On the Interpretation of the Absorption of Ultraviolet Light by Cellular Nucleic Acids

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EFFORTS TO EXTEND the field of quantitative biochemistry to the level of the single cell have been based largely on absorption spectrophotometry. This method has suggested a means of determining intracellular concentrations of specific substances by virtue of the proportional relationship between the number of molecules in the light path and the extinction at the wavelength characteristically absorbed by the compound in question. ($E = \log I_0/I = \varepsilon cd$).[1] This relationship, the Beer-Lambert law, usually holds for the absorption of light by gases and solutions, and—for the purpose of determining intracellular concentrations—is *assumed* to be true for the physical states encountered in living and dead cells (*8, 17, 18, 32, 33*). Conclusions based on this assumption have led to the development of a number of theories concerning the biochemistry of the nucleus, nucleic acid metabolism, protein synthesis, and metabolism of tumor cells (*8*).

However, it has been recently suggested (*12*) that the amount of light specifically absorbed by the substance under study may vary considerably with the degree of orientation imposed on the material. It was shown that when the following two conditions exist together, materials will fail to absorb more than one-half the incident light and thereby depart widely from the Beer-Lambert law: (a) the molecules absorb light by virtue of a distinctive electronic axis (i.e., one occupying a unique position in the structure of the molecule) and (b) the molecules exist in an oriented aggregate.

Most intracellular extinction measurements have been intended as determinations of intracellular nucleic acid content, and these data have figured largely in the development of the theories cited above. It would seem important, therefore, to determine to what degree the two conditions required for anomalous absorption apply to the nucleic acids occurring in cellular structures. As will be shown below, such an analysis suggests that the extinctions of intracellular objects at 260 mμ do not necessarily reflect their actual nucleic acid content, and that considerable caution must be used in interpreting such data.

Optical properties of nucleic acids.

The characteristic ultraviolet absorption of the nucleic acids is a property of their constituent purine and pyrimidine bases. According to the theory of color developed by Lewis and Calvin (*21*), the absorption of light is due to specific electronic oscillations within the structure of a molecule. Hence, for planar molecules such as the purines and pyrimidines, these oscillations can be resolved into lines in this plane.[2] As shown by Lewis and Calvin and by Ferguson (*13*), molecules which are nonsymmetrical with respect to the position of the groups responsible for their color have two fundamental electronic axes, both lying in the plane of the molecule. These axes are characterized by different wavelengths, the oscillation of lower energy representing the absorption maximum at the longer wavelength.

In some cases Lewis and his collaborators (see reference *12* for references) have localized these axes with respect to the dimensions of the molecule by determining the effect of the plane of polarization of the incident light on the extinctions yielded by molecules in rigid media. They have shown for such cases that the extinctions at the two characteristic wavelengths reach maxima at planes of polarization which are at 90 degrees to each other.

Unfortunately, comparable data for the nitrogen bases are lacking, and in discussing them one is forced to deal with assumptions based on analogy with the molecules studied by Lewis *et al.* The lack of symmetry of the nitrogen bases suggests that they possess two distinctive absorption axes lying in the plane of the molecule. Except for guanine, these substances yield a single maximum in the ultraviolet (see Hotchkiss, *16*) at 260–265 mμ in neutral solution. This maximum must represent either (a) the absorption

[1] E = extinction, I_0 = intensity of incident light beam, I = intensity of transmitted beam, c = concentration of absorbing material in moles/liter, d = thickness of the sample in cm, and ε = molar extinction coefficient.

[2] Such molecules may have a third axis representing electronic oscillations perpendicular to the plane of the molecule. The energy level of such oscillations is so high that this axis is characterized by light absorption at wavelengths too short to fall within the range involved in ordinary spectrophotometry of solutions. This type of absorption will therefore not be considered.

due to one of the two distinctive axes; or (b) absorption due to both axes.

