Counts on a number of droplets must be made and the data examined statistically in order to establish the reliability of the techniques used in specimen preparation for each material studied. Individual patterns suitable for counting represent volumes of the order of 10^{-8} ml of original suspension. Since it is desirable for assay purposes to have at least 10 organism particles in each droplet pattern, it is apparent that an approximate minimum concentration of 10^9 organisms/ml is required. Inasmuch as a volume of only 10 λ is required for spraying, however, a total of 10^7 particles is sufficient for making a numerical assay.

References

1. BACKUS, R. C., and WILLIAMS, R. C. *J. Applied Phys.*, **21**, 11 (1950).
2. LURIA, S. E., WILLIAMS, R. C., and BACKUS, R. C. *J. Bact.*, **61**, 179 (1951).
3. WILLIAMS, R. C., and STEERE, R. L. *J. Am. Chem. Soc.*, **73**, 2057 (1951).

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Schwarzschild-Villiger Effect in Microspectrophotometry

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Since Caspersson's pioneering work (*1*) in microspectrophotometry, this technique has yielded, especially in recent years, extensive applications in several modified forms (*2–4*), and has revealed many interesting problems in various fields of quantitative cytochemistry. The technique hitherto reported involves, however, a serious defect, which sometimes causes an error of large magnitude in the measured value of the light transmittance of a minute part of a cell nucleus, etc., thus leading to erroneous conclusions concerning the amount of its contents.

The defect in the usual measuring methods is that they are not free from the Schwarzschild-Villiger effect (*5, 6*). This effect, found by Schwarzschild and Villiger in 1906 in the microdensitometry of photographic plates, means that, in the measurement of the transmittance of a minute part of a photographic plate, the measured value would be unduly and sometimes seriously enhanced whenever the illuminating light flux is not limited to the part on the plate. The origin of the effect is readily conceivable. When the illuminating flux is spread over a wide area, including the minute part in question, the light passing through the surrounding portion may cause flare light, owing mainly to the internal reflections and scattering of the light in the image-forming optical system; and the flare light, being added to the image of the part in question, enhances the value of the measured transmittance. Thus the effect is especially prevalent when the transmittance of the part is low and that of its surroundings high, as in the case of the microdensitometry of the photographic image of a star or a spectral line. The circumstances are the same in microspectrophotometry, and the effect is serious in the case of a deeply stained cell nucleus embedded in a transparent background.

The main part of the flare light is caused by the internal reflections of light at each air-to-glass surface of the photomicrographic system, and the effect can readily be estimated quantitatively. If a light flux of one unit enters the lens system composed of m air-to-glass surfaces, all having the same average reflectance $\bar{r}$, the part $(1-\bar{r})/\{1+(m-1)\bar{r}\}$ of the flux is transmitted, of which the part $(1-\bar{r})^m$ is utilized for imagery, the effect of light scattering and absorption being neglected. Thus the ratio θ of the flare flux to the total transmitted flux is given by

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

which is the same as the ratio of intensity of illumination of the flare light (due to internal reflections only) on the image plane to the actual intensity of illumination on the same plane, provided the brightness of the object is uniform and its dimensions are sufficiently large—i.e., comparable with the effective focal length of the image-forming system. The values of θ for various values of m are shown in Table 1, for $\bar{r} = 0.05$

TABLE 1

m	θ (%) for $\bar{r} = 0.05$	θ (%) for $\bar{r} = 0.01$
1	0.00	0.00
2	0.25	.01
3	0.72	.03
4	1.40	.06
5	2.26	.10
6	3.27	.14
7	4.44	.20
8	5.73	.27
9	7.12	.35
10	8.62	.43
11	10.20	.52
12	11.84	.62
13	13.54	.72
14	15.29	.84
15	17.09	0.97

(for all usual optical systems) and $\bar{r} = 0.01$ (for coated optical systems).

If the number m of the photomicrographic system from the specimens to the image plane is 13, as is the case in our system—namely, 7 in an oil-immersed objective (f = 1.8~2 mm), 4 in a Huygenian eyepiece, and 2 in a reflection prism—and if the specimen is illuminated by a beam of light several millimeters wide, then the flare light caused by internal reflections only amounts to *ca* 13.5%, and the transmittance of a deeply stained nucleus having the true transmittance

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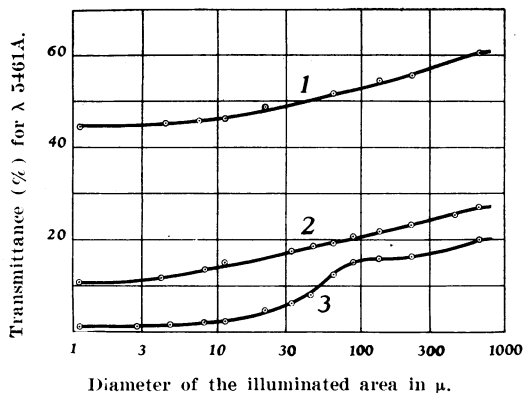

FIG. 1. 1—Isolated nucleus of rat liver (diam, 6.2 μ); 2—isolated nucleus of rat liver (diam, 4.4 μ); 3—first spermatocyte of rat testis (diam, 5.5 μ).

of, say, 1.0% must be measured as 14.5%, which is fourteen times as large as the true value. Thus the error in the value of the transmittance is 1350%!

