltmeter at different concentrations, C, of diazemuls below 15 mg/l. Further terms were attempted but did not significantly improve the fit. No effort was made to improve the fit for the readings of higher concentrations of Diazemuls since primary measurements on cataractous lenses always gave signals below 15 EDC.

Statistical characteristics of the non-pathological sample

The 95% confidence intervals for the means of the groups examined on different days were 0.584 ± 0.095 (n = 26) and 0.475 ± 0.077 (n = 25) EDC. These data implicate that there is no statistically significant difference of light dissemination between groups (test statistic = 1.83, $t_{49;0.975} = 2.01$).

There is no systematic difference of light dissemination between the right and the left lens as judged from a 95% confidence interval for the mean difference between the right and the left lens within animal, 0.001 ± 0.070 EDC.

The analysis of the estimated frequency distributions for the two groups of independent observations of lenses indicated a slight skewness towards higher values. The NED transform for each of the estimated distribution functions obtained for readings expressed in EDC were asymmetrical around 0 at the ordinate (Fig. 4). However, when the readings, C (EDC), were transformed to ${}_tC$ (${}_t$EDC) according to the following expression:

$${}_tC = \log_{10}(C + 1) \quad 4)$$

the NED transforms for the estimated distribution functions became approximately symmetrical around 0 at the ordinate (Fig. 4). The distribution test for light dissemination in lenses expressed in ${}_t$EDC (Fig. 5) did not show any significant deviation from a normal distribution (test statistic = 7.41, $\chi^2_{5;0.95} = 11.071$). For this reason all readings of

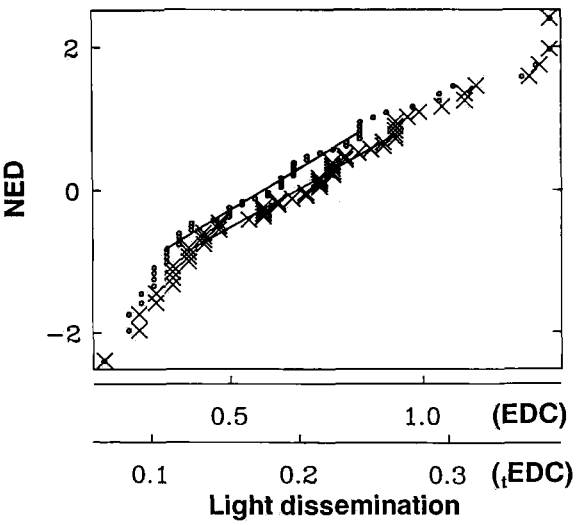

Fig. 4.
The normal deviate transform (NED) of an estimated distribution function for the light dissemination in a reference sample of non-pathological rat lenses, each observation being one lens from one rat. ○ = , C (equivalent Diazemuls concentration, EDC). × = transformed readings, $\log_{10}[C + 1]$ (${}_t$EDC). — = corresponding linear regressions for observations within 1 NED.

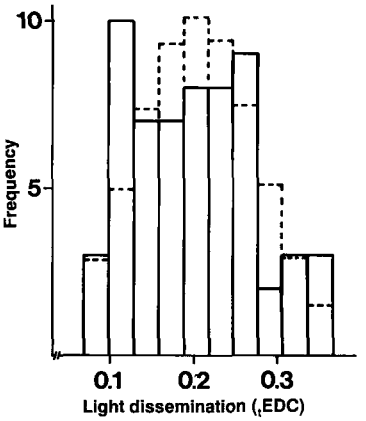

Fig. 5.
The estimated frequency distribution for the mean light dissemination in non-pathological lenses (—), one observation being one lens from one rat, and the expected frequency distribution (– –) as predicted by a corresponding normal distribution. n = 61, class range = 0.0298 ${}_t$EDC (transformed equivalent diazepam concentration).

Table 1.
Analysis of variance for the light dissemination in rat lenses.

