ment this will average out so that the result from each tube can be found by simple subtraction.

This system has the advantage of simplifying the operation as compared with use of the conventional counting systems. In addition, only one expensive counter amplifier, scaler, and chart recorder is needed to carry on work at several locations simultaneously. In the studies performed in our laboratories, it has been necessary to obtain a record of the counting rate for each minute throughout a 15–20-min period (*1, 2*). This requires a rather elaborate computer and chart recorder. A considerable financial saving and an increase in operating efficiency have been effected by the use of several wire recorders and only the one counter amplifier, computer, and chart recorder.

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Ultraviolet Microscopy and Microspectroscopy of Resting and Dividing Cells: Studies with a Reflecting Microscope[1]

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Recent advances in microscopy, including the development of achromatic and apochromatic reflecting optics (*7, 9, 21, 22*), make it possible to study many fundamental biological problems in a manner not heretofore possible (*2, 3*). In this investigation are described the methods and results of a study of living cells with a reflecting ultraviolet microscope. The limits of monochromatic exposure are estimated in terms of the total ultraviolet radiant energy which does not produce abnormal modification of cells in tissue culture. Ultraviolet microspectroscopy of living cells is carried out within those limits, but subject to the consideration that the extinction values may be affected by one or more of the following factors: (1) the absolute amount of absorbing materials (*12*); (2) the volume in which absorbing materials are distributed (*30*); (3) the loss of light by dispersion or refraction (*12, 30*); (4) the spatial orientation of absorbing materials (*13, 17*); (5) photochemical reactions which may accompany the absorption of light quanta (*25*) during image formation; and (6) chemical changes associated with the oxidation or reduction of absorbing materials themselves (*16*).

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[2] Fellow in Cancer Research of the American Cancer Society recommended by the Committee on Growth of the National Research Council.

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METHODS

Optical System. The optical system of the reflecting microscope[4] consists of an objective and a condenser, which are two identical lenses of a reflecting-refracting type (*21*). The numerical aperture is 0.72; the focal length is 2.8 mm; and the magnification is 53×. The objective is used alone or with a 4× amplifying-type eyepiece.

Light Source. The ultraviolet light source is a mercury arc (Type A-H4) with the outer bulb removed. In the illuminating system[5] the central image of the arc is focused and projected by aluminized mirrors so that the beam uniformly illuminates just the full aperture of the condenser. When both condenser and objective are in focus, the full aperture of the objective is filled with light in accordance with Köhler illumination.

Wavelength. The selection of wavelength is made by a set of monochromatizing transmission filters for the ultraviolet region (*1, 4, 23*). The principal pairs of mercury lines isolated by three filter combinations and observed with a quartz spectrograph are those at 253.7 and 265.2 mμ, at 275.3 and 280.4 mμ, and at 312.6 and 334.1 mμ.

Photographic Method. The ultraviolet images are recorded by the photographic method, which is the most satisfactory method of ultraviolet image conversion available at present (*24*). According to Caspersson (*13*), only the photographic method is useful for the measurement of the absorption spectra of very small (less than 1 μ) and irregular bodies, although its accuracy, of the order of 5%–10%, is inferior to that of photoelectric methods.

The emulsions which have been selected to compromise the mutually opposing requirements of high sensitivity and high resolution in the ultraviolet region are the Kodak 103-O UV (spectroscopic plate) and the Kodak 1372 (35-mm film). The relative speed of Kodak 103-O UV at 260 mμ is about four times that of Kodak 1372. The resolution of Kodak 103-O UV, on the other hand, is about one-third that of Kodak 1372.

Processing of the negatives is carried out under controlled conditions of temperature, time, and technique which give maximum contrast and reproducible gamma.

Microdensitometry. Microdensitometry of the processed negatives is carried out with a photoelectric microphotometer that has a mechanically positioned film and plate carrier, interchangeable illuminating apertures, and a device for viewing and centering an area for measurement. The radius of the illuminating aperture is chosen so that it will be less than or equal to one-third of the radius of the image area (*12*).

[4] Designed by Mr. David Grey of the Polaroid Corporation and constructed by the Bausch and Lomb Optical Company under the supervision of Mr. L. V. Foster (*18*).

[5] Designed by Mr. David Grey and constructed by the Research Department of the Polaroid Corporation.

