The Nature, Significance, and Evaluation of the Schwarzschild-Villiger (SV) Effect in Photometric Procedures*

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ABSTRACT

The Schwarzschild-Villiger effect has been experimentally demonstrated with the optical system used in this laboratory. Using a photographic mosaic specimen as a model, it has been shown that the conclusions of Naora are substantiated and that the SV effect, in large or small magnitude, is always present in optical systems. The theoretical transmission error arising from the presence of the SV effect has been derived for various optical conditions of measurement. The results have been experimentally confirmed. The SV contribution of the substage optics of microspectrophotometers has also been considered.

A simple method of evaluating a flare function $f(A)$ is advanced which provides a measure of the SV error present in a system. It is demonstrated that measurements of specimens of optical density less than unity can be made with less than 1 per cent error, when using illuminating beam diameter/specimen diameter ratios of unity and uncoated optical surfaces.

For denser specimens it is shown that care must be taken to reduce the illuminating beam/specimen diameter ratio to a value dictated by the magnitude of a flare function $f(A)$, evaluated for a particular optical system, in order to avoid excessive transmission error. *It is emphasized that observed densities (transmissions) are not necessarily true densities (transmissions) because of the possibility of SV error.*

The ambiguity associated with an estimation of stray-light error by means of an opaque object has also been demonstrated.

The errors illustrated are not necessarily restricted to microspectrophotometry but may possibly be found in such fields as spectral analysis, the interpretation of x-ray diffraction patterns, the determination of ionizing particle tracks and particle densities in photographic emulsions, and in many other types of photometric analysis.

I. INTRODUCTION

Microspectrophotometry has contributed significantly to advances in biology since the publication in 1936 of Caspersson's classic work in ultraviolet microscopy (3). Other optical methods such as visible light spectrophotometry (16, 19), phase (2) and interference microscopy (4), and microdensitometry with the x-ray absorption technique (5, 6) have all employed photometric procedures and have contributed information of importance to biology.

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Criticism indicating limitations or deficiencies of these methods has been made (7), particularly by Naora (9–12) who suggested that many of the reported studies using stained sections and visible light photometry have not included a consideration of a phenomenon, known to the astronomer as the Schwarzchild-Villiger (SV) effect, whereby photometric measurement of a small dark zone in a larger lighter area gives an erroneous, higher transmission value for the dark zone because of light scattered into the measuring beam. If Naora's criticism were true, serious reconsideration of many important, recently acquired concepts in biology would be in order.

In an evaluation of the x-ray absorption technique (5, 6) the SV effect was studied. The procedures used and the conclusions reached here may have pertinence to other photometric studies because of the necessity of evaluating properly the existence and significance of the SV phenomenon in photometric procedures.

II. THE SCHWARZCHILD-VILLIGER EFFECT

Schwarzchild and Villiger (18) measured the optical density of a series of photographically recorded images of the ultraviolet radiation of the sun's disc. They found on measuring the transmission of a very dark portion of a photographic plate in the immediate vicinity of a lighter region that a transmission error was observed. They assumed that bright light passing through the lighter region of the field was, in part, reflected at the objective of the observing microscope and some of the reflected light illuminated the upper surface of the dark portion of the plate and, thereby, added light to the amount transmitted through the dark area. The effect was strongly marked near the edges of the sun's image, leading to an error in the calculated radiation intensity of a −5 per cent when using a clean objective. A dust-covered objective, which diffusely reflected more light back on to the plate, gave errors as large as −50 per cent. The error was finally eliminated by placing over the plate a black disc possessing a small aperture only slightly larger than the measured area. Effectively, the size of the illuminating light beam had been reduced to that of the area whose density was to be measured.

In 1950 Swift (19) studied the absorption of light by Feulgen-stained nuclei by densitometry and interpreted the residual transmission error in terms of stray-light. Small regions of spherical nuclei were measured with the lamp diaphragm and the substage condenser diaphragm at various diameters. Swift stated that he obtained the density for different points on the sphere, as expected from geometrical considerations of the thickness, *only when the light source and substage condenser diaphragm openings were small.* When the diaphragms were wide open, transmission readings taken towards the periphery of the nucleus tended to be too high (similar to the original SV observation). With the source diaphragm reduced to 1 mm. diameter, much of the glare and scattered light from the surrounding field was reduced and the observed density was increased by as much as 30 per cent over that obtained with the wider lamp diaphragm (a transmission error of 100 per cent). Whether or not the stray-light error had become negligible at the 1 mm. opening was not stated. The measured density of nuclei was also found to increase as the numerical aperture (N.A.) of the condenser was decreased.

Naora (9) called attention to the SV effect and suggested that many investigators were illuminating too large an area of the specimen and, as a result, the microspectrophotometer data previously published were unreliable. Naora measured the transmission of small spheres of safranin, 2 to 30 μ in diameter, suspended in cedar oil. A constant light source image of 2 μ diameter, measured at the centre of the sphere, was employed. Using the Lambert-Beer $T = e^{-\epsilon C2r} = 10^{-D}$ (in which T is the transmission (20 to 90 per cent), C the concentration (0.03), ϵ the extinction coefficient, and the density $D = \epsilon C2r/ln_e10$), Naora determined the value of C as a function of the diameter ($2r$) of the spheres. He showed that a constant value of C was not obtained until the illuminated area was smaller than $\frac{1}{3}$ the diameter of the sphere. For a sphere of 2.2 μ diameter, with the 2 μ beam, a measured result of $C = 0.009$ was obtained. This value would correspond to an error of over 300 per cent in the density and it was interpreted by Naora as a result of the SV effect.

Later, Naora (10), among others, extended the concept of the SV effect into an equation giving the ratio, θ, of the "flare" flux to the total transmitted light flux:

$$\theta = \left[\frac{1-r}{1+(m-1)r} - (1-r)^m\right] \Big/ \frac{1-r}{1+(m-1)r}$$

in which m = the number of air/glass surfaces and r = the average reflectance of each air/glass surface within the lens system. The stray-light resulting from internal air/glass optical surfaces was designated as *optical flare.* Light reflected from the tube walls, and other non-optical surfaces, was defined as *mechanical flare.* The optical flare was considered to be responsible for the SV effect.

Naora calculated the value of θ as a function of m for $r = 0.05$ (uncoated surface) and $r = 0.01$ (coated surface). Using 13 optical surfaces from specimen to image plane ($m = 13$), θ was found to be 13.5 for uncoated and 0.72 for coated surfaces. Naora believed that this was the "saturated" SV effect for a light beam filling the whole of the objective field. Therefore, a specimen of true transmission $T = 1$ per cent would give an apparent transmission of 14.5 per cent. To test his hypothesis Naora measured the transmission of various spherical cell nuclei, 4 to 6 μ in diameter, using illuminating beams of from 1 to 650 μ in diameter. The results again showed that the correct transmission values were obtained only when the illuminated area was less than $\frac{1}{3}$ the diameter of the nucleus. Measurement with wider illuminated areas led to larger error.

The first result of Naora (9) gave an error of 300 per cent for a true transmission of 20 per cent when the illuminating beam and sphere were of equal diameter. In a later paper (10) a true transmission of 10 per cent gave an error of only 20 per cent when the nuclei to illuminating beam ratio was again unity.

The possible significance of this discrepancy will be demonstrated below.

The first denial of Naora's suggestions was made by Ornstein and Pollister (14). They stated that Swift (19) had prescribed conditions for the purpose of reducing glare to a low level, and that a total glare error of less than 3 per cent of the focused intensity could be assured. However, with Swift's maximum measured apparent density of 1.71 (*i.e.*, an apparent transmission of 1.95 per cent) a 3 per cent glare would result in a transmission error of 150 per cent. The true density (transmission) error may have been even larger. Actually, the extent of any flare error and the applicability of the Naora flare relation, θ, will depend entirely upon the operating conditions of measurement of the individual instrument. In the extreme case, as an upper limit, the θ equation provides, in the saturated condition, a reasonable basis for calculation, notwithstanding the objection of Ornstein and Pollister to the use of the θ relation on the basis of the glass-to-air surfaces not being parallel. Ornstein and Pollister also referred to the fact that Naora employed a condenser of large N.A. (1.25), as compared with those employed in former microdensitometry measurements, and suggested that part of Naora's large flare error arose from the use of a condenser with too large a numerical aperture. Swift used a condenser and objective of N.A. 1.4 but the condenser aperture was stopped down to 4 mm. and the actual N.A. was not specified. Under those conditions Swift found a decrease in the measured transmission as the angle of the illuminating cone was decreased. Using various condenser angles to produce a fixed 1 μ diameter light beam, Naora (11) subsequently found that the measured transmission of a single rat liver cell was independent of the illuminating cone angle between 63 and 110° (~N.A. 0.80 → 1.25).

In consideration of the SV problem Lison (8) re-examined his histophotometer for possible SV errors. Since an opaque object should give zero transmission with a SV-free instrument, Lison measured the transmission of heavily overstained blood cells and particles of lamp black, 1 to 20 μ in diameter, mounted in Canada balsam with a field diaphragm closed down to give an illuminated area in the object plane of 150 μ diameter with Köhler illumination. With the field photometric aperture diaphragm fully opened he obtained an apparent transmission of 2 per cent for the opaque test object, and Lison stated that even large changes in the condition of illumination did not produce a lower value. Two other objectives tested under the same conditions gave 5 per cent flare light.

Lison concluded that for his instrument the SV error was not important, since his measured transmissions were between 25 and 85 per cent. Closing the aperture of his condenser did not appreciably reduce the SV effect, thus confirming the observations of Naora (11).

III. CRITIQUE OF NAORA'S STUDIES

In Naora's original experiment (9) liquid spheres of extinction coefficient ϵ were dispersed in cedar oil, with an image of a field stop focused at the centre of each sphere. The Lambert-Beer relation was then used to determine the concentration C of the sphere of radius r. This equation was experimentally confirmed with a limitation that the size of the reduced image of the light source should be smaller than $\frac{1}{3}$ the diameter of the sphere. Divergencies in the calculated value of C, which appeared to be a function of the ratio of the illuminating beam diameter (IBD) to the object sphere diameter (OD) were interpreted as arising from the SV effect. For brevity this ratio will be referred to as the (IBD/OD) ratio.

The small spheres of diameter 2 to 30 μ were illuminated by a 2 μ diameter light image formed at the centre of each sphere. Naora stated that when the reduced image of the light source is formed at the centre of the sphere and when the dimension of the image is small compared with the diameter of the sphere, the optical length of any light beam through the sphere is equal to the diameter of the sphere and is independent of the illuminating cone angle (Fig. 1 *A*). Hence one can use the Lambert-Beer relation to determine T. This relation is certainly true for small images. But the condition of a 2 μ diameter beam at the centre of a 2.2 μ diameter sphere does not satisfy the postulated requirement of point-convergent light, and it is under this condition (of the light beam diameter approaching the spherical object diameter) that the SV error apparently became large. At the other extreme is the sphere illuminated by a parallel beam of light (Fig. 1 *B*). Actually, Naora's conditions of illumination will lie somewhere between the two extremes of parallel and point-convergent light (*vide infra*).

An examination of the transmission of a parallel light beam through an absorbing sphere of absorption coefficient k (Appendix A) shows that the observed transmission T is highly dependent on the value of r_1/r and kr. With $kr = 2.00$, the calculated transmission T is evaluated as a function of r_1/r in Fig. 2 *A*.

If one calculates a density $D = \log_{10}(1/T)$, (in which D corresponds to Naora's $\epsilon C2r/\ln_e 10$) he obtains the density curve (*3*) of Fig. 2 *B*. The measured density or transmission does not therefore assume a constant value until the light beam diameter is almost $\frac{1}{3}$ the sphere diameter. The observed variation in density could be interpreted as a flare error in the system. The ratio of density D to limiting density D^1 has been plotted as a function of r_1/r in curve (*2*) of Fig. 2 *B*. For comparison, the ratio of apparent concentration C to the limiting concentration C^1 derived by Naora is also plotted in this diagram, curve (*1*) Fig. 2 *B*. The limiting value of Naora's transmission corresponds to a density of approximately 0.7

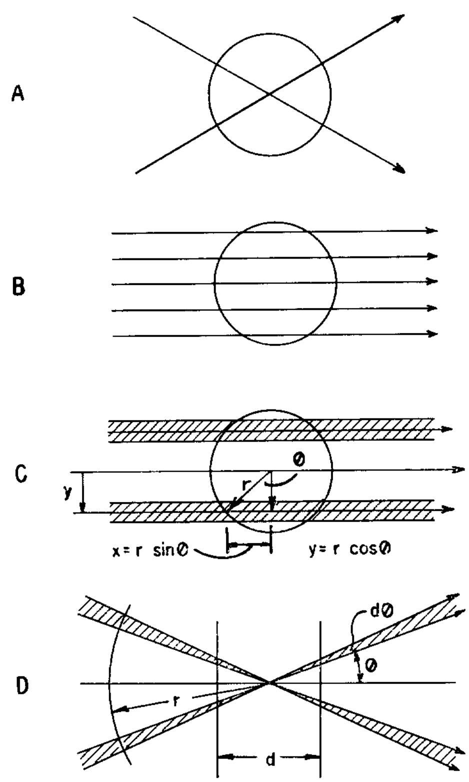

FIG. 1. Optical pathways of: *A*. A sphere illuminated by point-convergent light. *B*. A sphere illuminated by parallel light. *C*. A sphere illuminated by an annular element of a parallel beam at radius *y*. *D*. A parallel-sided section illuminated by point-convergent light.