If the first assumption is the correct one, the maximum at 260–265 mμ must represent one of the axes, and the second axis must account for an absorption maximum at a significantly shorter wavelength. The second maximum would probably lie in the range below 220 mμ and therefore be undetectable by spectrophotometry of solutions. If alternative (b) is the correct one, the axes must be characterized by nearly identical wavelengths. In the absence of critical data, it is of course impossible to choose between these alternatives, but on the basis of what is known about the optical properties of nonsymmetrical molecules, the second alternative seems the less likely one. Hence for the purposes of this discussion we shall assume that the absorption of the nitrogen bases (other than guanine) at 260–265 mμ is due to a distinctive electronic axis which lies in a unique position with respect to the dimensions of the molecule. If this assumption is incorrect, the analysis which follows is altered in certain details, which will be noted below.

The structure of nucleic acids has been discussed by Astbury (*1*) who concludes that the constituent nucleosides are arranged in a parallel stack, so that the entire molecule has the configuration of a roll of coins (where each coin is a nucleoside). He also suggests that each nucleoside is probably oriented in the same way with respect to the long axis of the molecule, thereby placing the rings of the nitrogen bases in complete alignment along the length of the molecule.

It follows, therefore, that the absorption axes of the nitrogen bases which account for the extinction of nucleic acids at 260 mμ must be rigidly oriented throughout the entire polynucleotide. Although the available data are insufficient to describe completely their position with respect to the dimensions of the nucleic acid molecule, these axes must lie in planes perpendicular to the long axis of the molecule. This is in accord with Schmidt's observation (*27*) that the birefringence of thymonucleate fibers is negative with respect to the long axis of the fiber, and the finding of Signer, Caspersson, and Hammersten (*30*) that flow birefringence is negative to the direction of flow. It also agrees with Caspersson's observation (*7*) that for maximum absorption at 257 mμ by partially oriented films of thymonucleic acid, light must be polarized in a plane perpendicular to the long molecular axis.

Similar results have been obtained by Butenandt *et al.* (*3*), who studied the absorption spectrum of flowing solutions of tobacco mosaic virus with plane polarized light. They found that the extinction at 260 mμ was at a maximum when the light was polarized in a plane perpendicular to the direction of flow (and therefore also perpendicular to the long dimension of the molecules oriented by flow), and concluded that the absorbing groups must occupy planes perpendicular to the long axis of the virus nucleic acid (which parallels the long axis of the virus particle itself).

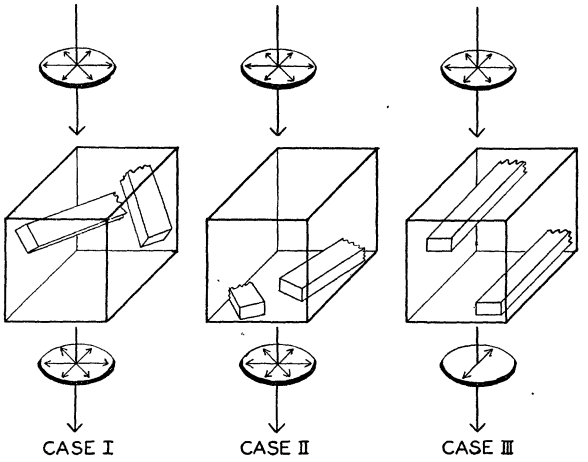

FIG. 1A. The effect of orientation of nucleic acid on the absorption of unpolarized light. The nucleic acid molecule is represented as an elongated oblong prism (only part is shown) whose smallest face is parallel to the planes occupied by the nitrogen bases. In Cases I and II light of all electric vectors is equally transmitted; in Case III only light with a vector perpendicular to the long axis of the molecule is transmitted. Since this represents 50% of the incident light, Case III gives a maximum extinction of 0.3. The dimensions of the molecule are arbitrary.

This visualization of the optical structure of nucleic acid molecules makes possible consideration of the effect of molecular orientation on the extinctions yielded at 260 mμ. Since the preponderance of the constituent nitrogen bases (i.e., except guanine) probably contribute to the absorption by virtue of a distinctive electronic axis, and appear to be rigidly ordered along the length of the polynucleotide, the orientation of these axes is determined by the position of the molecule itself.