Computation of Extinction. The graphic method of computation (*6*, *29*) is used to calculate the extinction ($E_\lambda = \log \frac{I_0}{I}$) for a central portion of an object. I_0 is the intensity of the monochromatic light (λ) incident upon the object and in practice is determined graphically from the photographic density of the image of the empty field in the neighborhood of the object. I is the intensity of the light transmitted by the object and is estimated graphically from the photographic density of the image of the object.

Intensity of Radiation. The intensity of monochromatic radiation is measured by an electronic photometer and a search unit with a phototube (RCA 935). The sensitivity of the search unit and photometer is determined in terms of ergs per second of monochromatic radiation at 253.7, 265.2, 302.2, and 312.6 mμ by comparison with a calibrated thermopile which is exposed to the same intensity of radiation. In addition, for the measurement of the intensity of radiation in small areas of the image plane, or for the comparison of the incident intensity at a reference position with the transmitted intensity at another position, a photomultiplier tube (RCA 1P28) is used in a search unit with a variable aperture. This direct photometric determination of intensity differs from the indirect photographic method, inasmuch as the photographic method measures intensity integrated with respect to time (*32*).

Tissue Culture. The technique of tissue culture departs somewhat from customary procedures. Fragments of tissue, usually numbering three per culture, are placed on a sterile, dry, ultraviolet-transmitting cover glass[6] to which is then added a drop of liquid medium containing *no* plasma. A metal slide with a central aperture is mounted to this cover glass. An additional cover glass, which is sealed to the other surface of the slide, completes the enclosure of the culture.

Ten ml of nutrient medium is made up of two parts by volume of a balanced salt solution (*20*), one part of a solution of penicillin (containing 250 units), one part of a solution of streptomycin (containing 250 γ), two parts of chicken embryo extract, and four parts of human placental serum. Cultures are kept in the lying-drop position at 37° C for 24 hr and then examined for healthy, singly spread cells which have migrated from the explant.

At the time of the microscopic study, the nutrient medium is replaced after several washings with a balanced solution of electrolytes which contains 0.1% glucose, and the culture is placed in the hanging-drop position. There is now liquid contact between the two cover glasses and space for air at the periphery. Except during photography, ultraviolet light is excluded by filters, and the adjustment of the focus of the condenser and the objectives is made in the green light of the mercury arc. Upon completion of the microscopic studies, which require about 30 min, the culture is renourished with its original nutrient medium and placed in the incubator in the lying-drop position until further observation.

This method of preparation and study is applied to fibroblasts and endothelial cells of the embryo mouse, Akm strain (*10*); to a transplantable sarcoma, MA 387 (*5*), of the adult mouse of the Akm strain; to fibroblasts of the embryo chicken; and to fibroblasts of the rat[7] in the 46th generation of passage.

Criteria for lack of injury during the period of observation, which varies from 4 to 18 hr, are the following:

1. The absence of abnormal morphologic change, such as increased optical opacity or refractivity in the nucleus and the cytoplasm, as observed and photographed in a phase contrast microscope (*34*).
2. No increase in the ultraviolet absorption of nuclear material at 260 mμ such as that characteristically associated with the injury of living cells (*8*, *26*, *30*).
3. The normal continuation of function, such as protoplasmic irritability, migration, and division, as evidenced by continued observations of particular cells.

Results

Ultraviolet Radiation. As a result of a study of the ultraviolet radiation of 25 tissue cultures derived from mesothelium, together with the extensive data of Mayer (*28*) for chicken fibroblasts, an estimate is made of the total ultraviolet exposure tolerated by living cells without injury. The order of magnitude of the intensities used is 1×10^3 ergs/sec/cm², and the time of exposure varies from a fraction of a second to several seconds.

TABLE 1

Estimate of Ultraviolet Radiant Energy Tolerated by Living Cells

Wavelength (mμ)	Radiant energy per unit area (ergs/cm²) Tolerated by living cells	Incident on emulsion (ε_F*)	Incident on cell (ε_C†)	Approx. no. of photographs possible
260	1×10^4	38×10^{-3}‡	0.53×10^3	18‡
275	1×10^4	44×10^{-3}‡	0.62×10^3	16‡
315	$> 1 \times 10^5$	32×10^{-3}‡	0.45×10^3	> 220‡
260	1×10^4	8.7×10^{-3}§	0.12×10^3	83§
275	1×10^4	6.9×10^{-3}§	0.097×10^3	103§
315	$> 1 \times 10^5$	6.4×10^{-3}§	0.090×10^3	> 1110§

* To produce a background density of unity.

