A Photographic Method for Measuring Ultraviolet Radiation Scattered from Microscopic Objects.* BY GEORGE T. RUDKIN AND SALLY L. CORLETTE. (*From the Institute for Cancer Research and Lankenau Hospital Research Institute. Philadelphia.*)‡

Light scattered from a microscopic object and thereby lost from an image-forming system can be one of the major sources of error in a microspectrophotometric measurement. The classical theoretical treatments of scattering by Rayleigh and by Mie have been used by Caspersson (1, 1 *a*) to determine boundary conditions under which the non-specific light loss can be expected to be negligible in measurements made on biological material in the ultraviolet region of the spectrum. It has been pointed out (2) that it is often difficult to assure conformance with one of his conditions, namely, that the relative refractive index between object and medium be less than 1.1. It is also objected that his calculation of the diameter of the smallest measurable area, 3 μ, does not take into account adequately the submicroscopic structure within that area (3, 5). These and other difficulties point to a need for a direct method of measuring scattered radiation whenever a new type of material is to be studied.

The drawbacks to extrapolation from extinction measurements made at wave lengths not absorbed into the region of the absorption peak have been amply discussed (2, 4). The construction and operation of a first class instrument for the measurement of the whole light-scattering pattern is a costly and time-consuming project. Ornstein (7) has used darkfield conditions for refractive index matching to minimize scatter in the visible region. The present report describes a simple and convenient way in which a darkfield mircoscope can be used to judge relative intensities of ultraviolet radiation scattered from a small part of a microscopic object.

For the present exploratory observations, darkfield conditions were obtained by means of an annular diaphragm (designed for phase contrast) at the back focal plane of a glycerin immersion quartz condenser. The hollow cone of radiation focused on the object by the condenser was included between the angles 22 and 24 degrees from the optical axis. The specimen was viewed through a 6 mm. focal length quartz objective which did not collect any of the radiation in the illuminating hollow cone, but did collect radiation coming from an object within 13 degrees (N.A. 0.35) from the optical axis. Thus, if we consider a tiny segment of the hollow cone as an incident beam, the objective could collect only rays scattered by the object between the angles 10 and 36 degrees from the axis of the *incident beam*. (See Text-fig. 1) (All angles are in quartz.) Some of the radiation scattered between these limiting angles was not collected by the objective.

The remainder of this report describes a method by which comparative measurements of the intensity of the collected scattered radiation can be made. Although the collection of all radiation scattered between known angles would enhance the value of measurements, the

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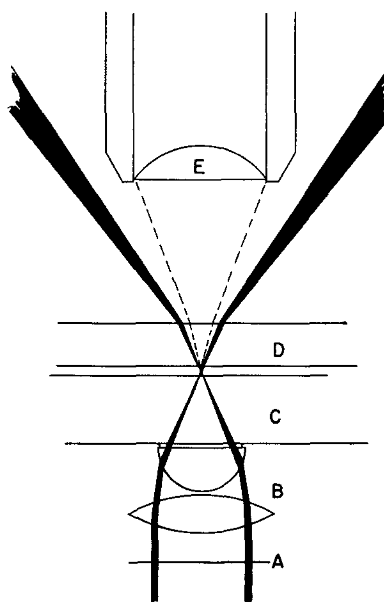
present arrangement is sufficient to demonstrate the utility of the method.

A visual check (by means of a fluorescent eyepiece) showed that the scattering detectable by this system was very sensitive to the index of refraction of the mounting medium, and that the index of the medium (glycerin) could be changed significantly by adding chloral hydrate or $ZnCl_2$, as first recommended by Köhler (8). Fig. 1 shows darkfield photomicrographs taken at 280 millimicra of a salivary gland chromosome of *Rhynchosciara angelae* in media of two refractive indices.

Measurements were carried out on salivary gland chromosomes of *Drosophila melanogaster*, fixed in 45 per cent acetic acid, squashed on quartz slides, and mounted in a glycerin solution (see reference 9). The refractive index of the mounting medium (95 per cent glycerin, 5 per cent saturated aqueous lanthanum acetate) was increased by the addition of zinc chloride. The values of n at 546 millimicra and 20 degrees C. (determined on a Pulfrich refractometer) for the media used are given in the column headings of Table I. Measurements given in the tables were performed on the same individual chromosomes after each successive change of mounting medium.