Hence we may consider the three cases of orientation of optical axes discussed in reference (*12*) in terms of the possible types of orientation of the nucleic acid molecule proper. These are diagramed in Fig. 1A. In Case I, the molecules, and the axes responsible for the absorption at 260 mμ, are randomly distributed in space. The relation between the number of molecules scanned (N) and the extinction (E) at 260 mμ is therefore a straight line, and the Beer-Lambert law is obeyed. In Case II the orientation is

at random within a series of parallel planes. The relation between E and N is again linear, but not identical with that of Case I. In Case III the molecules, and therefore the absorption axes, are completely oriented in three dimensions. Consequently, the relation of E to N is nonlinear above very low E values, and the system departs radically from the Beer-Lambert law. In this type of orientation, the fraction of incident light absorbed cannot rise above 1/2 and E cannot exceed 0.3. As a result, E becomes independent of the number of molecules scanned as N increases.

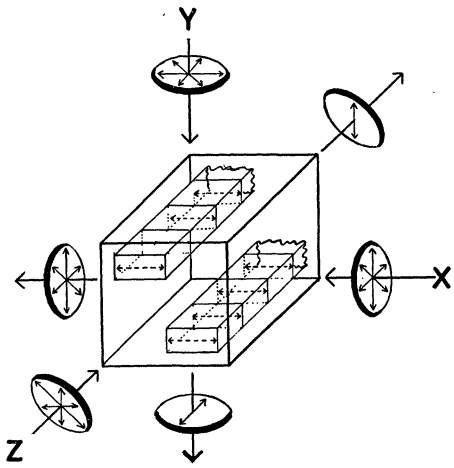

FIG. 1B. The absorption of unpolarized light impinging on an oriented aggregate of nucleic acid molecules from various directions. The nitrogen bases are represented as planes parallel to the front (smallest) face of the molecule; the dotted arrows in these planes represent the position of the distinctive electronic axis responsible for the absorption at 260 mμ. The dimensions of the molecule are arbitrary.

This description pertains to the situation in which the nucleic acid molecules are oriented with their long dimension perpendicular to the direction of the incident light beam. Since this relationship may not always be true of cytological preparations, the properties of oriented aggregates viewed from all possible directions need to be considered.

Fig. 1B describes the optical properties of a fully oriented nucleic acid aggregate when the incident light beam impinges on it from various directions. As shown in Fig. 1A, when the incident beam is perpendicular to the long dimension of the molecule and also to the distinctive absorption axes of the nitrogen bases (i.e., the beam parallels the Y axis), only light with an electric vector parallel to the planes of the nitrogen bases can be absorbed. Thus, the emergent beam is plane polarized and necessarily represents a minimum of 1/2 the energy of the incident beam. The limit of E is therefore 0.3, regardless of the number of molecules in the path of the beam.

The same effect occurs when the incident beam is propagated along the Z axis (parallel to the long dimension of the molecule), for here, too, the beam is perpendicular to the absorption axes. Thus, if the aggregate is examined from the direction of the Y axis, or the Z axis, or from any intermediate direction, the absorption effects typical of Case III occur.

When the incident beam is parallel to the X axis, and is therefore also parallel to the absorption axes of the nitrogen bases, the probability that any photon will be absorbed is zero, and the molecule is transparent (at 260 mμ) from this direction. Consequently E is zero for all N values. For beams impinging from directions between the X and Y axes, and between the X and Z axes, the relation between E and the number of molecules will be as follows: E cannot rise above 0.3, but (with increasing values of N) will approach this limit more slowly that it does when the beam is on the Y or Z axis.

Thus, if (as assumed) the absorption at 260 mμ by an oriented nucleic acid aggregate is determined by a distinctive absorption axis, extinctions greater than 0.3 cannot occur, regardless of the direction from which the aggregate is examined. Differences in the direction of the impinging beam only affect the rate at which this limit is approached with an increase in the number of molecules in the beam's path. This suggests that cytological preparations which contain oriented aggregates of nucleic acid may yield extinction values which do not follow the Beer-Lambert law and therefore are not necessarily proportional to the number of molecules scanned.[3]

The orientation of nucleic acids in cell structures.