(T = 20 per cent). Comparison with our calculations for $kr = 2$ (limiting $D^1 \sim 1.7$) shows a very similar deviation in the value of C/C^1 and D/D^1 from the limiting value of unity reached when $r_1/r \rightarrow 0.3$.

The relatively large SV error first noted by Naora in the spherical drop experiment may thus in part arise from the use of an inappropriate mathematical relationship in deriving the concentration C. For spherical specimens it is only when the ratio $r_1/r \rightarrow 0.3$ that the simplified Lambert-Beer law of $T = e^{-\epsilon C 2r}$ becomes applicable. For values of $r_1/r > 0.3$ the equation (1) derived in Appendix A should be used to calculate the constant value of k from the observed transmission T. On the assumption of a Lambert-Beer law we are thus led to the same general conclusion as Naora; *i.e., that the measured density or transmission would not assume a constant value until the light beam diameter is about* $\frac{1}{3}$ *the sphere diameter.* Evaluation of the transmission of convergent light through a parallel-sided section (Appendix B) shows that there is a counterpart inadequacy in the Lambert-Beer relation. It is shown in Appendix B that the observed transmission is a function of the illuminating condenser or limiting aperture of the optical system. Errors in apparent transmission may thus be produced by varying illuminating cone angles, which may then subsequently be interpreted as flare error.

We conclude that the controlling parameters, *i.e.*, area of illumination controlled by field stop and cone of illumination controlled by aperture stop, can produce apparent transmission errors of a magnitude dependent upon the object shape and the exactness of the mathematical relation used to relate observed transmissions to the derived quantity (such as dye content). On the assumption of a simple Lambert-Beer relation defining a constant transmission, convergent light will produce an apparent transmission error with a parallel-sided specimen; parallel light will produce an error with a spherical specimen. Critical considerations thus require that it is not enough merely to reduce the aperture stop in making transmission measurements of spherical objects. The ratio of beam/sphere diameters (IBD/OD) must also be reduced to at least 1:3, in this special case of $kr = 2$.

Both Swift (19) and Naora (9) appear to have approached these operating conditions but it is not possible to say that their measuring conditions were such as to ensure measurement of a true density based on a Lambert-Beer relation. Both authors found it possible to vary the measuring conditions and produce different results. The attainment of a constant value of measured concentration by Naora (9) for small ratios of IBD/OD, which agreed with the value derived from bulk concentration, suggests that no systematic error remained.

IV. STRAY-LIGHT DEFINITIONS (FIG. 3)

If it can be assumed that one has devised a measuring system such that a known, applicable, mathematical relation exists between the true transmission and the parameter required, *e.g.*, density of photographic image or dye content of stained nucleus, and it is further assumed that chemical factors (such as proportionality factors between stain and substance specific for that stain), optical distribution errors (inhomogeneity of dye distribution in the specimen), optical geometry (shape and thickness of specimen), and many other known variables have been corrected for, any further optical transmission error may be said to arise from "stray"-light. For our discussion it is necessary to review the classic definitions of optics. The phenomena may be classified as follows: *absorption*, conversion of light energy into thermal energy of a medium: *diffraction*, an apparent bending of a light beam around an obstacle; *scattering*, an abstraction of light energy from the direction of propagation and the re-emission of this energy in other directions; *refraction*, an abrupt change of direction of a light beam at the boundary between two media of different

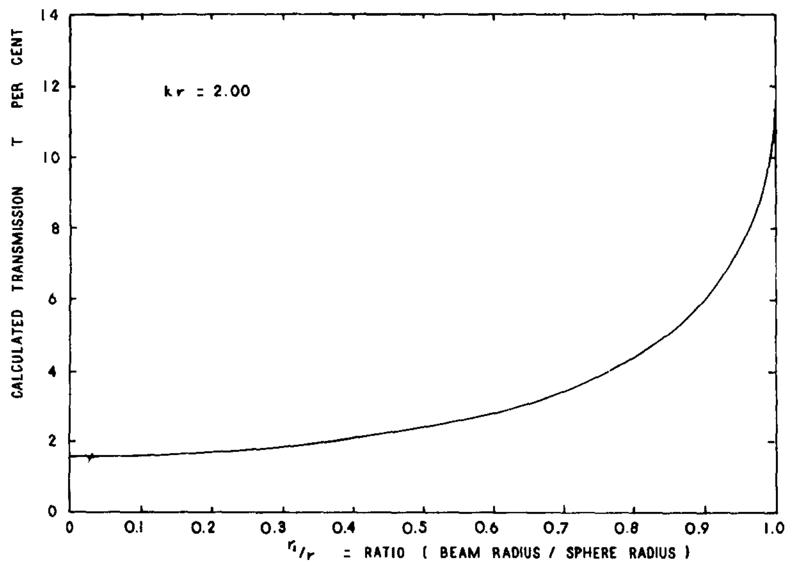

FIG. 2 *A*. Calculated transmission T of a parallel beam of light of radius r_1 through an absorbing sphere of absorption coefficient k and radius r as a function of r_1/r ($kr = 2.00$).

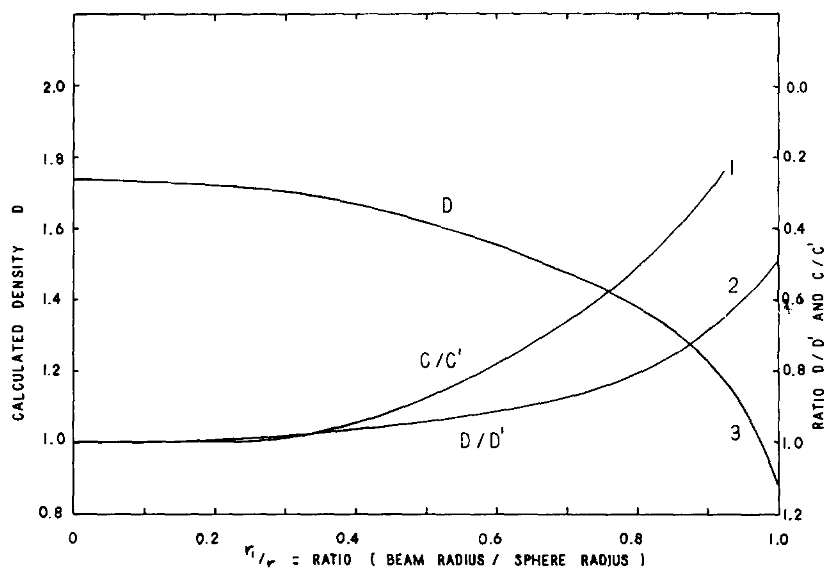

FIG. 2 *B*. (Curve *3*) Calculated density of a sphere of radius r illuminated by a parallel light beam of radius r_1 as a function of r_1/r ($kr = 2.00$). The density has been evaluated on the basis of a Lambert-Beer Law $D = -\log_{10} T$, in which T is the calculated true transmission given by the curve of Fig. 2 *A*. The variation in observed density could be interpreted as a flare error in the optical system. D approaches a constant limit of D^1 when $r_1/r \rightarrow 1/3$.

(Curve *2*) Ratio of apparent density D to limiting density D^1 as a function of r_1/r.

(Curve *1*) Ratio of apparent concentration C to limiting concentration C^1 derived by Naora (9) from measured transmissions and application of the simple Lambert-Beer Law $T = e^{-\epsilon C 2r}$. Naora interpreted this change in the concentration as a function of the IBD/OD ratio as arising from the SV effect. The similarity with curve (2) shows that it could have arisen from use of an inappropriate mathematical relationship used in deriving the concentration C.

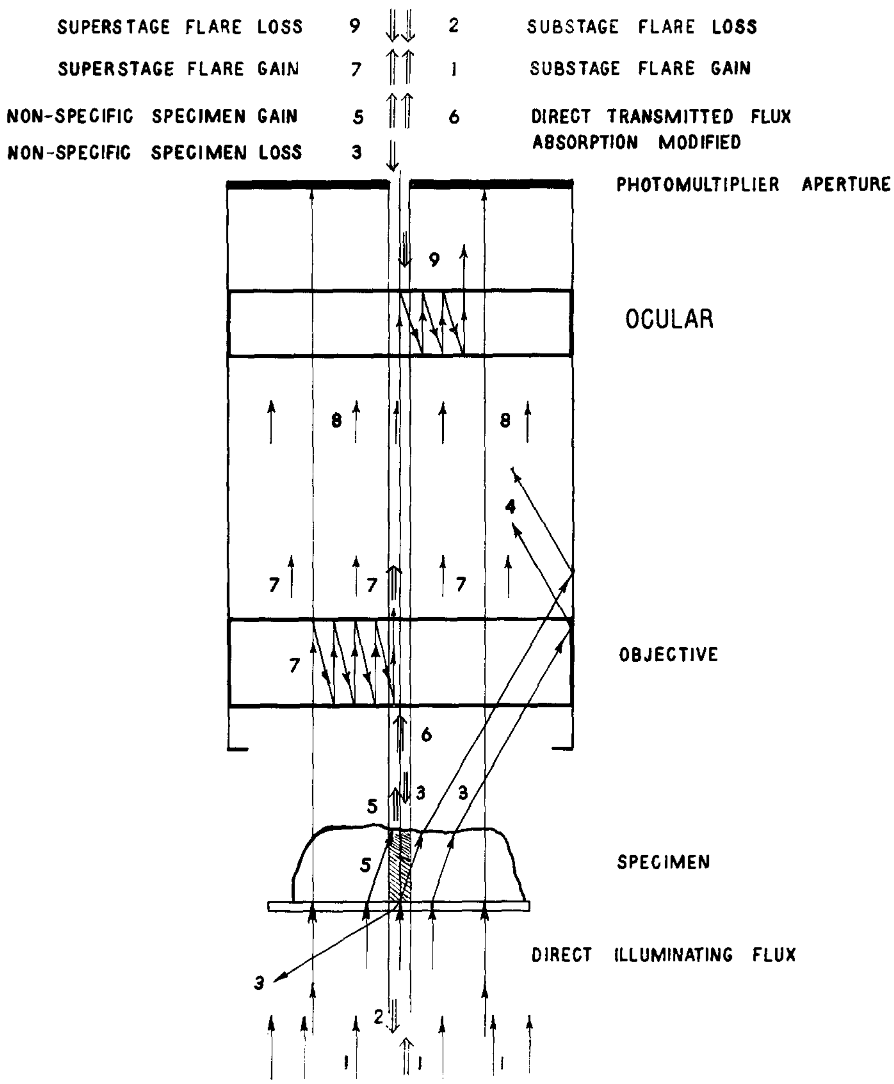

FIG. 3. Diagrammatic illustration of the transmission of light flux through an optical system and specimen showing the origin of stray-light components. The main flare components are represented by loss or gain flux vectors placed at the top of the diagram. *4* is an example of mechanical flare generation. *8* represents the flow of the compounded loss and gain flare flux through the optical system.

refractive indices arising from the different velocities of light in the two media; *reflection*, the turning back of a light beam into a medium through which it has originally traveled.

Light propagated through a uniform isotropic particulate medium can be represented by the intensity relations $I_d = I_0 e^{-\alpha d}$, in which I_0 and I_d are the intensities at distance zero and d, respectively, and α $(= \alpha_a + \alpha_s)$ is an apparent absorption coefficient. α_a defines a true absorption and α_s defines a scattering coefficient, and may be considered as including diffraction, refraction, and reflection losses as well. The light loss resulting from a finite value of α_s is often referred to in the examination of biological samples as the "*non-specific specimen light loss.*" It derives from the particulate nature of cellular material and the variations of refractive index within a specimen. It is possible that transmission measurements on an unstained blank can separate α_s from α_a. If not, the light diffracted, scattered, refracted, and reflected by the specimen out of the measuring light beam will lead to an unknown decrease in the transmission and must be considered a component of stray light. The *non-specific specimen light loss* x_1 will always *decrease* the apparent transmission, since the observed transmission will be $(I_0 - x_1I_0)\sigma/I_0 = \sigma(1 - x_1)$, in which σ is the true specimen transmission.

Scattered, diffracted, reflected, and refracted light lost from the measuring beam in traversing an optical system will be referred to as the *"flare light loss x_2."* It will produce no transmission error if present in the superstage optics since the light deflected out of the measuring beam had previously been attenuated in its transmission through the specimen. Hence the observed transmission is $(I_0\sigma - I_0x_{2sp}\sigma)/(I_0 - I_0x_{2sp}) = \sigma$ and is independent of the superstage flare loss term x_{2sp}. If the flare loss is present in the substage optics, however, the substage flare loss (x_{2sb}) increases the background illumination at the photomultiplier, leading to a transmission ratio $[(I_0 - x_{2sb}I_0)\sigma + yx_{2sb}I_0]/[(I_0 - x_{2sb}I_0) + yx_{2sb}I_0]$. It thus involves x_{2sb} and σ, and serves to *increase* the apparent transmission. y is a factor which takes care of the flare intensity translation from object plane to photomultiplier aperture.