TEXT-FIG. 1. Diagram (not to scale) of the microscope illuminating system for obtaining darkfield images in the ultraviolet region of the spectrum. *A*, annular diaphragm; *B*, condenser; *C*, slide; *D*, coverslip; *E*, objective. Longitudinal section of illuminating cone of radiation shown in black.

Relative intensities of incident and scattered radiation were measured by the photographic procedure outlined in earlier publications (1, 9, 10). The intensities employed for calibrating the plates with a rotating step sector were intermediate between those found in the

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bright- and darkfield images. Reciprocity failure was not expected to introduce a serious error into relative values.

Measurements were made from plates exposed as follows (see Fig. 2). First, a photomicrograph was taken in bright field with the condenser iris at minimum aperture (1 mm.) to establish the intensity incident on the object (Fig. 2 A), using a spot 0.125 x 0.25 mm. at the plate (corresponding to 0.5 x 0.1 microns at the object). Each darkfield photomicrograph of a chromosome was scanned along a path between two recognizable details and along two paths 0.8 microns (at the object) from the first one. Thus, the intensity of the radiation scattered by a given spot

TABLE I

Relative Intensity of Ultraviolet Radiation Scattered between 10° and 37° by Places on Salivary Gland Chromosome Bands in Media of Different Refractive Index, Expressed as per Cent of the Intensity of the Incident Radiation

275 millimicra					257 millimicra				
n^{20}_{546}	1.46	1.52	1.50	1.48	n^{20}_{546}	1.46	1.52	1.50	1.48
359 A	0.6	0.01	0.01	0.4	359 A	0.3	0.01	*	*
	0.5	0.01	0.01	0.3		0.02	0.01	*	*
	0.3	0.01	0.01	0.2		0.02	<0.01	*	*
359 B	1.7	0.3	*	*	359 B	1.7	0.5	*	*
	0.3	<0.1	*	*		0.03	<0.01	*	*
362 C	0.8	0.01	0.02	*	362 C	0.5	0.01	0.01	0.3
	0.02	0.01	0.02	*		0.02	<0.01	0.01	0.01
	0.01	<0.01	0.01	*		0.01	<0.01	<0.01	0.01
						0.01	<0.01	<0.01	<0.01
366 A	0.4	*	0.02	*					
	0.4	*	<0.01	*	366 A	0.3	0.02	*	*
	0.01	*	<0.01	*		0.01	<0.01	*	*
	<0.01	*	<0.01	*		0.01	<0.01	*	*
						0.01	<0.01	*	*

* Not measured.

called here the "monitored incident intensity." Second, the annular diaphragm was inserted into the optical system, the condenser iris opened wide, and a darkfield exposure was made on another part of the same plate (Fig. 2 B). The exposure time in darkfield was 60 times that in bright field; the final magnification was 240 diameters.

Per cent transmission of the plate was read with a recording densitometer could be measured at both wave lengths and in each medium. The value for the relative intensity was found graphically from the calibration curve. The ratio of the intensity of scattered radiation (darkfield) to the intensity of the radiation incident on the brightfield background was calculated.

In the tables this ratio has been corrected for the ratio between monitored (brightfield) and real intensity of in-

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cident radiation. This ratio was determined from plates exposed exactly as described above, but employing an objective that collected all of the radiation passing through the condenser annulus (6 mm. focal length, N.A. 0.75, half-angle 30 degrees).

The observed ratios are given as per cent in Table I. In the first column on the left the preparation is indicated by a number, the particular chromosome on it by a letter. Two to four places on each chromosome were measured; the place showing the most intense scatter was one of them. The columns are arranged in the order in which the mounting media were used. Thus, a chromosome scattered much less radiation after transfer from the lowest (second column) to the highest (third column) index medium. That the refractive index was the principal cause for reduction in scatter is shown by the increase in scatter after chromosomes were returned to media having lower indices (fourth and fifth columns).[1]

The effect of these mounting media on scatter shows that the particles making up the chromosome bands are fairly uniform in refractive index. If they were very heterogeneous in that regard, then only a portion of them could be matched by any given liquid and little difference between observations in different media might have been observed. It is possible that the absence of a striking change on going from the highest to the next highest refractive indices used here is an indication that the chromosome bands are not completely homogeneous, although the observed values are, for the most part, below the useful range of the measuring system employed.