† Calculated for a magnification of 53× by the relation, $\varepsilon_C = \varepsilon_F \times M^2 \times \frac{1}{T}$, where M is the magnification (53), and T is the ultraviolet transmission (0.2) of the microscope objective.

‡ Emulsion—Kodak 1372 (35-mm film).

§ Emulsion—Kodak 103-0 UV (plate).

The tolerated exposure, the product of intensity and time expressed as energy per unit area of cell, is 1×10^4 ergs/cm² at 260 and 275 mμ, and in excess of 1×10^5 ergs/cm² at 315 mμ (Table 1, column 2). Within these limits of exposure the living cell undergoes no abnormal

[6] Corning Glass Works, *Vycor*, No. 791.

[7] The original strain was obtained from Dr. H. P. Thompson of the Rockefeller Institute for Medical Research.

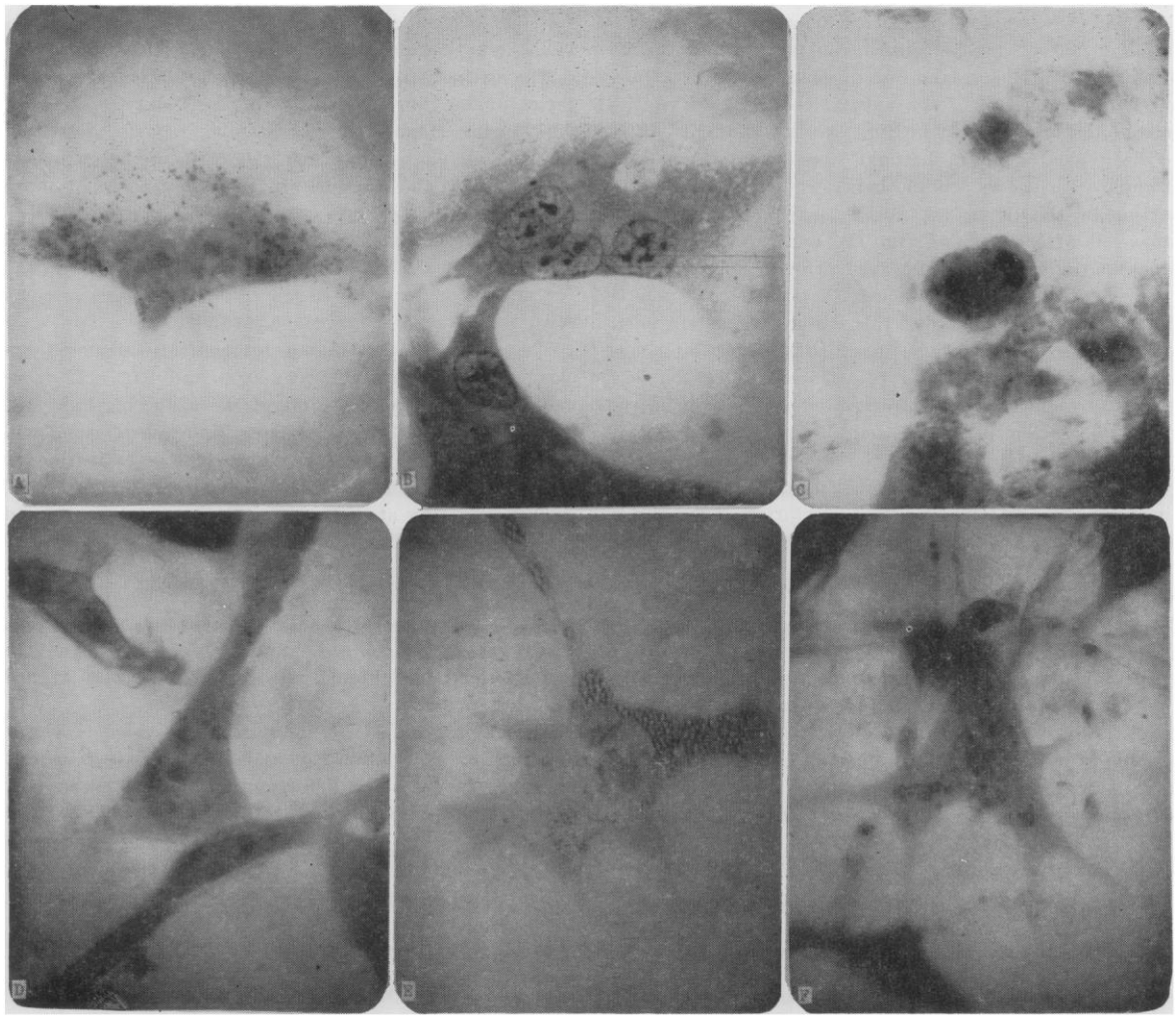

FIG. 1. Ultraviolet photographs of cells: A, rat fibroblast, living; B, rat fibroblast, fixed; C, rat fibroblast, living, mitosis; D, chicken fibroblast, living; E, mouse sarcoma, living; F, mouse sarcoma, living. All photographed at 254–265 mμ. Magnification × 600.

change in size or shape; the transparency of the interphase nucleus is unaltered in visible and in ultraviolet light; the mitochondria are intact; there are no degeneration granules in the cytoplasm; and normal function is not manifestly interrupted. However, in view of technical errors in the measurement of the absolute exposure together with biological variations which are likely to occur in a more extensive study, the limits of exposure are subject to a tenfold variation in order of magnitude.

The foregoing data are useful in predicting the number of photographic images that may be recorded in the ultraviolet region without injury to living cells. In column 3 of Table 1 is given the radiant energy per unit area of emulsion required to produce a photographic density of unity. At 260 mμ the order of magnitude is 38×10^{-3} ergs/cm² for Kodak 1372 and 8.7×10^{-3} ergs/cm² for Kodak 103-O UV. The radiant energy incident on a cell when a photographic image with a background density of unity is formed at a magnification of 53× is given in column 4 of Table 1. At 260 mμ the order of magnitude is 0.53×10^{3} ergs/cm² of cell for Kodak 1372 and 0.12×10^{3} ergs/cm² for Kodak 103-O UV. It is to be noted that the radiant energy incident upon the cell and required for the formation of the photographic image is directly proportional to the square of the magnification at the image plane and inversely proportional to the ultraviolet transmission of the microscope objective.