While there is considerable information on the orientation of molecular aggregates in cellular structures, decisive data on the orientation of nucleic acids proper are rather sparse and contradictory. Most of the evidence is summarized in the exhaustive reviews of Schmidt (*27*), Frey-Wyssling (*14*), Schmitt (*28*), and Monné (*22*), and unless otherwise indicated, ref-

[3] If the nucleic acid absorption at 260 mμ is *not* due to a distinctive electronic axis, these relationships are altered as follows: (1) Case I and Case II yield identical straight lines. (2) In Case III, the limit of E is 0.3 when the incident beam parallels the Y axis or the X axis or any direction between these two. When the beam impinges along the Z axis, the relation of E to N is linear and the Beer-Lambert law is obeyed. For positions between the X and Z axes, and the Y and Z axes, the relation of E to N is not linear, but approaches the extreme (in which the limit of E is 0.3) as the X and Y axes are approached. Thus, even if it is not assumed that the nucleic acids are characterized by a distinctive absorption axis at 260 mμ, oriented aggregates will not follow the Beer-Lambert law except in the rare case when the molecules are examined from a direction exactly parallel to their long dimension.

erences relative to the discussion which follows may be found in these sources.

That preparations of extracted nucleic acids may form fully oriented aggregates is evident from Caspersson's observations (*7*) of dichroism in thymonucleate gels. Such aggregates are also birefringent, the sign of this effect being negative with respect to the long axis of the aggregated molecules. This provides a method of distinguishing birefringence due to nucleic acids from that due to aggregates of parallel protein chains, for the sign of the latter is positive with respect to the long axis.

Practically all cytological structures may be birefringent under the proper conditions. The effect is frequently due to parallel orientation of protein fibers with their long axes perpendicular to the light beam. This orientation appears to persist in structures such as muscle fibers, chromosomes (in condensed stages), myofibrils, and cilia. In undifferentiated living cytoplasm birefringence is common but highly variable in degree, and may be elicited by dehydration, mechanical stress, and chemical treatment. Here, too, protein fibers seem to be frequently responsible for the optical property.

On purely theoretical grounds, these results suggest that the nucleic acids in cell structures may exist as aggregates so oriented as to elicit the absorption anomalies. Astbury and Bell (*2*) have shown that the internucleotide spacing of the nucleic acid molecule is practically identical with the spacing of side-chains along the protein polypeptide skeleton. This suggests that the nucleoprotein complex probably involves a side-by-side binding of the thread-like protein molecules with the equally elongated nucleic acids. This would mean that in any structure containing nucleic acids these would be oriented in the same way and to the same degree as the proteins with which they are combined. This occurs in tobacco mosaic virus (*3*).

Direct evidence on this possibility is difficult to obtain from measurements of birefringence. In a few cases, such as sperm heads, the nucleic acid content is sufficiently great to give the entire structure negative birefringence, indicating that the nucleic acid is in an oriented state. In most structures, however, the nucleic acids are present in small concentrations in comparison with protein. The negative birefringence caused by the nucleic acids thus cannot counteract the large opposite effect due to the protein present. As Caspersson (*7*) has pointed out, the birefringence expected from the usual concentrations of nucleic acid lies close to the limits of the method. Hence, the failure to detect negative birefringence, where the proteins are themselves oriented, cannot be taken as evidence that the nucleic acids are not oriented.