Our refractive index values are limited to the mercury lines at 546 mμ and 436 mμ. Extrapolation by the dispersion formula of Kordes (11) gives values of n close to 1.6 for our most concentrated $ZnCl_2$ solution at 275 and 257 mμ. This can be taken as a minimum estimate of the refractive index of the chromosome bands in the middle UV until higher index UV-transparent fluids are obtained.

The values given in the table are observed percentages. Some of the scattered radiation would have been absorbed before it left the chromosome and some of the incident radiation would have been attenuated in its course through the chromosome before it was scattered. The true scattering, corrected for absorption, must be higher than we observe. As a first approximation we can adjust the observed values upward by a factor equal to the reciprocal of the transmission of the object. For example, the maximum value in the table is 1.7 per cent. The per cent transmission found in that particular chromosome band at 275 mμ was 61.5, whereupon the corrected value is 2.7 per cent. Since only the densest bands scattered enough radiation to be conveniently measured under our conditions, the values of this correction factor would fall within a two-fold range for all of the tabulated cases and would, of course, be constant for any one place on a chromosome. Thus, none of the conclusions we have drawn would be altered by absorption at the wave lengths at which the measurements were made. Correction for absorption would be necessary in a system which collects all of the radiation scattered between two chosen angles from the incident beam, or when the

[1] The same observation indicates that the mounting media did not extract significant amounts of refractile material from the preparation. This was confirmed by measurements of the ultraviolet absorption of the same chromosomes in each of the media.

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amounts of scattering at different wave lengths or by different objects are to be compared.

It can be concluded, however, that the refractive index of the most concentrated solution of $ZnCl_2$ is very close to that of the chromosomal material at the two wave lengths used. Observation of the Becke line at low condenser apertures shows that the index in the solution is slightly lower than the index in chromosome bands.

The index has been directly matched at the wave lengths used in our laboratory (and in others (1, 4, 9)) for the localization and determination of nucleic acids and proteins in intracellular structures. The direct observation avoids assumptions regarding anomalous dispersion in the vicinity of absorption maxima.

Bibliography

1. Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73,** suppl., no. 8.

1 *a*. Caspersson, T., Cell Growth and Cell Function, W. W. Norton, New York, 1950.

2. Glick, D., Engström, A., and Malmstrom, B. G., *Science*, 1951, **114,** 253.

3. Scott, J. F., *Lab. Inv.*, 1952, **1,** 73.

4. Nurnberger, J. I., *in* Analytical Cytology, (R. C. Mellors, editor), New York, McGraw-Hill Book Company, 1955.

5. Walker, P. M. B., *in* Physical Techniques in Biological Research, (G. Oster and A. W. Pollister, editors), New York, Academic Press Inc., 1956.

6. Svensson, G., *Exp. Cell Research*, 1954, **6,** 529.

7. Ornstein, L., *Lab. Inv.*, 1952, **1,** 83.

8. Köhler, A., *Z. wissensch. mikr.*, 1904, **21,** 129 and 275. See also Köhler, A., and von Rohr, M., *Z. Instrumentenk.*, 1904, **24,** 341.

9. Rudkin, G. T., Aronson, J. F., Hungerford, D. A., and Schultz, J., *Exp. Cell Research*, 1955, **9,** 193.

10. Thorell, B., Studies on the Formation of Cellular Substances During Blood Formation, London, Henry Kimpton, 1947.

11. Kordes, E., *Naturwissensch.*, 1956, **43,** 52.

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EXPLANATION OF PLATE 259

FIG. 1. Photomicrographs of a salivary gland chromosome of *Rhynchosciara angelae* taken with the apparatus described in the text. In Fig. 1 A the mounting medium is glycerin with lanthanum acetate (see text). In Fig. 1 B the medium has been removed in 95 per cent alcohol and replaced with a 63 per cent solution of chloral hydrate in the glycerin, raising n at 540 mμ, 20°C. from 1.477 to 1.508. In Fig. 1 C the chloral hydrate solution has been replaced by the original mounting medium. Some chromosome fragments were lost from the preparation during the replacement. All photographic conditions were kept as constant as possible in the preparation of both negatives and prints. (λ: 280 mμ; Zeiss 6 mm., N.A. 0.35 monochromat 2570 A, Zeiss 10 $\times$ ocular, 840 diameters at plate).