Source of variation	Degrees of freedom	Mean square ($\times 10^{-1}$ $_t$EDC)^2	Expected mean square
Animals	60	1.52	$\sigma^2_\epsilon + n\sigma^2_B + bn\sigma^2_A$
Sides	61	0.490	$\sigma^2_\epsilon + n\sigma^2_B$
Measurements	122	0.031	σ^2_ϵ

σ^2 = the expected variance for the indexed source.
A = animals, B = sides (right and let eye), ϵ = measurements.
a = number of animals = 61, b = number of sides within animal = 2,
n = number of measurements within side = 2.

light dissemination expressed in EDC have been transformed in accordance to Eq. 4 in the following analyses. The estimated mean and the estimated standard deviation for light dissemination in rat lenses, as expressed by the adopted transform (Eq. 4), were 0.2035 $_t$EDC and 0.0751 $_t$EDC, respectively.

Tolerance limit for non-pathological

Adopting the estimated mean and standard deviation the 95% tolerance limit for non-pathological light dissemination, was found to be 0.35 $_t$EDC corresponding to 1.24 EDC.

Sources of variation

The results of the analysis of the sources of variation, in the determination of light dissemination in non-pathological rat lenses, has been outlined in Table 1. The variance components in a random measurement of the light dissemination in a lens were estimtated to 2.57×10^{-3} ($_t$EDC)^2 for animals, σ^2_A, 2.30×10^{-3} ($_t$EDC)^2 for sides, σ^2_B, 0.31×10^{-3} ($_t$EDC)^2 for measurements, σ^2_ϵ. A 95% confidence interval for the mean, μ (Eq. 2), light dissemination for lenses was estimated to 0.191 ± 0.016 $_t$EDC corresponding to [0.50;0.61]EDC.

Discussion

Measurements of light dissemination in the lens

Investigations of lens toxicity demand a suitable method for the differentiation between non-pathological and pathological lenses. Existing methods for classifying cataracts have been developed with the aim to correlate subgroups of cataracts to toxic doses of cataractogenic agents or to biochemical changes associated with cataract.

Since it is the intensity of light disseminated forwards in a lens that impairs vision in the cataractous eye and the intensity of light disseminated backwards is lower (Bettelheim & Ali 1985) the measurements of the present study were focused on light disseminated forwards.

The pixel representation of a frontal view of a lens imaged against a white background, has been adopted for the establishment of a tolerance limit for statistically not significant cataract (Chylack et al. 1988b). In that study only variability in the measurement method was considered since the aim was to detect changes in a specific lens. In the present system the goal was to define a tolerance limit for non-pathological light dissemination, that is applicable for a randomly selected lens, and therefore the variability among lenses from different animals was necessary to take into account.

Hazards in the standardization of the measurements

Due to the strong wavelength dependence of the intensity of scattered light, it is crucial that the temperature of the source used for illumination is kept constant. This was accomplished by feeding the lamp with a fixed voltage. Furthermore, since the intensity of scattered light depends on the size of the scattering particle and the refractive index gradient between the scattering particle and the surroundings, slight variations in these parameters may cause variability in the standard. However, the selected standard is a pharmaceutical preparation manufactured under strict control and preliminary measurements of light dissemination on dis-

tinct batches of Diazemuls did not show any significant difference.

The adopted model for light dissemination, measured as a function of Diazemuls concentration (Eq. 1), assumes an ideally diluted solution without absorption. At very high concentrations a reduction of the intensity of scattered light is expected (Benedek 1971; Delaye & Tardieu 1983). However, in the data plotted in Fig. 3 the concentration of Diazemuls was lower than that giving rise to a maximum intensity of light scattered. The reduction observed at very high concentrations of Diazemuls may, to some extent, have been caused by absorption. Furthermore, at high concentrations of scattering particles, secondary scattering will diminish the power of light scattered forwards to less than a linear fraction of the total power of light scattered and the adopted model (Eq. 1) no longer holds.

The model for the regression used to derive the parameters of Eq. 1, assumes that there are only measurement errors. However, the variability of the light dissemination detected at a certain concentration of Diazemuls is also dependent on the dilution error. Yet, in the present investigation the dilution error was negligible in relation to the error for determination of light dissemination .

The derived expression for light dissemination from different concentrations of standard (Eq. 2), described most of the experimental data reasonably well. However, at a very low concentration of Diazemuls the estimated regression indicated slightly higher relative light dissemination than was actually recorded (Fig. 3). This deviation probably originates from a greater weight of the higher values over that of the lower values in the regression analysis. A polynomial function was fitted for the conversion of direct relative readings to EDC because the deviation from the regression equation occurs at around the level of light dissemination as expected in lenses from untreated animals.