Scattered, diffracted, reflected, and refracted light may enter the measuring beam from the relatively unobstructed regions of the larger illuminated field surrounding the measuring area. It, too, consists of a component associated with the optical system, referred to as the *"flare light gain x_3,"* and a component associated with the specimen, referred to as the *"non-specific specimen light gain x_4."* Both the former, developed in either the superstage or the substage optics, and the latter can produce an increase in the apparent transmission of the specimen, since in both cases the deflected light component, $+x_3I_0$, for example, ultimately appears at the photomultiplier as an increase n the background illumination. The observed transmission ratio will then be $(I_0\sigma + y\,x_3I_0)/(I_0 + y\,x_3I_0)$ and again the error will involve both σ and x_3.

The flare components of stray-light may be subdivided into a component arising from reflections at mechanical or non-optical surfaces, defined as *"mechanical flare"* (11, 12), and an optical component, *"optical flare,"* arising from scattered, diffracted, refracted, and multiply-reflected light at and between air/glass optical surfaces or at defects within a glass lens. The only significant optical flare component is, therefore, one which produces an increase in the apparent transmission. The total integrated substage and superstage flare light which appears at a photomultiplier aperture will subsequently be referred to as the flare light. The non-specific specimen light gain, which is light gained from the illuminated field surrounding the measuring area, may be considered a special case of optical flare arising in the specimen. It is referred to by Ornstein and Pollister (21) as "specimen glare." The reduction of non-specific specimen light loss and gain in biological specimens by choice of mounting medium, is also discussed by these authors.

The algebraic sum of the error-producing components: flare light loss, non-specific specimen light loss, flare light gain, and non-specific specimen light gain, constitutes the stray-light of the system, for a given measuring condition and specimen.

The stray-light components which are independent of the specimen, *i.e.*, the flare light, are sometimes referred to as "glare." The flare light error involves the system of apertures of, and the method of using, a given optical system. Mechanical flare light may be eliminated by means of a blackened objective (1). In the past authors have estimated the extent of the flare error by measuring the apparent transmission of an opaque object (17). A diagrammatic representation of the transmission of a light beam through an optical system and specimen based on the foregoing discussion is shown in Fig. 3.

It is stated by Ornstein and Pollister (21) that the major (superstage) flare light component arises from glare generated at the upper interface of the specimen and mounting medium. Reduction of this surface reflectivity, by choice of the correct mounting medium, combined with the use of oil immersion optics then leads to a reduction in flare. For cytological specimens, flare from this cause should certainly be reduced by adopting the procedure recommended in reference 21. For x-ray microradiographs, reflections between the objective and the highly reflecting silver grains of the mounted photographic emulsion will probably remain a contributing mechanism of flare light even when oil immersion optics are used. Qualitative tests made with our equipment, using both correctly mounted cytological and photographic specimens, with oil immersion did not result in significant change in the total flare light observed at the photomultiplier.

It is, therefore, our opinion that, whilst every reasonable effort should be made to reduce all flare components, the main emphasis should be in experimentally demonstrating that the residual flare light of an optical system will not produce a flare error for the type of measurement and specimen under consideration. It is for this reason that we develop the concept of an experimentally determinable flare function in the following paper.

Ornstein (13) and Patau (15) devised a ratio method for the elimination or reduction of the distributional and stray-light error in microspectrophotometry. They proposed the measurement of the density of a dye material by a method involving two wavelengths, so chosen as to produce extinction coefficients differing by a factor of 2. We conclude (Appendix C) that the ratio method only reduces the stray-light error to one which is directly proportional to the stray-light, and does not eliminate the stray-light error as claimed by both Ornstein and Patau. It suffers from the further disadvantage that the measuring conditions necessitated by the method are instrumental in producing stray-light. Furthermore, in the density range for which the ratio method can be used (extinctions E_{max} not exceeding 0.6 (15)) the flare light error is usually small.

V. THEORETICAL EVALUATION OF THE SV EFFECT

Naora (10) considered the problem of a uniform beam of parallel light propagating through m surfaces, each of reflectivity r. The direct flux issuing from the m'th surface is (for unit incident flux at the 1st surface) $(1 - r)^m$. The total exit flux is

$$(1 - r)^m \left(\frac{1}{1 - r^2}\right)^{m-1} \rightarrow \frac{1 - r}{1 + (m - 1)r}.$$

The flare light flux is thus

$$(1 - r)/(1 + (m - 1)r) - (1 - r)^m$$

and Naora's θ, which is the ratio of flare light flux to total light flux, is

$$\theta = \left[\frac{1 - r}{1 + (m - 1)r} - (1 - r)^m\right] \Bigg/ \left[\frac{1 - r}{1 + (m - 1)r}\right].$$

He referred to θ as the maximum or "saturated" flare light error. The derivation is based on the concept of total flux and parallel light and hence does not involve either the area of the illuminating beam or the value of the optical image field. It represents a theoretical mode of operation of an optical system. In practice the light paths will not be parallel. The intensity of the focused direct image will thus be raised, while the intensity of the diffused flare light will be lowered. The modifications of the θ equation that we have derived below involves the optical magnification, the area of the beam, and the diameter of the image field. Since it is intensity and not light flux that is generally measured, the simple saturated flare light error θ derived on the basis of parallel light is not applicable to a practical optical system except (as we later demonstrate) as a special case.

Propagation of a Non-Parallel Beam of Light through m Air/Glass Surfaces, Each of Reflectivity r:

Fig. 4 illustrates the possible cases to be considered.

A = total area of an illuminating beam, incident on a specimen and measured in the plane of the specimen.
B = size of the image field of the optical system, measured in the image plane.
C = size of image formed by focused direct light, measured in the magnified image plane.
E = size of the photomultiplier aperture in the image plane.
F = size of object in the object plane.
M = superstage magnification = C/A.
σ = the true transmission of the object.

In all cases some flare light will be lost in traversing the non-parallel optical system (14). This could be allowed for by using an enhanced value of the flare light illuminated image field B defined above. Since we do not calculate an absolute flare error but restrict ourselves to relative changes of flare light as a function of the illuminating beam and specimen size, this point has not been examined further. The total flux incident on the specimen plane = A. (Unit light flux per unit area.) The direct flux transmitted = $A\ (1 - r)^m$. This forms an image of intensity (flux/unit area) = $(A/C)(1 - r)^m$ in the image plane. The flare light intensity (flux/unit area in the image plane)

$$= (A/B)\left[\frac{1 - r}{1 + (m - 1)r} - (1 - r)^m\right]$$

$$= (A/B)(R) \quad \text{in which}$$

$$(R) = \left[\frac{1 - r}{1 + (m - 1)r} - (1 - r)^m\right].$$

Hence the ratio of flare light intensity to direct image intensity measured in the image plane is

$$= (C/B)(R)/(1 - r)^m.$$

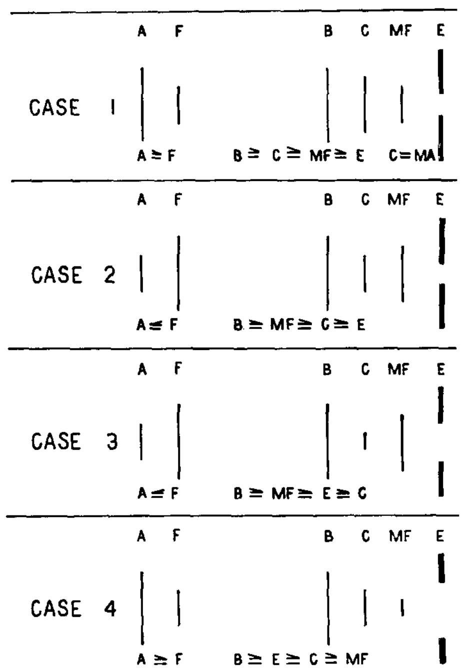

FIG. 4. Optical conditions defined by the special cases of flare light error discussed in the text.

The ratio of flare light intensity to total intensity in the direct image region of the image plane

$$\theta = \frac{A}{B}(R) \Big/ \left[\frac{A}{B}(R) + \frac{A}{C}(1-r)^m\right].$$

It follows that the saturated condition of Naora is the special case obtaining when $C = B$ (*i.e.* when $MA = B$, since the superstage magnification $M = C/A$). Increasing A beyond the value of B/M will have no effect, since all the exit pupil is then filled with direct light. For A less than B/M, C is less than B, and the error in θ is reduced.

Case 1:
$A \geq F, \quad B \geq MA \geq MF \geq E \qquad (MA = C)$

For unit flux per square centimeter entering the object plane at area A, the direct light intensity measured in the image plane $= (A/C)\,(1-r)^m\sigma$. The flare light intensity in the image plane

$$= \left(\frac{A-F}{B}\right)(R) + \frac{F\sigma}{B}(R).$$

We observe a reading on the photomultiplier corresponding to a transmission of direct plus flare light

$$= \frac{A}{C}(1-r)^m\sigma + \left[\left(\frac{A-F}{B}\right)(R) + \frac{F\sigma}{B}(R)\right].$$

The flare error (flare light/total light) is

$$= \left[\frac{A-F}{B} + \frac{F\sigma}{B}\right](R) \Big/ \left[\frac{A}{C}(1-r)^m\sigma + \left[\frac{A-F}{B} + \frac{F\sigma}{B}\right](R)\right].$$

The flare error is seen to diminish with decreasing A, for a constant F and B, until it reaches a minimum with $A = F$. The apparent transmission (defined as the ratio of the received light with and without the specimen present) is measured as

$$= \left[\frac{A}{C}(1-r)^m\sigma + \left[\frac{A-F}{B} + \frac{F\sigma}{B}\right](R)\right] \Big/ \left[\frac{A}{C}(1-r)^m + \frac{A}{B}(R)\right].$$

The transmission error is zero when $A = F$. When $A > F$, the smaller the value of σ the larger the error. Changing E has no effect on the error providing $A \geq F$ and $MF \geq E$.

Case 2: $A \leq F, \quad B \geq MF \geq C \geq E$

Here the direct light intensity in the image plane is $(A/C)\,(1-r)^m\sigma$. The flare light intensity in the image plane is $(A\sigma/B)\,(R)$.
The apparent transmission is measured as

$$= \left[\frac{A}{C}(1-r)^m + \frac{A}{B}(R)\right]\sigma \Big/ \left[\frac{A}{C}(1-r)^m + \frac{A}{B}(R)\right] = \sigma$$

the true transmission, and zero transmission error is obtained for all values of $A \leq F$.

Case 3: $A \leq F, \quad B \geq MF \geq E \geq C$

The total direct plus flare light received by the photomultiplier aperture E in the presence of the specimen is $(A/C)\,(1-r)^m\sigma + (EA/B)\sigma\,(R)$. In the absence of the specimen the light received $= (A/C)\,(1-r)^m + (EA/B)\,(R)$. The received light ratio is, therefore, the true transmission σ and no transmission error results.

Case 4: $A \geq F, \quad B \geq E \geq C \geq MF$

The light received by the photomultiplier in the presence of the specimen is

$$(F/A)(1-r)^m\sigma + \frac{A-F}{A}(1-r)^m + \left[\frac{A-F}{B} + \frac{F}{B}\sigma\right](R)\,E.$$

In the absence of the specimen the light received is $(1-r)^m + (A/B)\,(R)\cdot E$.
The apparent transmission is thus

$$\left((1-r)^m\left[\frac{F}{A}\sigma + \frac{(A-F)}{A}\right] + \left[\frac{A-F}{B} + \frac{F}{B}\sigma\right](R)\,E\right) \Big/ \left[(1-r)^m + \frac{A}{B}(R)\,E.\right]$$

And again if $A = F$ the ratio reduces to the true transmission σ and no error in the transmission results. For $A > F$ an error is present.

It is apparent that in all the cases discussed, corresponding to possible optical modes of measurement, flare light is present in the system. For $A > F$ the flare light is of the form $\left(\frac{A-F}{B} + \frac{F\sigma}{B}\right)(R)$. When $A \leq F$, it reduces to $(A/B)\sigma\,(R)$. In this condition the flare light is produced after passing through the absorbing specimen of true transmission σ. Transmission measurements then take into account the flare light absorption and yield the true transmission.