It is not surprising, then, that the only unequivocal evidence for orientation of nucleic acids comes from studies of sperm heads. Schmidt (*27*) detected a strong negative birefringence in this case, and concluded that the nucleic acid molecules were oriented

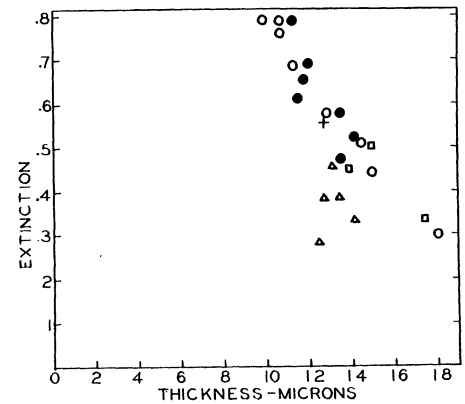

FIG. 2. The relation between extinction at 257 mμ and the thickness of nuclei of spermatocytes of *Gomphocerus*. Data taken from Table 1 of reference *5*. Triangles represent nuclei in early leptotene; open circles—late leptotene; plus sign—synizesis; closed circles—pachytene; squares—diplotene.

in lines parallel to the long axes of the protein fibers. This was confirmed by Caspersson's observation of dichroism at 257 mμ in the sperm bundles of a locust (*7*).

The evidence for the orientation of nucleic acid in chromosomes is contradictory. Schmidt (*27*) found that the chromomere discs of salivary gland chromosomes have a weak negative birefringence, and concluded that the nucleic acid was oriented as in sperm heads. However, Frey-Wyssling (*14*) concludes from quantitative determinations of the optical properties of the chromomeres that only a small percent of the nucleic acid molecules are oriented. Furthermore, Caspersson (*7*) failed to find the dichroism at 257 mμ expected from Schmidt's conclusions. Caspersson suggests that the insect salivary gland chromosome is not of typical composition, and he does not consider these data sufficient to rule out the probability that nucleic acids are oriented in most chromosomes. Negative birefringence has been demonstrated in chromosomes of a number of animal and plant cells, as well as in "resting" nuclei. This property is variable in degree, and marked changes (including changes in sign) may result from environmental effects and fixation procedures.

Extinctions yielded by cellular nucleic acids.

Since both conditions required for the appearance of nonlinear relations between E and N (i.e., absorption due to a distinctive axis, and orientation)

may apply to the intracellular nucleic acids, it is of interest to examine the data obtained from such objects for evidence of this anomaly.

Demonstrations that intracellular extinctions at 257–260 mμ do indeed follow the Beer-Lambert law

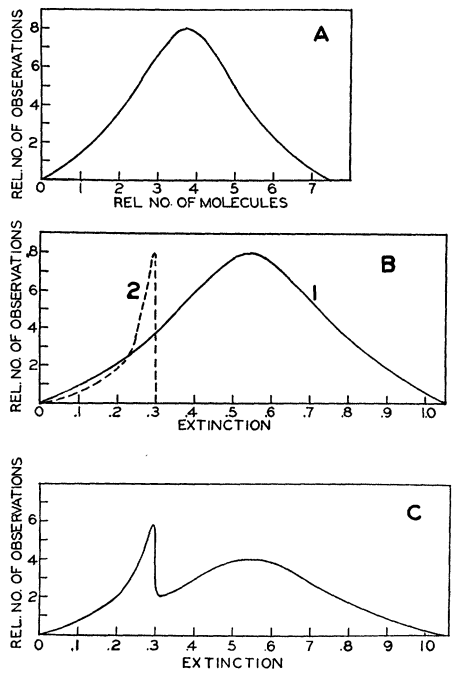

FIG. 3. The effect of orientation on the distribution frequency of extinction values. Fig. 3A is an arbitrarily drawn normal distribution curve for the actual number of nucleic acid molecules in a significantly large population of similar cellular structures. Fig. 3B shows the distribution curves for the extinction values calculated from the relative number of molecules according to the E vs. N equation for unoriented material (solid line) and oriented material (broken line). Fig. 3C is the distribution curve expected from a population containing equal numbers of oriented and unoriented objects.