The approximate number of photographs permitted without exceeding the radiant energy tolerated by living cells is given in column 5, Table 1, and is obtained by dividing the data in column 2 by the corresponding data in column 4. At 260 mμ at a magnification of 53×, approximately 18 photographs may be taken with Kodak 1372 and 83 with Kodak 103-O UV. These results are considered to be first order approximation.

Ultraviolet Photomicrography. Ultraviolet photographs of living cells taken at 260 mμ are shown in

Fig. 1A, C–F, which are positive prints with a magnification of 600 ×. The low optical contrast (relative to the density of the background) of nuclear structures at 260 mμ is characteristic of the natural condition of resting cells of the type studied in Fig. 1A, C, D, and E.

The typical changes brought about by fixation, for example with acetic acid-alcohol, is indicated by a comparison of the living rat fibroblast, Fig. 1A, with the same cell after fixation, Fig. 1B, and with other cells which had migrated into the field prior to fixation. The ultraviolet absorption of nuclear material at 260 mμ is increased after fixation, and this is due, at least in part, to a decrease in the volume in which the absorbing materials are distributed, and to an increase in the loss of light by dispersion and refraction (*30*). The ultraviolet absorption of cytoplasmic material at 260 mμ undergoes a different change. Formed elements in the cytoplasm such as the mitochondria in Fig. 1A, which dimensionally are at the limit of resolution of 0.2 μ for the objective lens, absorb much ultraviolet light in the living cell. These elements are dispersed in the fixed cell (Fig. 1B), and if initially present in abundance, then contribute to the ultraviolet absorption of the cytoplasmic ground substance.

As indicated in Fig. 1C, there is a characteristically high optical contrast (relative to the density of the background) in nuclear and chromosomal material at 260 mμ in living, dividing cells during certain stages of mitosis. As will be shown, the degeneration of cells is also accompanied by an increase in optical density of nuclear material.