Inclusion of the Substage Flare Light:

From the above case analyses it is apparent that it is only necessary to fulfil the condition of $A \leq F$

and the flare light transmission error will be eliminated, even though the flare light is still present. It must be remembered, however, that flare light generation has also been occurring in the substage condensing optics as well. So that instead of directing a clearly defined beam of area A onto the specimen of area F, the field of the superstage objective is being illuminated with a light flux of intensity, measured in the specimen plane, of the form (for unit flux per cm.[2] leaving the lamp diaphragm)

$$(C^1/A)\cdot(1 - r^1)^n \text{ for the direct light}$$

$$\text{and } (C^1/B^1)\cdot(R^1) \text{ for the substage flare light.}$$

In which

- A = area of illuminating direct beam measured in the specimen plane,
- B^1 = area of image field of substage optical system measured in the specimen plane,
- C^1 = area of illuminated substage field stop (lamp diaphragm) ($C^1 = AM^1$),
- M^1 = reduction power of substage system ($M^1 > 1$),
- n = number of air/glass surfaces in substage optics, each, of reflectivity r^1,

$$(R^1) = \left[\frac{(1 - r^1)}{1 + (n - 1)r^1} - (1 - r^1)^n\right].$$

At the photomultiplier, in the image plane of the superstage optics, the direct light intensity will be

$$\approx \frac{C^1}{A}(1 - r^1)^n \frac{A}{C}(1 - r)^m.$$

The substage flare light intensity at the photomultiplier is

$$\approx \frac{C^1}{B^1}(R^1)\frac{B^1}{B}.$$

The superstage flare intensity at the photomultiplier, arising from the direct light transmitted through the superstage optics (for $A \leq F$) is

$$\approx \frac{C^1}{A}(1 - r^1)^n \frac{A}{B}(R)\sigma.$$

By keeping $A \leq F$ and σ small, we can ignore this superstage component and consider only the effect of the substage flare reaching the photomultiplier.

After transmission through the specimen ($A \leq F$) the direct light intensity at the photomultiplier becomes

$$\approx (C^1/C)\ (1 - r^1)^n\ (1 - r)^m\sigma.$$

When $MA \geq E$ and $A \leq F$ we have the total substage flare flux received at the photomultiplier

$$\approx (C^1/B)\ (R^1)\ E$$

and the total transmitted direct flux

$$\approx (C^1/C)(1 - r^1)^n\ (1 - r)^m\sigma\ E.$$

The flare error (flare flux/transmitted flux)

$$\approx \frac{C(R^1)}{B(1 - r^1)^n(1 - r)^m\sigma} \approx \text{constant} \cdot A/\sigma.$$

$$(\text{Since } C = MA.).$$

This is the observed transmission error, and is thus a linear function of the illuminating beam area A.

In this case the error does not involve the ratio of A/F but only the magnitude of A. Reducing the beam size reduces the error.

When $MA \leq E$ and $A \leq F$ we receive at the photomultiplier a total flare flux

$$= (C^1/B)\cdot(R^1)\ E$$

and a total transmitted direct flux

$$= (C^1/C)\cdot(1 - r^1)^n\cdot(1 - r)^m\sigma C.$$

The flare error is then

$$\approx \frac{C^1(R^1)E}{B} \bigg/ \frac{C^1}{C}\cdot(1 - r^1)^n\cdot(1 - r)^m\,\sigma C.$$

$$\approx \text{Constant}\cdot(E/\sigma)$$

and again this is the observed transmission error.

For the special case of $MA \leq E$ and $A \leq F$, reducing A in the presence of substage flare, therefore, will not produce a diminishing flare error. A systematic error proportional to the photomultiplier aperture E will consequently appear in the measured transmission. *The constancy of any transmission measurement as a function of illuminating beam diameter does not, therefore, necessarily imply the absence of flare error.*

The Flare Function:

In practice the substage flare light can be more adequately described as $f(C^1/B^1)\cdot(R^1)$ instead of the simple form $(C^1/B^1)\cdot(R^1)$ previously used. The unknown function of C^1/B^1 can then be written

$$f\left(\frac{M^1A}{B^1}\right) = f_1\left(\frac{M^1}{B^1}\right)\cdot f_2(A)$$

in which f_1 is a constant of the substage optics and f_2 is a variable in A. Provided $MA \geq E$ and $A \leq F$ the percentage error in σ is

$$\approx \frac{100\cdot f_1 f_2(R^1)B^1\,C}{BC^1(1 - r^1)^n(1 - r)^m\,\sigma} \approx \text{Constant}\cdot\frac{f_2(A)}{\sigma}.$$

In practice, therefore, it will be advisable to make transmission measurements with $MA \geq E$ and $A \leq F$, to assume $\Delta\sigma$ per cent = constant$\cdot f_2(A)/\sigma$ and to determine experimentally the form of f_2 as a function of the beam size A.

Only in this way can one determine if the A chosen, as distinct from the A/F ratio, will be sufficiently small, for a given σ, to reduce the substage flare error to a negligible value.

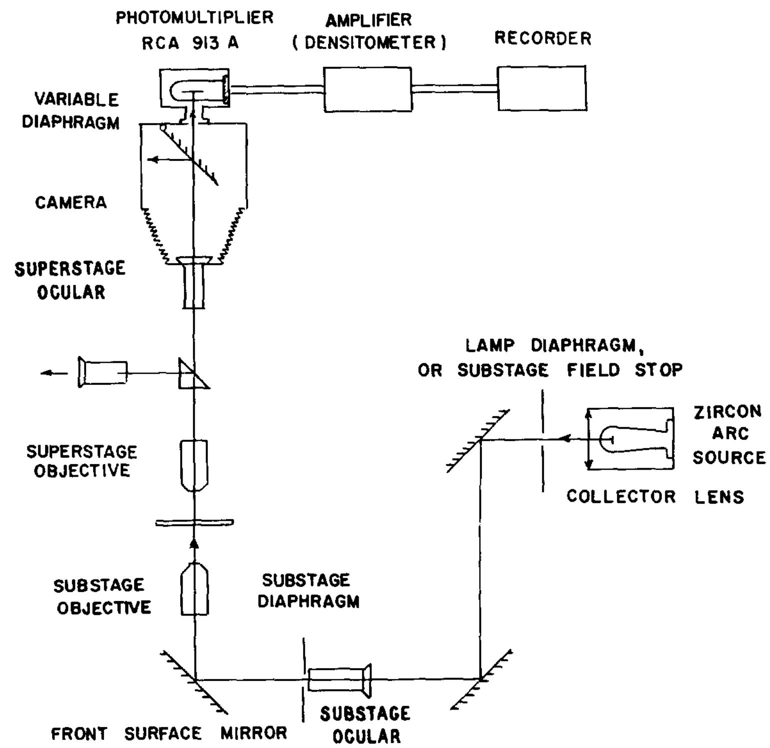

FIG. 5. Block diagram of the optical system.

Merely making $A/F \leq 1$ takes care of the superstage flare error only.

VI. EXPERIMENTAL TECHNIQUE

Optical Equipment (*Fig. 5*):

The optical system used was a Leitz Pamphot photomicrographic unit with a modified illuminating system with the addition of a substage inverted ocular and objective so arranged as to produce a reduced image of the illuminated lamp diaphragm in the plane of the specimen. A Sylvania zirconium 100-watt arc served as the light source. The lamp diaphragm acted as a variable diameter field stop (0.1 to 2.6 cm. diameter). A substage diaphragm situated between the ocular and objective reduced the mechanical flare of the substage optics. The latter diaphragm was usually set at 3 to 5 mm. diameter, in which case it did not function either as a field or aperture stop for direct light.

Various combinations of substage and superstage objectives and oculars were employed. The substage objective mounting was provided with fine control adjustments in the vertical and horizontal directions to provide optical alignment. A two-dimensional horizontal motor drive, coupled to the specimen stage, permitted automatic scanning of the specimen when required. A speed reduction unit allowed a velocity selection of the stage of either 125 or 25 microns per minute with respect to the stationary illuminating beam.

The magnified image of the specimen, illuminated by the reduced secondary light source image of the lamp diaphragm focused in the plane of the specimen, could be viewed on a viewing screen in the conventional photomicrographic manner. An image-plane variable stop, situated in the camera housing, allowed access of a selected area (0.1 to 2.6 cm. diameter) of the final image to the cathode of a photomultiplier tube (type RCA 931A). The output voltage of the photomultiplier amplifier (Ansco model 12 densitometer) was then fed to a Leeds and Northrop recorder. A continuous record of apparent transmission could thus be obtained. Experiments were carried out with combinations of the following uncoated optical components.

Superstage

Objective

x 3.2	Bausch and Lomb	28 mm.	N.A. = 0.08
x 10	Spencer	16 mm.	0.25
x 25	Leitz	4.6 mm.	0.50

Ocular

x 10 Leitz
x 2 Leitz

Substage

Objective

x 21 Bausch and Lomb 8 mm. 0.50

Ocular

x 6 Bausch and Lomb

Optical conditions used in a particular experiment are given in the legends.

Calibration of the Densitometer:

It was necessary to establish the precise form of the photomultiplier response as a function of the light flux received and to use a calibration curve for the determination of density values. The calibration curves were obtained by the use of standard photographic density step wedges and the basic circuit and optical arrangement shown in Fig. 5. The range of the photomultiplier circuit was extended by attaching a 10 ohm shunt across the output terminals of the densitometer. This

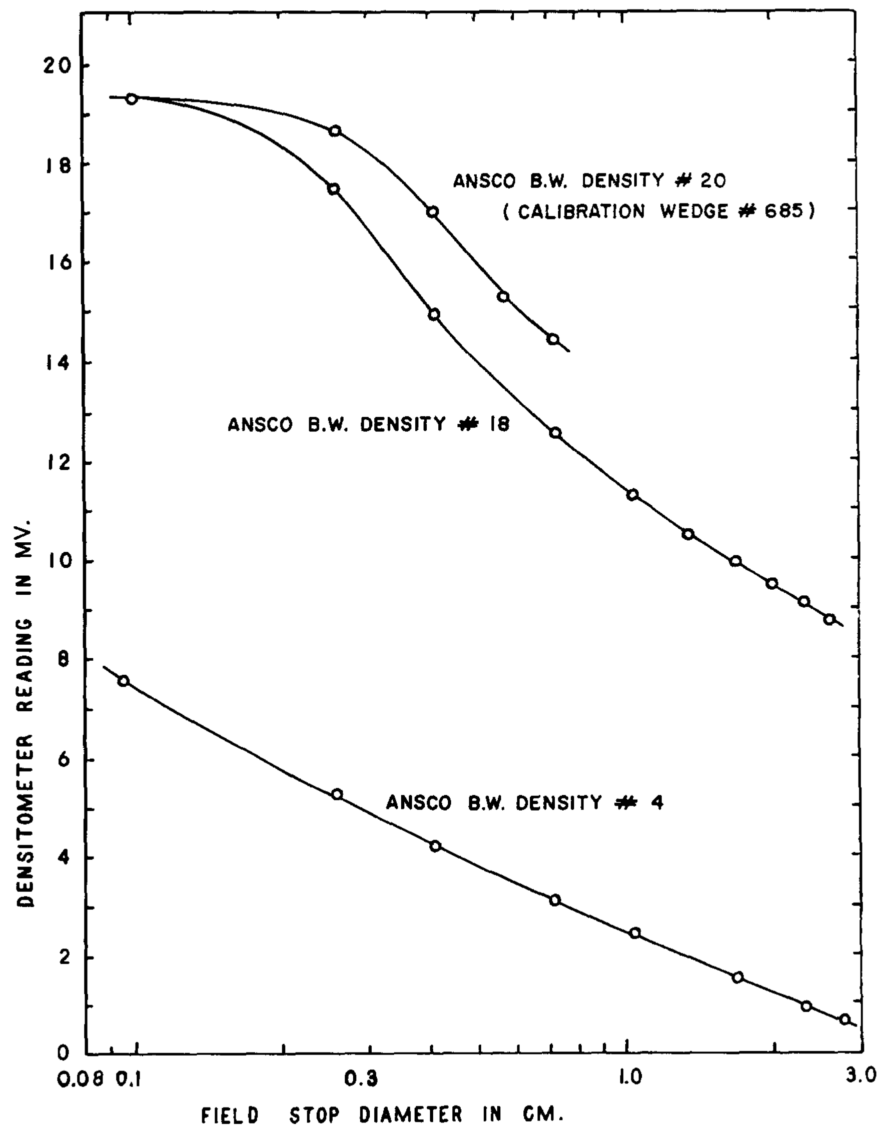

FIG. 6 *A*. Photomultiplier response as a function of field stop diameter. Photomultiplier aperture fully open at 2.6 cm. diameter. A substage field stop diameter of 2.6 cm. ~ 95 μ in the object plane. Substage obj. $\times$ 21, oc. $\times$ 6. Superstage obj. $\times$ 3.2, oc. $\times$ 2. The low power superstage optics were chosen to ensure collection of all direct light by the photomultiplier at maximum light beam diameter.

gave an effective photomultiplier light flux corresponding to unshunted outputs from 0 to 19.2 mv. Above 10 mv. output the response of the densitometer was non-linear and was corrected to obtain linear output signal differences corresponding to light-transmission ratios. The zero point of the densitometer was always adjusted to give a dark current unshunted output voltage of 19.2 millivolts. The measured curves (Figs. 6 *A* and *B*) were used to convert all densitometer millivolt readings, first to a corresponding linear value, and second to a direct density.