would dismiss the possibility that the orientation effect is of any consequence. As far as the writer is aware, there are only two published descriptions of a direct test of this relationship in cells, and neither involves naturally-occurring solid structures. Pollister and Ris (*25*) have shown that the extinction yielded by the colored substance produced in the Millon test for proteins is directly proportional to the thickness of the test section. Since the structure of the compound is unknown, it is impossible to say whether it is sufficiently nonsymmetrical to produce the orientation effect. There is no evidence that the colored substance becomes oriented by whatever structural organization exists in the proteins tested. Commoner (*11*) found that the extinction due to anthocyanins and flavones dissolved in intracellular fluid vacuoles is proportional to the vacuole thickness. In this case, the orientation effect is not expected, since the absorbing substance is in solution and therefore completely unoriented. It should be noted that both of these studies test only Lambert's law (linear relation between E and sample thickness). The relation of E to the number of molecules per unit sample thickness was not determined.

One of Caspersson's papers (*5*) does include a table relating E at 257 mμ to the thickness of nuclei of *Gomphocerus* spermatocytes in various stages of prophase. These data have been plotted in Fig. 2, and strikingly fail to follow the Beer-Lambert laws. Indeed, the extinction appears to be inversely proportional to the sample thickness.

In the absence of experimental demonstration that the extinction resulting from the nucleic acids of cell structures is actually proportional to the number of molecules scanned, the validity of interpreting such measurements according to the Beer-Lambert law remains an open question. From certain of the extinction data in the literature, it is possible, however, to obtain an indirect test of the linearity of the E to N relationship. Although this method cannot be applied to all the available extinction data, the results obtained from those cases susceptible to this type of analysis suggest that the orientation effect may play an important role in determining intracellular extinctions due to nucleic acids.

This analysis is derived from a consideration of the curve relating E to N for the case of fully oriented nucleic acid molecules. (See Fig. 1 of paper *12,* page 43.) This curve shows that *all* oriented objects containing more than a relatively small number of nucleic acid molecules yield the value $E = 0.3$. Since all such objects give the same E value, regardless of nucleic acid content, it is to be expected that the frequency distribution of E values for a significantly large group of structures containing nucleic acid will be distorted by this phenomenon.

This is illustrated in Fig. 3. Assume that in any large population of cytological structures, the *actual* nucleic acid content (N) of each object in the group follows some normal Gaussian distribution curve. An example of such a curve, arbitrarily drawn, is shown in Fig. 3A. Now if this population of objects is examined spectrophotometrically, the E values obtained will yield a frequency curve *the shape of which depends on whether or not the objects contain oriented material.* If all objects in the population are unoriented, E is proportional to N for all values of N. Curve 1 of Fig. 3B is the frequency distribution of the E values calculated from the population shown in Fig. 3A, according to this proportionality.

On the other hand, if the whole population is com-

posed of fully oriented structures, E is proportional not to N but to the expression $0.3\text{-}\log_{10}(e^{-\alpha N}+1)$.[4] If E values are calculated from the N values of curve 3A according to this relationship, and plotted against the corresponding frequency, the distribution curve 2 of Fig. 3B is obtained. It is impossible for such a population to yield any E values above 0.3. If the population contains equal proportions of unoriented and fully oriented structures, the distribution curve shown in Fig. 3C will result (obtained by adding curves 1 and 2).

Fig. 3C shows that if a significant fraction of a population of cytological elements contains fully oriented nucleic acid molecules, a rather unusual type of frequency curve will be found for the E values obtained. There will be a tendency for the values between $E=0.2$ and $E=0.3$ to predominate, and the frequency of observations will drop sharply as E rises above 0.3. At higher E values a secondary maximum may appear. If the proportion of oriented objects is very low, this second maximum may be the dominant one, but in any case the curve shows an unusual number of values between 0.2 and 0.3, with a sharp drop just above this range.

This general relationship will not be altered by differences in the direction from which a cytological structure is viewed, for as shown above, the value of $E=0.3$ is limiting, regardless of the direction of the incident beam. If the assumption made previously, that the absorption at 260 mμ is due to a distinctive axis, is not true, then the relative frequency with which values around 0.3 would occur would be reduced. However, values in the range $E=0.2$–0.3 would still occur with an unusual frequency, and the qualitative effect on the distribution frequency will not be altered.