Ultraviolet Microspectroscopy. The extinctions at 260 and 315 mμ in the nuclear and the cytoplasmic material of living and fixed cells are given in Table 2. A range of extinctions is given for nuclear material in order to indicate the order of magnitude of optical heterogeneity. The extinction at 260 mμ for the nuclear material of healthy, resting fibroblasts of the rat, chicken, and mouse, endothelium of the mouse, and sarcoma of the mouse, varies between 0.12 and 0.43. When corrected for the unspecific dispersion of light, as indicated below, the extinction varies between 0.12 and 0.33.

The extinction at 260 mμ for the chromosomal material of rat fibroblasts in mitosis is of the order of 0.97 during stages thus far studied, namely metaphase through anaphase, in which the chromosomes are condensed (*30*) and presumably spiralized in relation to their resting state.

The extinction at 260 mμ for nuclear material in fixed fibroblasts of the rat varies between 0.29 and 0.61, and for nuclear material in degenerating and fixed sarcoma cells, the value lies between 0.59 and 0.82.

The extinction at 315 mμ for centrally disposed nuclear material varies between 0.00 and 0.09. If these values are taken as measures of the unspecific light dispersion, the Rayleigh extinction (*11*, *33*) at 260 mμ will be approximately twice the value of the extinction at 315 mμ, i.e., it will vary between 0.00 and 0.18.

The extinction at 260 mμ for the cytoplasmic ground substance of resting fibroblasts and endothelium varies between 0.12 and 0.18, whereas the extinction for cytoplasmic formed elements, such as mitochondria, is undoubtedly much greater but not accurately measurable. After fixation, the mitochondria are dispersed, and the extinction of the cytoplasmic ground substance at 260 mμ is 0.28.

The interpretation of the extinction value, 0.85, at 260 mμ in the juxtachromosomal material of a rat fibroblast in mitosis is deferred until further study. It is the supposition that some of the absorbing material is either derived from mitochondria (*27*) and is dimensionally below the limits of resolution of the microscope, or is related to the formation of the spindle.

TABLE 2

EXTINCTION ($E\lambda$) AT 260 mμ AND 315 mμ IN NUCLEAR AND CYTOPLASMIC MATERIAL OF LIVING CELLS AND CELLS IN ALTERED STATES

Culture	Nucleus*		Cytoplasm*		Nuclear diam (μ)	Remarks
	E_{260}	E_{315}	E_{260}	E_{315}		
Rat fibroblasts	0.24–0.36	0.01–0.05	0.18	0.05	16.3 × 14.2	Resting cell (Fig. 1A)
Rat fibroblasts	0.29–0.61	.....	0.28	...	16.1 × 12.1	Same cell, fixed (Fig. 1B)
Rat fibroblasts	0.97†	0.10†	0.85‡	0.10‡	.....	Mitosis (as in Fig. 1C)
Chicken fibroblasts	0.22–0.35	0.00–0.05	0.21	0.00	21.6 × 14.2	Resting cell (Fig. 1D)
Mouse fibroblasts	0.25–0.40	0.07–0.09	0.12	0.00	19.4 × 11.8	Resting cell
Mouse endothelium	0.35–0.38	0.05–0.07	0.17	0.09	19.0 × 14.6	Resting cell
Mouse sarcoma	0.12–0.28	0.00–0.08	0.06	0.05	13.8 × 13.8	Resting cell (Fig. 1E)
Mouse sarcoma	0.37–0.43	0.00–0.05	0.27	0.05	18.0 × 14.2	Resting cell (Fig. 1F)
Mouse sarcoma	0.59–0.70	0.05–0.12	0.40	0.07	18.4 × 11.8	Same cell, degenerated
Mouse sarcoma	0.73–0.82	0.07–0.16	0.62	0.12	15.2 × 10.4	Same cell, dead

* Area of measurement = 1.5 μ².
† Chromosomal material.
‡ Juxtachromosomal material.

The extinction at 260 mμ for the cytoplasmic material of healthy, resting sarcoma cells varies between 0.06 and 0.27 and increases with the degeneration or death of the cell.