Hazards in the measurements of lens light dissemination

Since the light dissemination in an opacified lens varies with dissemination angle (Bettelheim & Chylack 1985) comparisons to the present measurements can only be obtained with a similar illumination device and equivalent imaging optics. The dependence of the recorded response on the sensitivity of the photo diode and the amplification is overcome by the conversion of readings to EDC (Fig. 3).

When light dissemination is measured in a lens, a fraction of the illuminating light and the light from secondary light dissemination, respectively, is refracted at the anterior and at the posterior surface of the lens and homogeneously within the lens. This changes the vergence of the incident as well as the exiting light and therefore alters the power of light collected in the photo diode. Consequently, the response in the measuring device deviates systematically from the response evoked by an equivalent power of light dissemination originating from the calibration solution. However, this difference is consistent for a homogeneous population of measured lenses.

The findings that the skew frequency distribution for light dissemination expressed in Diazemuls concentration equivalents, C, was made approximately symmetrical through transformation according to $\log_{10}[C+1]$ (Fig. 4), and that the transform did not deviate significantly from a normal distribution (Fig. 5), suggest that all readings sin concentration equivalents should be transformed before treatment with normal distribution statistics.

Significance of light dissemination detected in lenses

According to microradiographical data on the protein distribution in the non-pathological lens there are no local sharp shifts of refractive index in the lens matter confined to volumes bigger than the wavelength of light (Philipson 1969). It is therefore probable that small particle scattering and Mie scattering are the main sources of light dissemination in the non-pathological lens. On the assumption that the concentration of scattering centers is low, the measured power of light disseminated forwards is exponentially dependent on the concentration of scattering centers (Eq. 1). If the recordings of light dissemination in the lens are analyzed according to the model that was adopted for the measurements of the standard (Eq. 1), it is necessary to generalize the exponential term to $e^{-(k_1C_1 + k_2C_2..,k_nC_n)}$. In this expression C_i refers to different species of light scattering centers and k_i signifies the corresponding scattering coefficients.

To interpret the intensity of light disseminated from an ocular lens, to a quantity of disturbed anatomy is not only difficult but also hazardous. A

small alteration of the water balance within the tissue changes the refractive properties (Barer & Joseph 1954) and may induce considerable light dissemination. The current device measures a fraction of the integrated amount of light disseminated forward from a probed lens. Information about the distribution of light disseminating zones within the lens is therefore lost. It is anticipated that these basic measurements will be easy to analyze statistically.

Similarly, with all other methods for cataract detection, the presently described method does not give direct information on the disturbance of the image formation. The recording of light dissemination is a measurement of a quantity which interferes with the image formation.

Estimated tolerance limit for non-pathological

The currently adopted registration of the condition of the lens on a continuous scale enables formulation of a normal distribution. The estimated mean and standard deviation of light dissemination expressed in $_{1}$EDC were adopted to settle a tolerance limit for a non-pathological lens. The selected probability for classification of a non-pathological lens as pathological was 5% and implicates a high specificity but a low sensitivity for detection of lenses with pathological light dissemination. Consequently, only a small number of individuals are needed to detect pathological light dissemination in an experimental group.

Significance of the sources of variation

The analysis of variance for light dissemination in estimations on the reference sample of rats (Table 1) elucidated the variability among animals and the variability among sides to be the main sources of variation. The significance of the measurement error in determinations of means for animals is small. This is indicated by the fact that the mean square for measurements constitutes a small fraction of the mean square for animals (Table 1). The probably random antero-postero orientation of the lenses during measurement contributes to the variation among individuals (Table 1). If increased precision is required, a greater number of animals have to be used.

Applicability of the developed method

Data obtained in experimental toxicology are supposed to fit a continuous dose-effect function or a quantal dose response relation (Mays 1988). An adequate model for the development of light dissemination in lenses has to be determined for each toxic agent. The currently derived tolerance limit for non-pathological light dissemination may be applied as a threshold for pathology if a continuous dose-effect function is applicable, and as an objective basis for the quantal classification if the dichotomous dose-response model is the appropriate. The developed method is objective and permits the large number of observations needed in toxicological risk assessment studies. It is therefore anticipated that the presented method will be a powerful tool in toxicological risk assessment studies.

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