Model Specimens:

As a provisional model for the investigation of stray-light, an ordered mosaic of 286 micron diameter holes etched into a subsequently blackened metal foil (Fig. 7) was placed in the object plane and an opaque area between the holes illuminated. The stray-light recorded on the photomultiplier was then measured as a function of the diameter of the illuminating light beam (Fig. 8). To eliminate edge effects of the object, a series of photographic negatives of the hole-mosaic object was

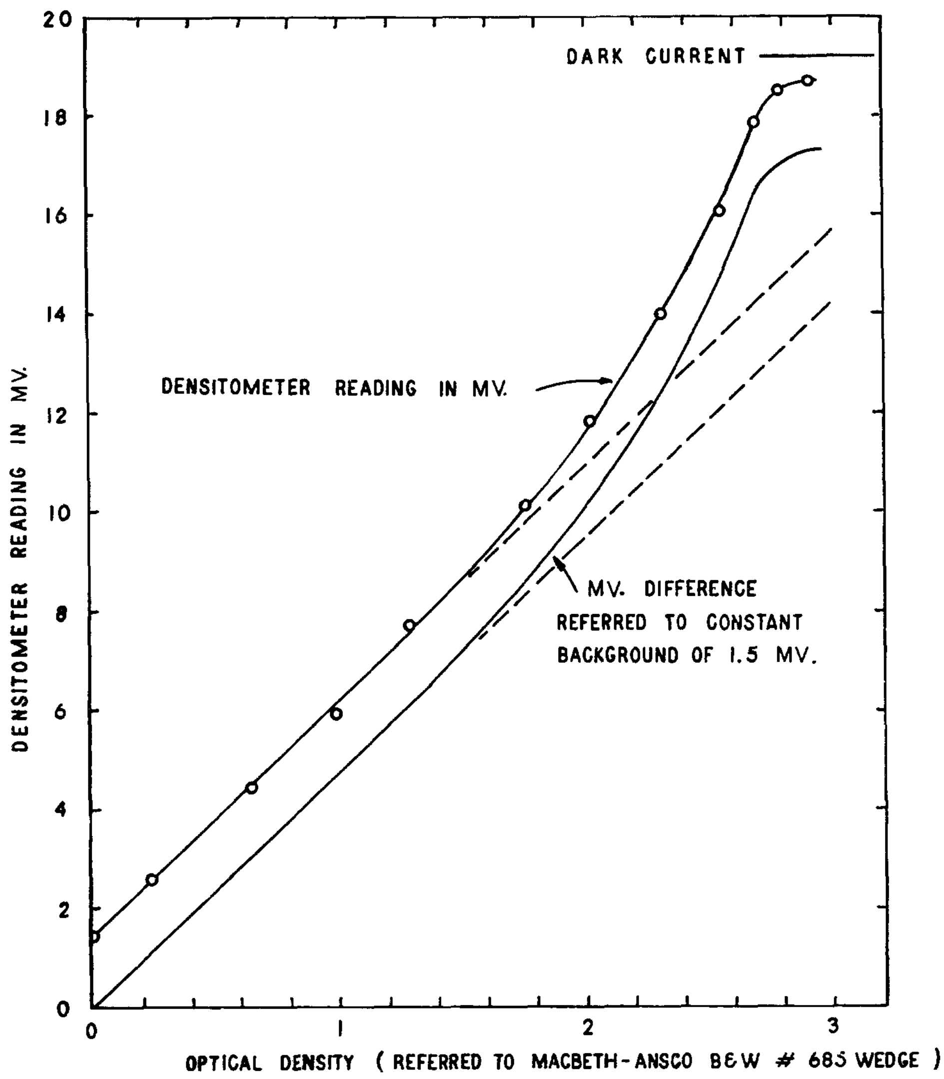

FIG. 6 *B*. Photomultiplier response as a function of optical density of a Macbeth-Ansco photographic wedge (B and W No. 685 referred to the Zircon arc). Instrument zeroed on dark current at 19.2 mv. (10 mv. with 10 ohm shunt). A substage field stop diameter of 1.36 cm. ~ 50 μ diameter light beam in the object plane. A photomultiplier aperture of 0.09 cm. diameter produced a constant 4 μ diameter measuring area in the object plane. Substage obj. × 21, oc. × 6. Superstage obj. × 10, oc. × 10.

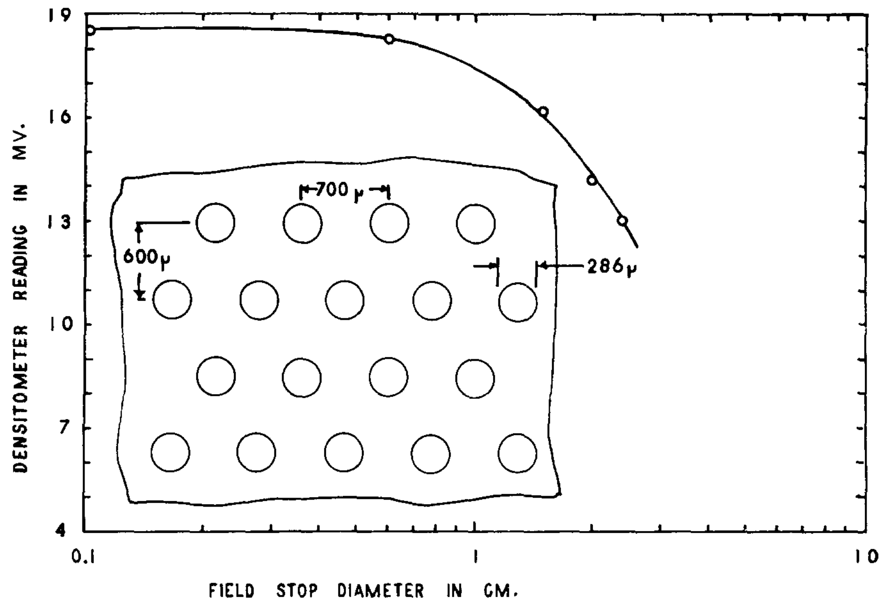

FIG. 7. Photomultiplier response to stray-light as a function of illuminating beam diameter (IBD) incident upon an opaque portion of an ordered array of 286 μ holes set in a blackened metal screen placed in the object plane (inset). Photomultiplier aperture = 2.6 cm. diameter. Substage obj. × 21, oc. × 6. Superstage obj. × 3.2, oc. × 10. A substage field stop diameter of 2.6 cm. produced a 95 μ IBD in the object plane. Photomultiplier zeroed at dark current level of 18.5 mv. Increase of field stop diameter is accompanied by increase of stray-light.

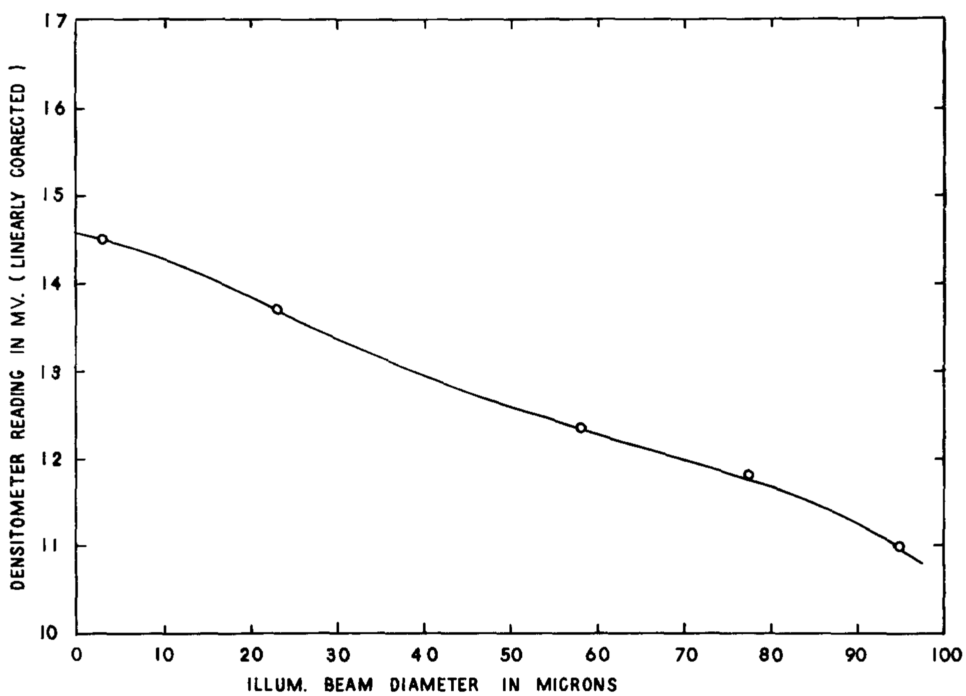

FIG. 8. Photomultiplier response to stray-light of Fig. 7 corrected for non-linearity and normalized to a dark current zero level of 19.2 mv. Marked stray-light reading as a function of beam size arose from edge diffraction effects.

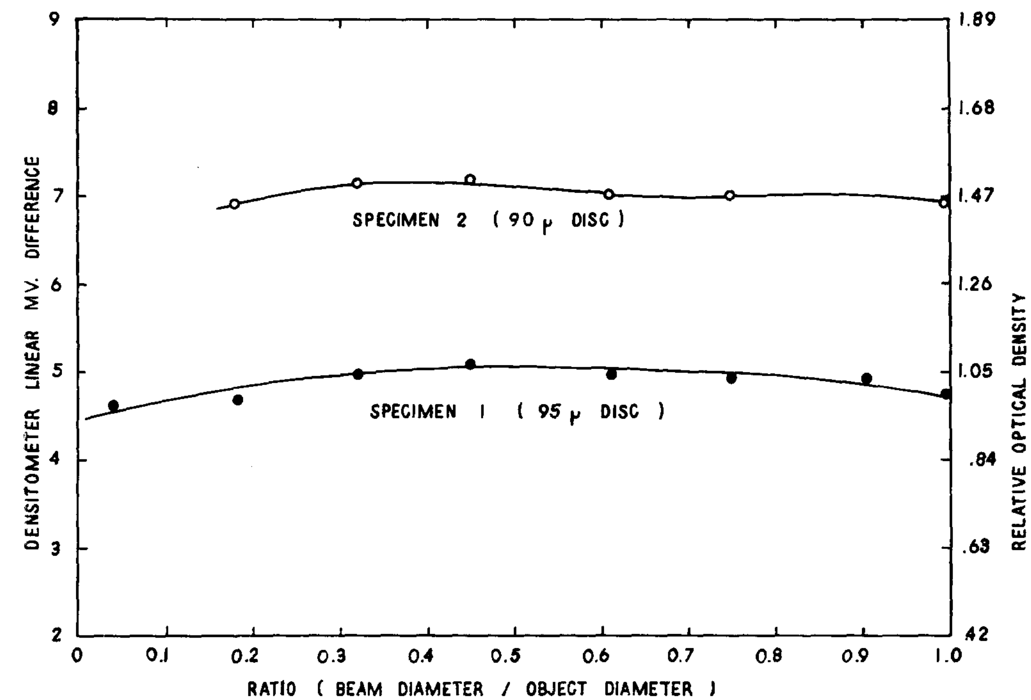

FIG. 9. Optical density plot of photographic image discs corrected for photomultiplier non-linearity and expressed as a function of IBD/OD ratio. Substage obj. × 21, oc. × 6. Superstage obj. × 3.2, oc. × 10. Photomultiplier aperture = 2.6 cm. diameter. The results substantiate the apparent absence of an SV error for the special case where $MA \leq E$ and $A \leq F$ as discussed in the text.

prepared. Various sizes and intensities of the disc pattern were made on Lippman film, using a fine grain developer. Uniformity of the grain distribution within the dark region of each disc chosen for measurement and sharp definition of the disc edges were obtained. In these photographs the holes appeared as an ordered array of dark discs situated in a transparent background. The prepared films were finally mounted on glass slides for densitometric measurements.

VII. EXPERIMENTAL RESULTS

Measurement of Optical Density as a Function of Operating Conditions:

Fig. 9 shows the optical density plot, corrected for photomultiplier non-linearity, of two typical dark 90 μ diameter photographic image discs as a function of the illuminating beam diameter/object diameter ratio (IBD/OD) (photomultiplier aperture in the image plane = 2.6 cm. in diameter). All readings were taken in complete darkness. The densities are referred to the background transmission of each photographic specimen. In this experiment $MA \leq E$, $A \leq F$ (Fig. 4). The flare light error will be, to a first approximation, a constant governed by the size of the photomultiplier aperture. The SV-error distribution as a function of IBD is as beam-size independent as one might expect if the concept of substage flare alone is considered. A slight dependence on the illuminating beam diameter is, however, present. *The result could, at this stage, be interpreted as indicating the absence of SV error.*

A second experiment was made with the photomultiplier aperture closed down to 0.09 cm. diameter. With the substage field stop fully open, a light beam image of 2.15 cm. diameter was obtained at the photomultiplier image plane. The density, corrected for non-linearity, of specimens 1 and 3 showed marked dependence on IBD (Fig. 10). No measuring range was reached over which a constant density existed as a function of IBD even with such a small IBD/OD ratio of 0.2. The light flux at densities higher than ~2.4 was insufficient to drive the photomultiplier tube under the circumstances employed.