As stated previously, the estimate of the total ultraviolet, radiant energy that is tolerated by living cells without manifest injury is based upon a study of a number of tissue cultures derived from embryonic mesothelium, together with the more extensive data of Mayer (*23*) for chicken fibroblasts. The methods of the two

investigations, however, are dissimilar. In the work of Mayer the cells were in a medium containing a plasma coagulum and embryo extract, whereas in the present study at the time of radiation the nutrient medium is replaced by a simple electrolytic solution containing glucose but neither plasma nor embryo extract. Measurements of the ultraviolet transmission of the culture medium made according to an estimate of the amounts and proportions of plasma and embryo extract used by Mayer indicate that this medium transmits only 20% of the incident light at 260 mμ and 275 mμ, and 100% at 315 mμ. Thus, in the original investigation of Mayer, the cells probably received about one-fifth as much radiation at 260 and 275 mμ as had been estimated. Despite these discrepancies in methods, the orders of magnitude for the total radiant energy tolerated by living cells of the type studied at 260, 275, and 315 mμ agree within a factor of ten, which is the probable limit of error of the measurements.

The qualitative aspects of the ultraviolet microscopy of living cells are in general similar to those reported by others (*8, 30*). There are in the literature, however, no quantitative studies of nuclear material such as those described in the present communication.

The type of distributional analysis described by Commoner (*17*) cannot be applied to the present study, for the data are insufficient in number. It is noteworthy, however, that in the nuclear material of healthy, living cells the extinctions at 260 mμ, when corrected for the unspecific dispersion of light, are in the region of 0.3. Furthermore, in mitosis during metaphase and anaphase, the corrected extinctions at 260 mμ in nuclear material increase to values in considerable excess of 0.3. Among several possible interpretations (*17*), such an increase in extinction is suggestive of a change from an oriented to a random distribution of nucleic acids. This change may be related to a decrease in the degree of orientation of the major ultraviolet chromophore, the —C=C—C=N— system of the pyrimidine ring (*15*), in the desoxyribose nucleic acids of chromosomal fibrils which are presumed to be more spiralized in metaphase and anaphase than in the resting state. However, direct observations of the spatial orientation of nucleic acids in the chromosomal material of living cells are not yet reported, and the data for fixed chromosomes (*14, 19, 31*) are inconclusive (*17*).

In summary, an estimate is made of the total ultraviolet exposure tolerated by cells in tissue culture without injury. The value, expressed as energy per unit area of cell, is 1×10^4 ergs/cm^2 at 260 and 275 mμ, and in excess of 1×10^5 ergs/cm^2 at 315 mμ. Microspectroscopic studies of living cells are carried out within these limits of exposure. It is noteworthy that in the nuclear material of healthy, resting cells the extinctions at 260 mμ, when corrected for the unspecific dispersion of light, are in the region of 0.3, while in certain stages of mitosis the extinctions in nuclear material increase to values in considerable excess of 0.3.

Addendum

Developments of significance in relation to this work have occurred since this paper was written:

1. Methods have been developed which increase the reflectivity of the mirrors in the microscope objective (Foster, L. V., personal communication).

2. An achromatic monochromator has been utilized to illuminate the microscope with band widths of the order of 50 mμ, or less.

3. Preliminary studies of fixed cells with plane-polarized ultraviolet light gave no evidence of orientation of chromophore at 260 mμ.

4. Spectrographic recordings of the ultraviolet absorption of living cells have been made at *many* wavelengths *simultaneously*, but not without eventual injury to the cell.

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Cataloguing of Infrared Spectra[1]

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A brief method for matching an infrared spectrum, in the 2–16-μ region, of an unknown sample to a large number of known spectra has proved satisfactory for nucleic acid derivatives, and may well be an aid in many of the empirical uses of infrared data. The method is analogous to the ASTM–AXRED X-Ray Powder Pattern Card File as devised by Hanawalt and co-workers (*1*).