The error is further enhanced by the flare light because of the scattering of light at lens surfaces that are not perfectly polished or are contaminated, or contain minute bubbles. These flares cannot be evaluated by simple mathematical treatment, but it is certain that their effect is more serious in lenses of smaller dimensions, as in microscopic objectives of high magnifying power.

In order to verify the above statement and to get the true value of the transmittance, we have performed the following experiment upon cell nuclei of spherical form, 4 μ–6 μ in diameter, and differing in their transmittance of light.

The magnified image of the specimen (×2000) is formed at the image plane of a photomicrographic system, and a fixed diaphragm of 2 mm diameter, placed at the image plane, leads the light corresponding to the small part of the specimen 1 μ in diameter to a photomultiplier tube. The illuminating system under the specimen forms the image of a uniformly luminous iris diaphragm at the center of the specimen, the diameter of the image being variable from 1 μ to 650 μ. For smaller imagery an oil-immersed microscope objective is used as a condensing lens, as described in our previous report (*7*). The transmittances of each of the three specimens were measured by this apparatus, and in each case the diameter of the illuminated area was varied from 1 μ to 650 μ. The results are shown in Fig. 1. In cases of small illuminated areas, the measured transmittances of the specimens become constants which must be considered to be the real transmittances of the nuclei. When the illuminated area is broadened, the measured values show a gradual increase, but they tend to a constant saturated value, which must be the sum of the true value and the flare light discussed above. Although the maximum diameter 650 μ in our experiment is not sufficient to cause the fully saturated flare, the enhanced value exceeds the flare ratio shown in Table 1, where we can attribute the difference to the flare caused by the scattering of light. The irregularity in the curve for specimen No. 3 can be explained as the effect of other nuclei existing in the same field, as shown in Fig. 2.

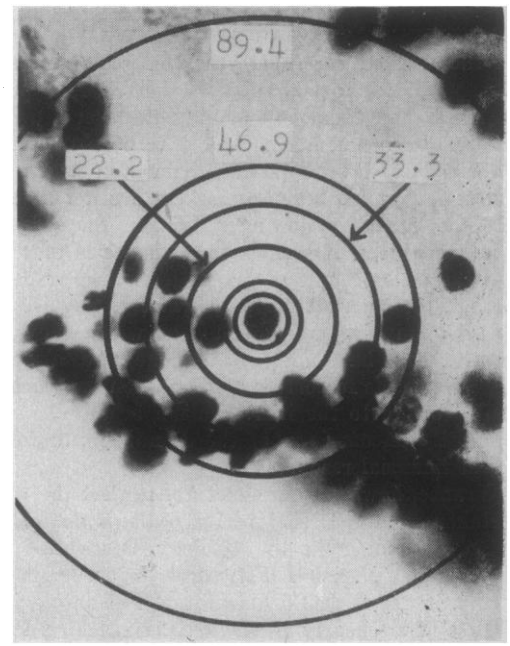

FIG. 2. Nucleus at center of circles is specimen No. 3. Number attached to each circle indicates its diameter in μ.

These results indicate that the true value of the transmittance can be measured only when the diameter of the illuminated area is less than (preferably one third) the diameter of the nucleus.

The Schwarzschild-Villiger effect can be reduced by antireflection treatment of all air-to-glass surfaces of the lens system. If we assume that the value of r be reduced to 0.01 by the coating and that no scattering of light occurs at these surfaces, the flare ratio θ, as shown in the last column of Table 1, amounts to only 0.72% in our system, which means 72% error in the example mentioned above, where the actual transmittance is 1.0%. The effect can be further reduced by using an all-reflection type microscope objective instead of the ordinary refraction type, in combination with a coated eyepiece. However, the scattering of light at each surface is not negligible in the measurement of low transmittance, so that the need for a small illuminated area can never be neglected, even after the improvements in the apparatus just proposed.

References

1. CASPERSSON, T. *Skand. Arch. Physiol.*, **73**, Supp. 8 (1936).
2. POLLISTER, A. W., and MOSES, M. J. *J. Gen. Physiol.*, **32**, 567 (1949).
3. SWIFT, H. H. *Physiol. Zoöl.*, **23**, 169 (1950).
4. LISON, L. *Acta Anat.*, **10**, 333 (1950).
5. SCHWARZSCHILD, K., and VILLIGER, W. *Astrophys. J.*, **40**, 317 (1906).
6. KOANA, Z. *J. Illum. Eng. Inst.* (in Japanese), **26**, 371 (1943).
7. NAORA, H. *Science*, **114**, 279 (1951).

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