To verify that a constant plateau of density existed, measurements were continued on larger discs, of some 500 μ diameter (specimen 5). IBD/OD ratios as low as 0.006 could now be

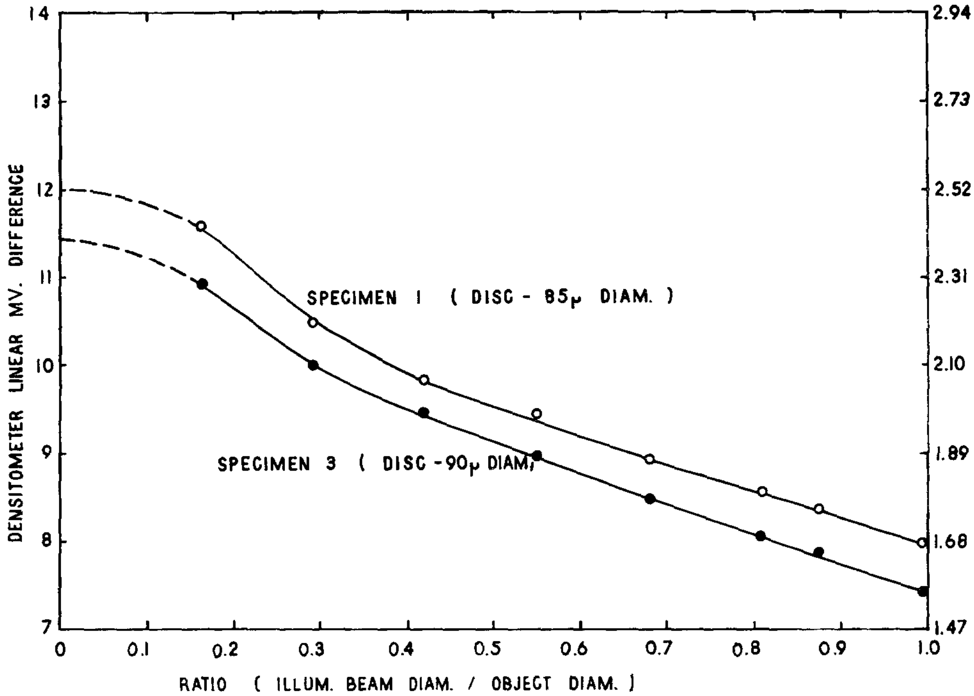

FIG. 10. Optical density plot of measurements on very dense discs corrected for non-linearity and expressed as a function of IBD/OD ratio. A substage field stop of 2.6 cm. gave a light beam of 95 μ diameter in the object plane. Photomultiplier aperture = 0.09 cm. (equivalent to a 4 μ diameter measuring area in the object plane). Superstage obj. × 10. oc. × 10. Substage obj. × 21, oc. × 6. A very marked SV error as a function of the IBD/OD ratio is apparent. Compare with the *apparent* SV-error-free results of a special case shown in Fig. 9.

reached. The results, shown in Fig. 11, indicated that a constant plateau of density could be obtained with suitable IBD/OD ratios. The limiting value of the ratio is dependent on the true density of the object and the extent of the SV error. For specimen 5 the true density was approximately 1.1; hence the plateau of constant density probably extended out to IBD/OD ratios as large as 0.5.

Reduction of the Substage Flare Light:

It has been shown in section 5 that the superstage optics cannot produce an SV error for IBD/OD ratios less than unity. It follows that the principal component of our SV error must originate in the substage optics, thus being a function only of the ratio A/B^1. The worn surfaces of the three reflecting front-faced mirrors included in the substage optics were re-silvered and the density measurements of specimens 1 and 3 repeated (Fig. 12). Even at the extremely high densities involved (~2.9) the plateau of true density was approached at IBD/OD ratios of less than 0.3 and it was obvious that a considerable improvement had been made by the addition of the new mirrors and resultant reduction of substage flare (compare Figs. 10 and 12). To illustrate the magnitude of the improvement, a 77.5 μ and a 30 μ diameter formvar-coated copper wire embedded in acrylic resin wire were scanned with illuminating beams of different diameters. The photomultiplier output voltage has been recorded as a function of the width of IBD (Figs. 13 *A* and *B*), using the old and new mirrors respectively. The maximum millivolt recording for each width of illuminating beam in Fig. 13 has been plotted in Fig. 14 against the corresponding IBD/OD ratios for both wires, referred to a constant direct light intensity of 1.74 mv. From these figures it is apparent that there is a significant substage flare as indicated by the *reduction of the substage flare light resulting from a re-silvering of the substage mirrors. In addition, there is also a pronounced SV effect which is a function of illuminating beam diameter.*

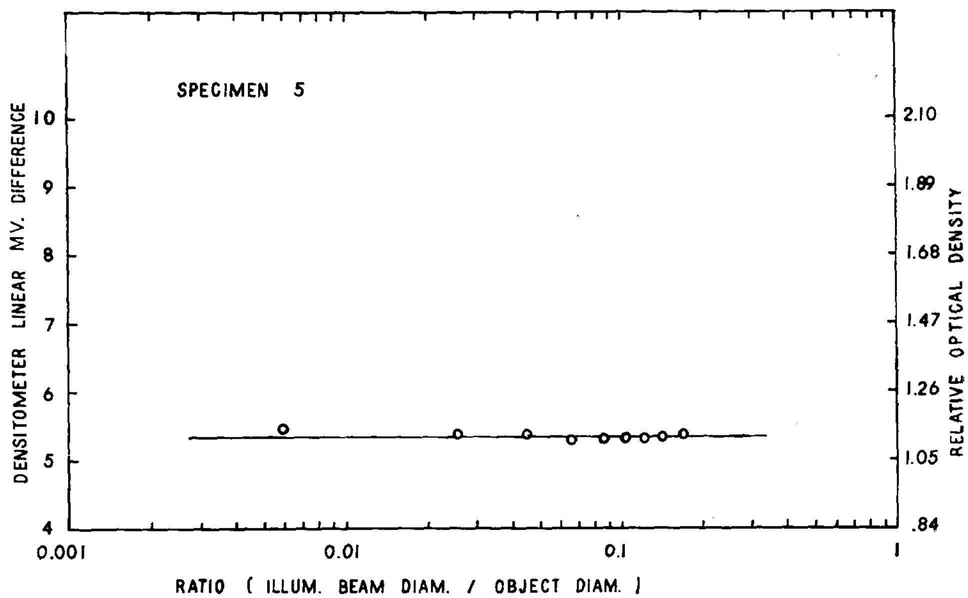

FIG. 11. Optical density plot of 540 μ diameter disc as a function of IBD/OD ratio. Optical conditions as given in Fig. 10. Photomultiplier aperture fully closed at 0.09 cm. diameter (equivalent to 4 μ diameter measuring area in the object plane). A substage field stop of 2.6 cm. produced a light beam of 95 μ diameter in the object plane. Attainment of true SV-error-free condition by reduction of IBD/OD ratio and use of correct optical conditions.

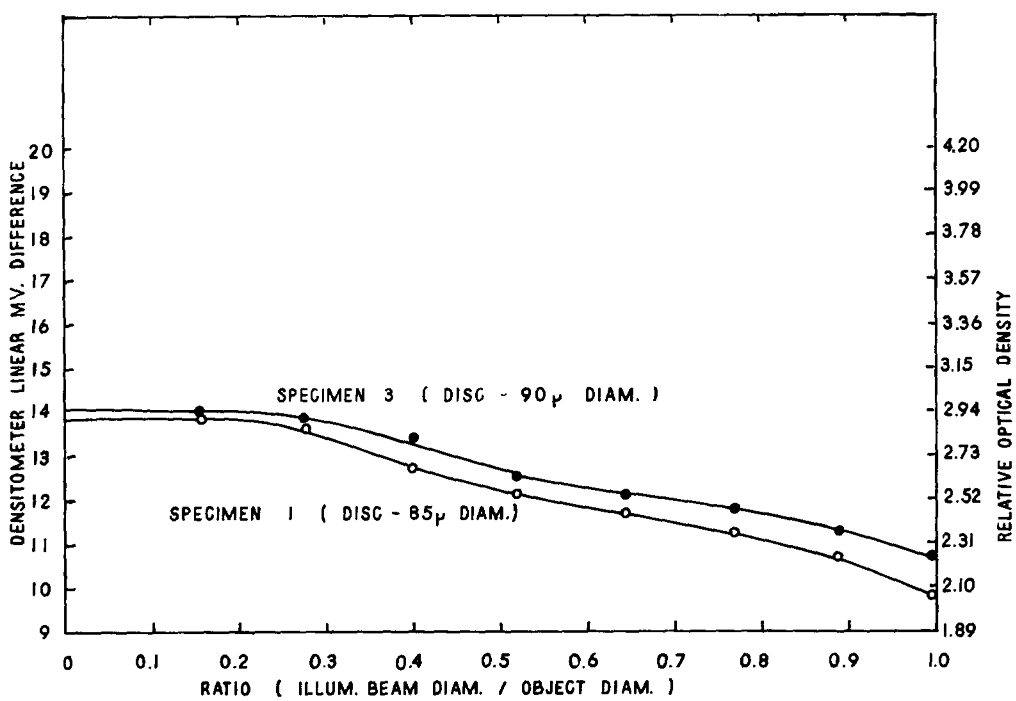

FIG. 12. Optical density plot of photomultiplier response to high density measurements using re-silvered substage mirrors to reduce substage flare. Substage obj. × 21, oc. × 6. Superstage obj. × 10, oc. × 10. Photomultiplier aperture fully closed (= 4 μ diameter measuring area in the object plane). A substage field stop of 2.6 cm. produced a 95 μ diameter beam in the object plane. Reduction of the substage flare error is apparent when results are compared with those of Fig. 10. The latter show no plateau is obtained at minimum IBD/OD ratio as does the former.

Ambiguity Associated with the Estimation of Stray-Light Using Opaque Objects:

In the ideal error-free optical system there would be no transmission of light if an opaque object were illuminated. The linearly corrected results shown in Fig. 14 indicate that, for the 77.5 μ diameter wire, the limit of the photomultiplier sensitivity is reached at an IBD/OD ratio of

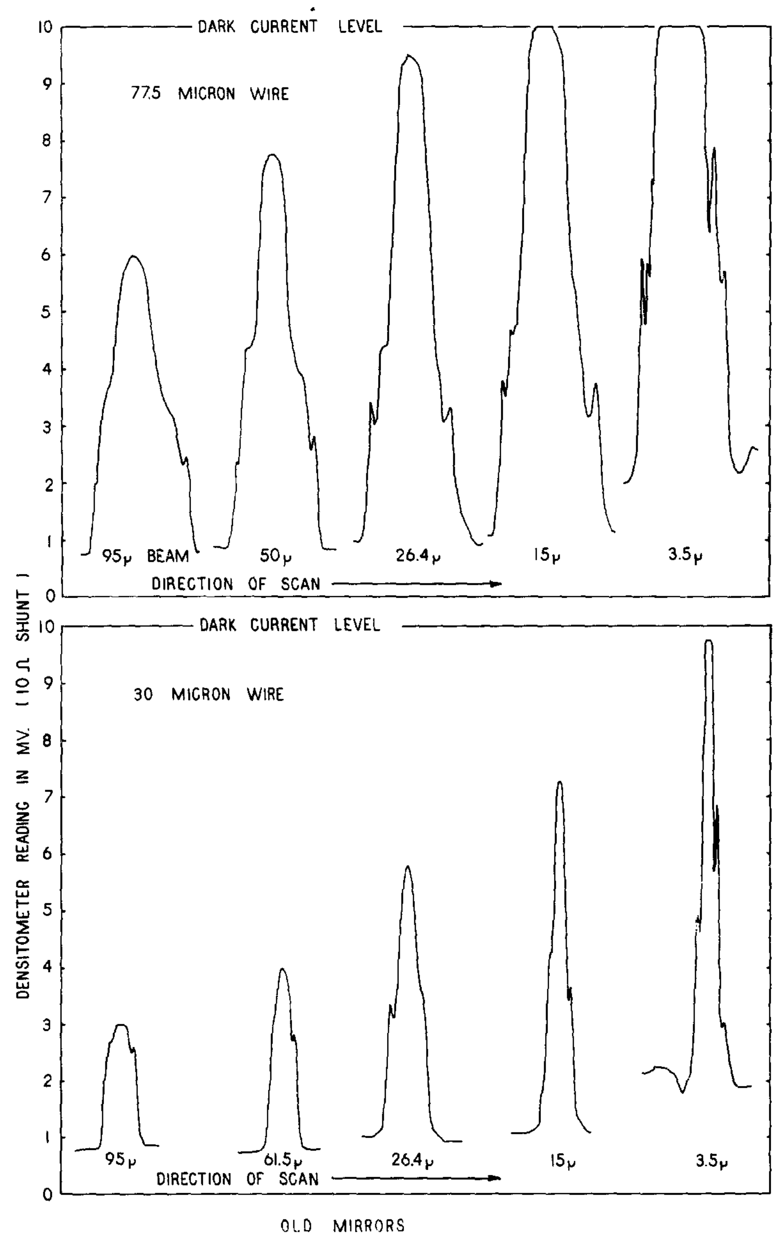

FIG. 13. Photomultiplier response as a function of IBD during scans of 77.5 μ and 30 μ diameter (OD) formvar-coated copper wires embedded in acrylic resin. *A*. Original worn substage mirrors. *B*. Newly re-silvered substage mirrors.