For the infrared spectra, the cataloguing procedure is as follows: A smooth base line is drawn on the high transmission side of the absorption bands. This affords a scattering correction. (See Fig. 1 for a solid sample

λ(μ)	9.15	9.48	11.97b
E/E₁	1.0	0.7	0.5

1-D arabinosyl uracil
(from Dr. Irving Goodman)
Capillary mineral oil mull

Curve 81198

λ(μ)	E/E₁	λ(μ)	E/E₁
2.83	0.4	9.15	1.0
3.01	0.5	9.48	0.7
5.84	0.3	9.81	0.1
5.98	0.6	10.57	0.3
6.99	0.3	11.04	0.2
7.67	0.3	11.97b	0.5
7.87	0.5	12.28	0.1
8.11	0.5	13.24	0.1
8.30	0.3	15.2bb	0.1
8.65	0.4		
8.98	0.7		

FIG. 2. The infrared file card for 1-D arabinosyl uracil.

bands (excepting those of mineral oil for mulled samples), chemistry, method of preparation, etc., are recorded on the card (see Fig. 2); the spectrum may be printed on the back. The cards are then filed according to wavelength of the strongest band.

To identify an unknown, its spectrum is run and its card tabulated, then the file is searched at the wave-

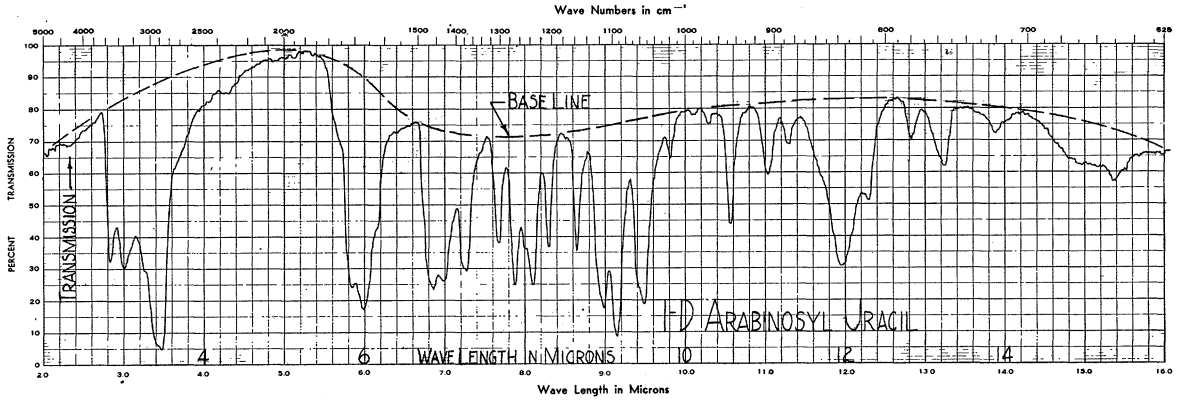

FIG. 1. The infrared absorption spectrum of 1-D arabinosyl uracil, with base line drawn in.

mulled in mineral oil. Visibly transparent samples have a flatter base line.) The extinctions, log ($T_{\text{base line}}/T_{\text{absorption peak}}$), are then determined for the three strongest bands in the region 9.00–15.00 μ. This is primarily the "backbone" vibration region, bands here being more characteristic of the whole molecule than the side group bands of shorter wavelength. The extinction of a band overlapped by other bands is calculated by subtracting from the observed extinction the extinctions of the overlap bands extrapolated. These three bands are then tabulated as to wavelength in order of decreasing extinction, and the ratios of the extinctions of the bands to the extinction of the strongest are tabulated. If two have the same extinction, the one of shorter wavelength is tabulated first. The data for each molecule are put on a 3 in.×5 in. card, the wavelengths of the three strongest bands being prominently placed in the upper left of the card, as with the x-ray cards. Other strong length of the strongest band (in the 9–15-μ region), plus or minus 0.04 μ to allow for wavelength errors. The inclusion of a secondary set of cards, on which the second strongest band is listed first, and filed accordingly, also allows for intensity errors from uncertainties of base line or from instruments of markedly different slit widths. When the three strongest bands are matched, other tabulated bands of the known and unknown are compared and then the spectra are directly matched and possibly rerun under identical conditions. Bands of widths greater than 0.1 μ within 2% of the absorption maxima are designated with a "b," greater than 0.2 μ with a "bb." Improvement in purity or sample preparation may permit resolution of several bands here, so that these values should be considered with caution. Simple mixtures may be identified by successive elimination of bands, though adjacent bands of the components may be unresolved. Chemical compounds, however, have bands in the 9–15-μ region quite different from those of the free components.

As compared to the various punch card systems, such

[1] Supported by a fellowship from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.