FIG. 2. Photomicrographs of a chromosome of *Drosophila melanogaster* taken (Fig. 2 A) with the condenser iris at minimum aperture (transmitted light) and (Fig. 2 B) under dark-field conditions (see text). (λ: 275 mμ; Zeiss 6 mm., N.A. 0.35 monochromat 2570 A, Cooke 5 $\times$ ocular, 240 diameters at plate).

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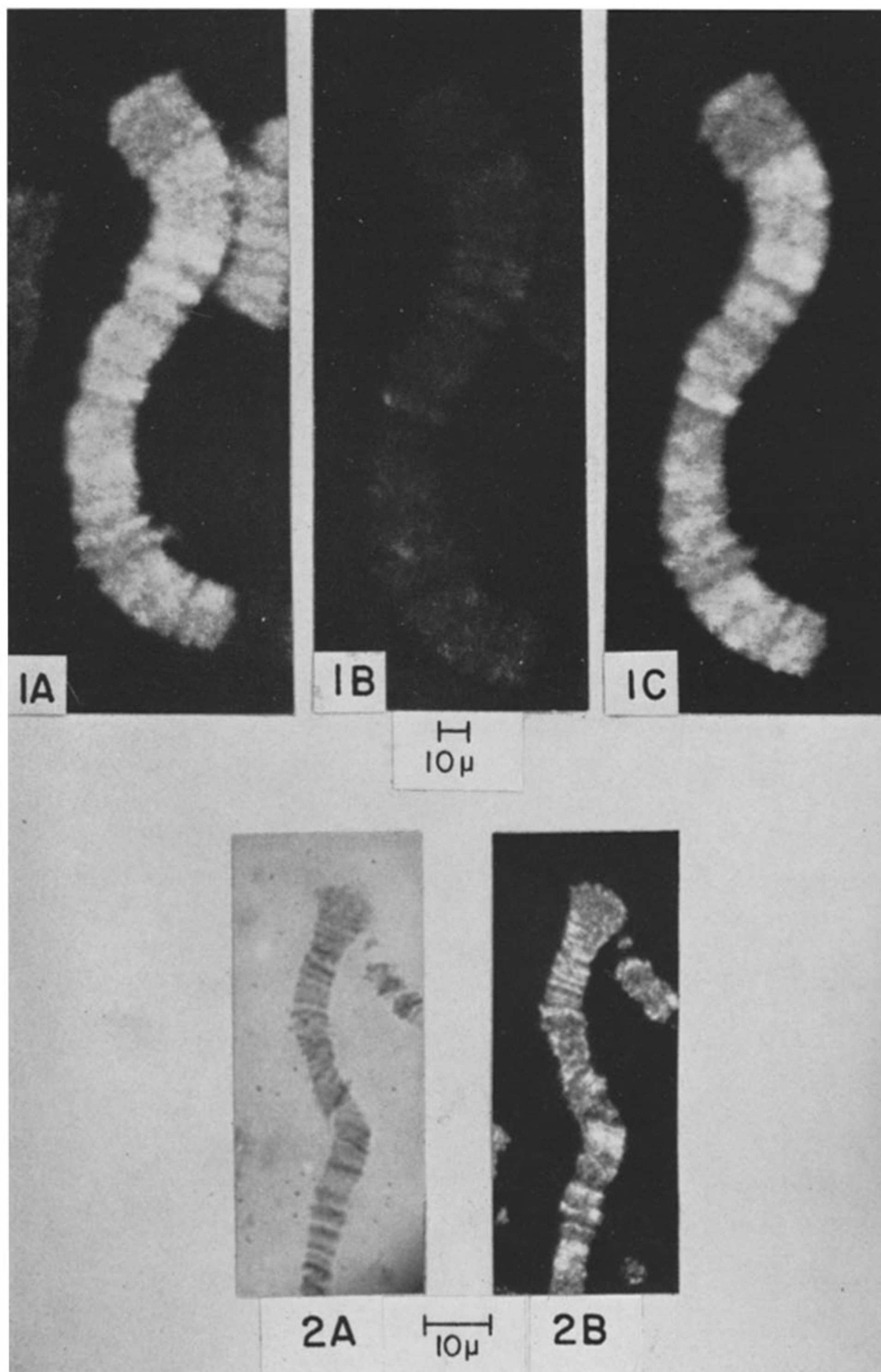

(Rudkin and Corlette: Method for measuring ultraviolet radiation)

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