Substage obj. × 21, oc. × 6. Superstage obj. × 10, oc. × 10. The photomultiplier aperture of 0.09 cm. corresponded to a 4 μ diameter measuring area in the object plane. The large SV error resulting from the use of large IBD/OD ratios is evident. The effect is more pronounced with the small wire. Newly re-silvered mirrors which reduces the substage flare leads to a reduction in the total flare error.

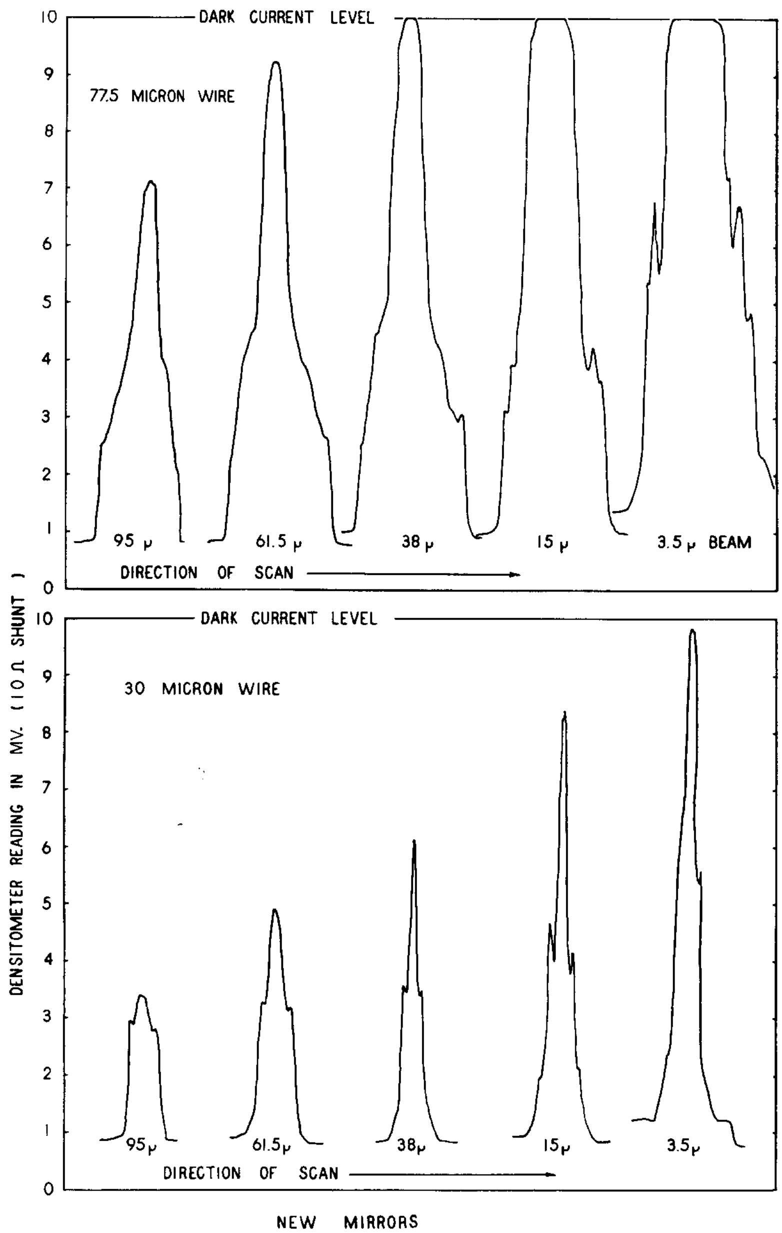

FIG. 13 *B*

0.35 (0.045 with the old mirrors), with an IBD of approximately 26 μ. For ratios below 0.35 the flare light reading is below the dark current of the photomultiplier.

Using the 30 μ diameter wire, the dark current of the photomultiplier is not reached, even down to an IBD/OD ratio of 0.1, with an IBD of 3 μ. Since dark current can be reached under the same conditions (same IBD/OD ratio) by replacing the 30 μ wire with the 77.5 μ wire it must be assumed that diffraction effects are present in the case of the narrower wire.

It is for these reasons that a flare error analysis made by measuring the stray-light obtained in the presence of an opaque object can be so misleading, unless the stray-light relationship between the IBD and OD is studied, for any point on the curves of Fig. 14 could be arbitrarily chosen for a stray-light error determination.

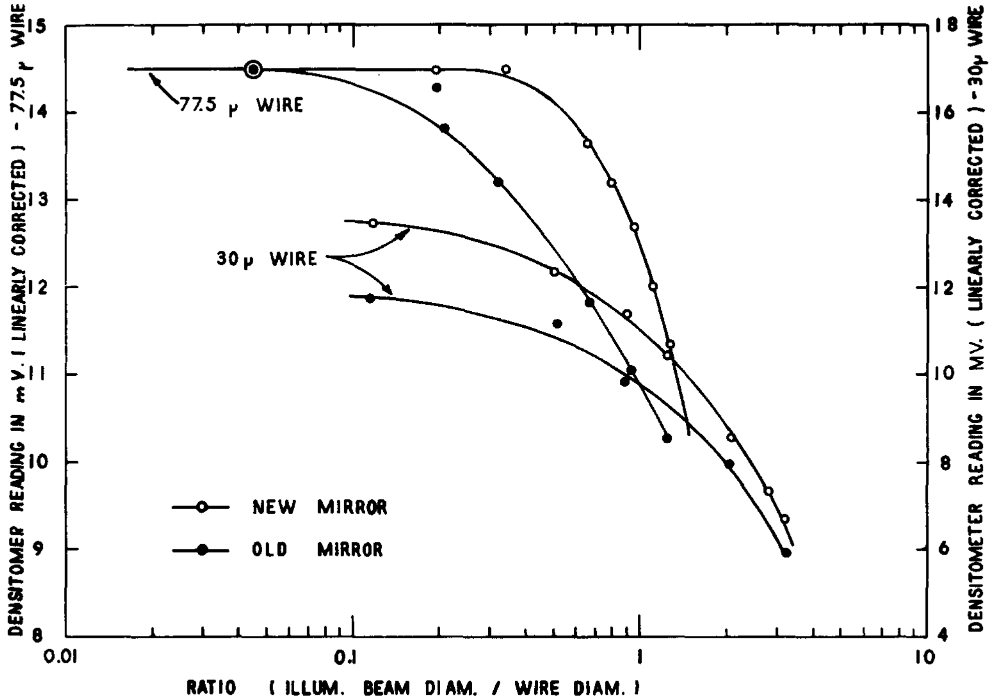

FIG. 14. Maximum photomultiplier response corresponding to the minimum stray light as a function of the IBD/OD ratio obtained from the wire scanning results of Fig. 13. Curves have been corrected for non-linearity and normalized to a constant direct light intensity of 1.74 mv. The reduction of the substage flare light resulting from the re-silvering of the substage mirrors and the well developed SV effect are clearly apparent.

VIII. MAGNITUDE OF THE SV ERROR AND EVALUATION OF THE FLARE FUNCTION $f(A)$

A knowledge of the IBD/OD ratio has been shown to be necessary but not sufficient for the determination of SV error. The ratio of the illuminating beam diameter to the substage image field diameter measured in the specimen plane or, alternatively, the beam diameter itself must also be included. Furthermore, it must be ensured that the image plane aperture is smaller than the direct light image. To investigate a system for SV error, the illuminating beam diameter should be varied and measurements made to be certain that a constant value of the observed density results, after allowing for any geometrical transmission correction applied to the specimen because of its shape. When this is established no SV error is present in the system, under the given conditions of measurement. Should a variation of observed density be encountered, the beam diameter must be reduced or optical surfaces coated, until a constant density is obtained. The system will then be operable in a SV-error-free condition.

A plot of the transmission or density as a function of IBD/OD can reveal the extent of flare error as a function of true density. Assuming the true transmitted light $= \sigma I_0$ and the flare light $= f(A)AI_0$ $(MA \geq E)$ the percentage error in transmission is $f(A)A/\sigma$. Defining the optical density $D = \log_{10}\sigma$, then

$$D = -\frac{1}{2.3} \cdot \frac{1}{\sigma} \cdot d\sigma = -\frac{1}{2.3} \frac{1}{10^{-D}} f(A)\, A.$$

Hence the percentage error in density

$$= \frac{\Delta D}{D} \cdot 100 = \frac{100}{2.3} \cdot \frac{10^D}{D} \cdot f(A)\, A$$

$$= \frac{100}{2.3} \cdot \frac{10^D}{D} \cdot f(A)\pi d^2/4$$

in which d = the diameter of the illuminating beam measured in the specimen plane.

Knowing $\Delta D/D$ from the measured SV error curves obtained with specimen 3 (Fig. 12) the flare function $f(A)$ has been calculated as a function of the beam diameter d and the IBD/OD ratio (Fig. 15).

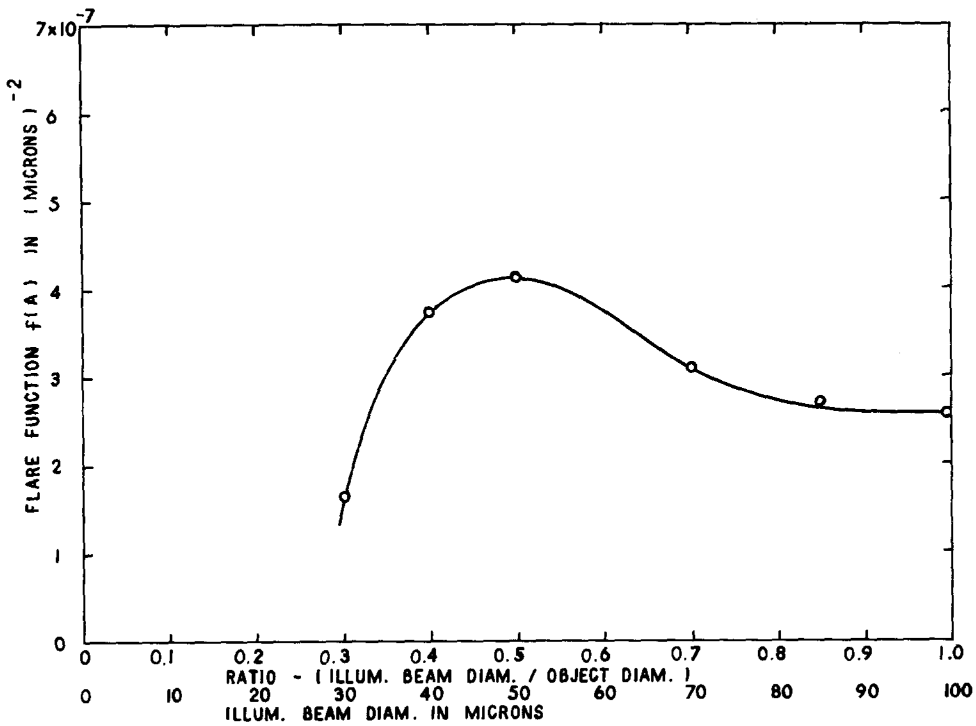

FIG. 15. The SV-flare function $f(A)$ as a function of IBD/OD ratio. The plotted curve has been evaluated from the measured density results obtained with the 90 μ diameter disc of specimen 3 (Fig. 12) of true density 2.94. Evaluation of the flare function provides a measure of the magnitude of the SV error present. This flare function has been used to calculate the percentage SV error in density shown in Fig. 16.

The values of $f(A)$ so derived have been used to calculate the percentage error in the density curves of Fig. 16 arising from the SV effect, as a function of true density and the IBD and OD ratio. For the particular uncoated optical system used in these experiments any microspectrophotometric measurement can, therefore, be made on specimens of unit density using IBD/OD ratios of unity with a resultant density error of 1 per cent. For lower densities, or with smaller beam diameters, the error will be even less. For heavily stained cells or for densitometric measurements on specimens approaching density 2, a 5 per cent error results even when using an IBD/OD ratio of unity. At a density of 3 the error rises to 30 per cent. Under such conditions it would be imperative to reduce the IBD/OD ratio to a value consistent with the accuracy required. (For an example of the effects of the residual SV error in a well designed modern densitometer see Altman and Stultz (1).)

A simple evaluation of the flare function $f(A)$ thus provides one with a measure of the SV-error condition of an optical system. From the values of $f(A)$ determined the SV error appropriate to a particular density measurement can then be estimated in advance. Should the SV error be found excessive for the range of densities to be measured, reduction of the flare light is in order. Effective reduction can be made by decreasing the IBD/OD ratio, by coating the superstage and substage optical surfaces, by improvement of the quality of all reflecting surfaces, and the elimination of mechanical flare. The final error arising from flare light should always ultimately be estimated by density measurements made as a function of the IBD/OD ratio.

Note Added in Proof:

A recent paper (22), which has come to our attention since the present paper was submitted for publication, experimentally demonstrates the necessity of reducing the measuring area, illuminated area, and condenser N.A. in order to diminish the flare error. With an India ink droplet of 4 μ diameter, a 2 μ measuring diameter, and a 7 μ illuminating area, the authors obtained an apparent transmission of 4.21 with condenser N.A. = 1.4, 1.58 at N.A. = 0.4, and 1.17 at N.A. = 0.25. This should be compared with Naora (11), who

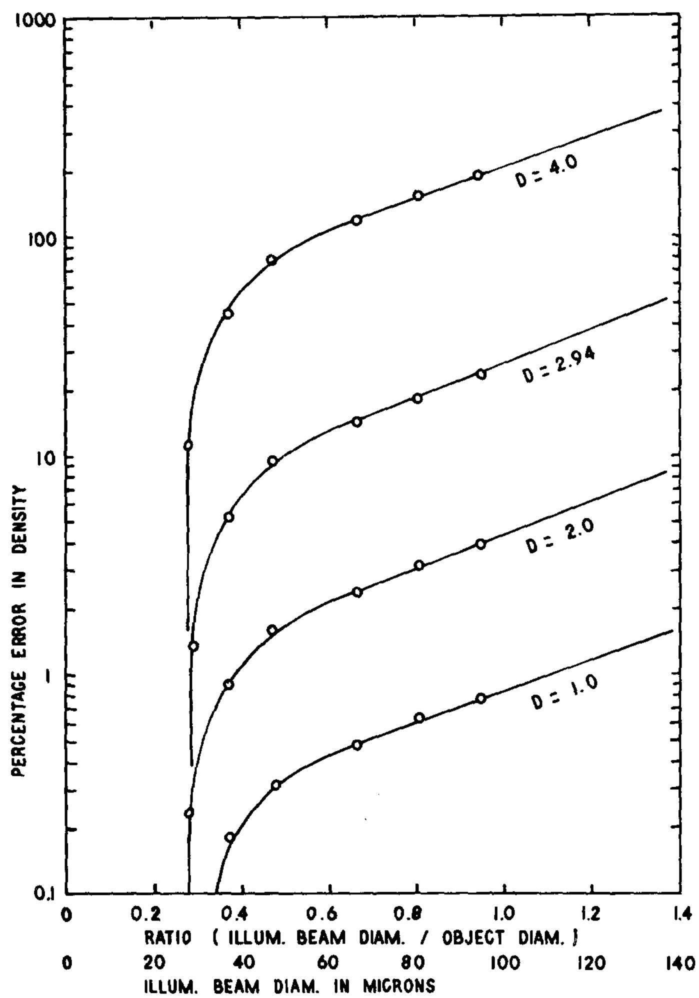

FIG. 16. Percentage SV error in density as a function of the true density and the IBD/OD ratio for the optical conditions given in Fig. 12. The curves enable a measurement to be made with a definable SV error.

found the flare error independent of the illuminating cone angle (N.A. = 1.25 to 0.8) and Lison (8), who also found that closing the condenser aperture did not reduce the flare error. It confirms the wisdom of measuring the flare light in terms of the flare function, derived in the present paper, and hence knowing the extent of the flare error *for the measuring conditions adopted.*

Pogo and Cordero Funes (22) conclude from a study of Feulgen-stained cells that their results are in accordance with those of Naora; that in microspectrophotometric measurement correct values can only be obtained by the use of equipment and measuring techniques producing minimum flare.

APPENDIX A

Transmission of a Parallel Light Beam of Radius r_1 through an Absorbing Sphere of Radius r:

The relationship between the ratio of the diameter of a parallel illuminating beam of light to the diameter of an object sphere (r_1/r) and the observed transmission is evaluated, because, when the ratio of light beam diameter to spherical object diameter (IBD/OD) approaches unity, the illumination condition is considered to approximate parallel illumination (Fig. 1 *B*) rather than the point-convergent illumination (Fig. 1 *A*) postulated by Naora (9).

Let k = the absorption coefficient of the sphere material (k = Naora's ϵC). Consider the transmission of an annular element of the beam at radius y as it passes through the sphere (Fig. 1 *C*). The area of projected annulus at radius $y = dA = 2\pi y\, dy = 2\pi r \cos\theta\, r \sin\theta\, d\theta$. After absorption in a thickness of cylinder $2x$,

$$\text{emergent flux} = dI = I_0 e^{-2kx} dA$$

$$= I_0 e^{-2kr} \sin\theta \cdot 2\pi r^2 \cos\theta \sin\theta\, d\theta.$$

Putting $\sin\theta = z = (1 - \cos^2\theta)^{1/2} = [1 - (y/r)^2]^{1/2}$

$$dI = I_0 e^{-krz} \cdot 2\pi r^2 z\, dz.$$

The total emergent integrated light flux of the whole beam

$$= I = \int_0^{r_1} (I_0\, e^{-2krz}\, 2\pi r^2\, z)\, dz.$$

Hence $I/I_0 = -2\pi r^2 \left[e^{-2krz} \cdot \left(\frac{1}{(2kr)^2} + z/(2kr) \right) \right]_{z_2}^{z_1}$

in which $z_1 = 1 - (r_1/r)^2]^{1/2}$

$z_2 = 1$

I_0 = intensity per unit area of the incident light beam

I = total integrated light flux of beam of radius r_1, after traveling through an absorbing sphere of radius r.

The ratio $I/(\pi r_1^2 I_0)$ = total incident light/total transmitted light

= transmission T

$$= -2\frac{r^2}{r_1^2} \cdot \left[e^{-2krz_1}\left(\frac{1}{2kr}\right)\left(\frac{1}{2kr} + z_1\right) - \frac{e^{-2kr}}{2kr}\left(\frac{1}{2kr} + 1\right) \right] \quad (1)$$

in which $z_1 = [1 - (r_1/r)^2]^{1/2}$.

It is thus T that is measured and k that is calculated. The calculated T is shown as a function of r_1/r in Fig. 2 *A*. Incorrect interpretation of this transmission can lead to a variation of transmission with illumination condition incorrectly ascribed to an SV error.

APPENDIX B

Transmission of Convergent Light through a Parallel-Sided Section:

The effect of the illuminating cone-angle of convergent light used to measure the transmission of a parallel-sided specimen or tissue section must be considered (Fig. 1 *D*). The incident light flux through the element of solid angle shown is $I_0 2\pi r^2 \sin\theta\, d\theta$, in which I_0 is the flux per unit area at distance r from the centre of the specimen. Absorption in a thickness d is $e^{-kd/\cos\theta}$.

The ratio

$$\frac{\text{Total flux through absorber}}{\text{Total flux without absorber}} = \frac{\int_0^u I_0\, 2\pi r^2 \sin\theta e^{-kd/\cos\theta}\, d\theta}{\int_0^u I_0\, 2\pi r^2 \sin\theta\, d\theta}$$

$$= \frac{\int_0^u \sin\theta e^{-kd/\cos\theta}\, d\theta}{\int_0^u \sin\theta\, d\theta} = \text{observed transmission } T.$$

For parallel light this reduced to $I = I_0 e^{-kd}$, in which k = the absorption coefficient, and u = the half-cone angle of the convergent light.

The transmission is thus a function of the numerical aperture of the illuminating condenser or limiting aperture of the optical system. Uber (20) has evaluated this transmission as a function of kd and limiting angular aperture $2u$. Increasing $2u$ from 42° to 89° with $kd = 1$ raises the error in k from 4 per cent to 17 per cent.

Again, incorrect interpretation of this transmission can, therefore, lead to a variation of transmission, as a function of the illumination, incorrectly ascribed to an SV error.

This may account for some of the discrepancies in the literature concerning the effect of condenser angle on flare error (8, 11, 22).

APPENDIX C

The Two-Wavelength Method of Ornstein (13) and Patau (15):

For comparison with Patau, the density $D = -\log T$ is referred to as the "extinction" E. One may then define an extinction coefficient k^1, in which $D = k^1Cd$, C being the concentration of an absorbing specimen of thickness d. Hence $k^1C = k/\ln_e 10$ and $k^1 = \epsilon/\ln_e 10$. We consider an object of area A and of transmission σ situated in an illuminated field, illuminating a photomultiplier aperture corresponding to an area B in the object plane. $B \geq A$. Let the incident light intensity $= I_0$. Without the object present, the total light flux $= I_0 B = I_{\text{inc}}$. With the object present, the total light flux $= I_0 A\sigma + I_0(B - A) = I_T$. The total relative field transmission $T = (I_0 A\sigma + I_0(B - A))/I_0 B = (A(\sigma - 1) + B)/B$. For the object alone $I/I_0 = \sigma$, in which I = intensity transmitted through the object alone. The extinction E of the object $= -\log_{10}\sigma = k^1Cd$ in which k^1 = the extinction coefficient of the object, C the dye concentration, and d = the thickness (when assumed uniform). The mass of dye in the object, of volume Ad, is thus $M = EA/k^1$ milligrams. Putting

$L = (I_{inc} - I_T)/I_{inc} = 1 - T$, which defines the light loss in traversing the field of area B, we have $L = -A(\sigma - 1)/B$ or $\sigma = 1 - BL/A$.

Hence $M = -(A/k^1) \log(1 - BL/A)$

$= (-A \ln(1 - BL/A))/(k^1 \ln 10)$

$= -KA \ln(1 - BL/A)$ (in which $K = 1/(k^1 \ln 10)$)

Which $\approx KBL$, for $|BL/A| \ll 1$.

This relation is only true, since $B/A \geq 1$, for very small values of the light loss L, *i.e.*, high transmissions. Patau, therefore, replaces the equation $M = KBL$ above by the relation, $M = K_1BL_1C_1 = K_2BL_2C_2$ in which C_1 and C_2 are constants. Choosing $K_1/K_2 = 2$, *i.e.*, $k_2^1 = 2k_1^1$ by selecting two-wavelength λ_1 and λ_2, which produce two extinctions E_1 and E_2 of the dye staining the object, such that $E_2(\lambda_2) = 2\,E_1(\lambda_1)$, he evaluates the constant C_1 as being $C_1 = (1/(2 - Q))\cdot \ln[1/(Q - 1)]$. When $K_1/K_2 = 2$, $Q = L_1/L_2$, and $C_2 = 2Q\cdot C_1$. Measurement of K_1, K_2, L_1, L_2, and B then yields the value of M.

The derivation of the above relation involves assumptions about the uniformity of the dye distribution and the relation between M and the light loss L. As Patau points out, these assumptions are only valid for extinctions E_{max} not exceeding 0.6. As we have shown, for such extinctions the SV error can be reduced to a negligible amount. It is true that with the Patau method the stray-light error x $(= I_{stray}/I_{10})$ produces

$$L^1 = 1 - T^1 = 1 - \left(\frac{I_{11} + xI_{10}}{I_{10} + xI_{10}}\right) = \frac{I_{10} - I_{11}}{I_{1.}(1 + x)} = \frac{L}{(1 + x)}$$

in which

I_{11} = the true total transmitted flux
I_{10} = the true total incident flux
L = true light loss
L^1 = apparent light loss.

Hence $M \propto 1/(1 + x)$; and the computed dye content error is $(-x)$. As opposed to this the direct method yields a result of

$$M \propto E = -\log \sigma^1 = -\log\left(\frac{I + xI_0}{I_0 + x\,I_0}\right) = -\log(\sigma + x)$$

and the dye content error is $(-x/\sigma)$. σ^1 defines an apparent transmission for the object.

At first sight it, therefore, appears advantageous to utilize the Ornstein and Patau ratio method as a means of eliminating the flare light error, *e.g.*, given 2 per cent of flare light (*i.e.*, $x = 0.02$ above) the maximum error in the ratio method determination of the dye content M is -2 per cent. In the direct method a 2 per cent flare light would produce a maximum error (corresponding to the maximum density of 0.6 permitted on the ratio method) of -8 per cent in the dye content. For densities below 0.6 the error is reduced for the direct method and remains constant for the ratio method. For densities greater than 0.6 a comparison cannot be made, the limitation being imposed by the necessary condition of $|BL/A| \ll 1$.

The fallacy of the above comparison lies in the statement of flare error. In the ratio method much of the light is transmitted through regions not occupied by the absorbing object, and the postulated measuring conditions, of $B \geq A$ and the illuminating beam $\geq B$, are conducive (as we have shown) to large flare errors. Furthermore, the ratio of intensities actually measured is $(A(\sigma - 1) + B)/B$, as compared with the larger ratio σ measured by the conventional method. Finally, in the direct method, ideally, when the illuminating beam is smaller than the specimen, the superstage flare light becomes not xI_0 but $x\sigma I_0$, and the flare error disappears. In practice, this situation is not obtained. Nevertheless, under the conditions evaluated in this paper the flare error, for densities of order unity, can be reduced to less than 1 per cent. We can conclude that the ratio method only reduces the effect of flare light error to one which is directly proportional to the flare light, and does not eliminate it as claimed by both Ornstein and Patau. Furthermore, the ratio method does not offer a realizable reduction in stray-light error, since the measuring conditions necessitated by the method are instrumental in producing a high level of flare light. Also, in the density range for which the ratio method can be used the flare light error is usually very small. The principal advantage of the ratio method remains in its ability to reduce the distribution error.

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