This type of analysis can be applied to extinctions obtained from cell structures if the following conditions are met: (1) The data must comprise a significantly large series of extinction measurements made on similar structures, and (2) the size of the field scanned must be so small as to cover a homogeneous area within the structure (if the field is large, differences in the spatial organization of various areas included in it may obscure the orientation effect). If such data yield frequency distributions (i.e., of E values) which are distorted by a predominance of values in the range $E=0.2$–0.3, it is suggested that the group of structures examined contain a significant number of objects with oriented nucleic acid. The E values obtained in these sets of data are therefore not necessarily proportional to nucleic acid content.

The method is applicable to the following published studies on extinctions due to cellular nucleic acids, all of which are based on scanning areas of the order of 1 square micron and include at least 29 measurements on similar material: Thorell (*32*), 132 E values for erythroblasts; Thorell, Bing, and Fagraeus (*33*), 117 E values for plasmacytes; Hydèn and Hartelius (*18*), 30 E values for normal rabbit neurones, and 28 values for neurones of animals treated with malononitrile; Hydèn (*17*), 46 E values for rabbit neurones; Caspersson and Santesson (*9*), 30 E values for various tumor cells; Caspersson (*6*), 29 E values for various regions of nuclei and chromosomes.[5] Details concerning the conditions of these measurements are given in Table 1.

TABLE 1

SOURCE OF DATA PLOTTED IN FIGS. 4 AND 5

Reference	Type of cell	Thickness in μ	Condition	No. of exfinction values at 257–260 mμ		
				cytoplasm	nucleus	total
Thorell (*32*)	Rabbit erythroblasts	10–16	living & fixed	143	8	151
Thorell, Bing, and Fragaeus (*33*)	Rabbit plasmacytes	10–16	living	117	0	117
Hyden (*17*)	Rabbit neurones	5	fixed	28	25	53
Hydèn and Hartelius (*18*)	Rabbit neurones—normal and malononitrile-treated	5–10	fixed	58	0	58
Caspersson (*6*)	Insect salivary gland cells	2–3	fixed	0	29*	29
Caspersson and Santesson (*9*)	Tumor cells	5	fixed	22	8	30

* Includes 16 readings on chromosome structures, 3 on nuclear sap, 10 on nucleoli.

The frequency distributions obtained from the data presented in each of these studies are shown in Figs. 4 and 5. The extinction values were taken from tables published in these studies, or read off the published absorption spectra. The tabular values are for extinctions at 257 mμ; the other values are for 260 mμ, at which the absorption spectra usually indicate a measured point.

Of the seven sets of measurements, three (Figs. 4B, 4C, and 5, solid curve) yield distribution curves which show definite maxima at $E=0.2$–0.3. One curve (Fig. 4D) which shows a maximum at this range is not sig-

[4] α = absorption coefficient per molecule.

[5] Consideration of a significantly large set of measurements of neurones by Gersh and Bodian (*15*) has been excluded because the scanning area used was so large as to include a heterogeneous section of the cell.

nificant in itself because of the smallness of the population, but is presented for the sake of completeness. Two sets of data (Figs. 4A and 5, broken curve) show maxima at E values significantly greater than 0.3. The set of data on nuclear structures (Fig. 4E), while too small to be statistically significant, shows a tendency for E values less than 0.3 to predominate.

The occurrence of several sets of measurements with well-defined maxima at 0.3 suggest that the orientation effect has probably influenced the values obtained. It is, of course, possible that these maxima reflect actual nucleic acid contents (unoriented) which correspond to the extinction of 0.3, and that the occurrence of this particular number of nucleic acid molecules in the cytoplasm of erythroblasts varying in thickness from 10–16 μ, in sections of neurones 5–10 μ thick, and in 5-μ sections of various tumor cells is purely fortuitous.

Detailed analysis of these data suggest that the latter explanation is not admissible, and that the maxima shown in *all* the distribution curves can be more reasonably accounted for by the orientation effect than by actual differences in the nucleic acid content of the objects studied.

In the first place, it is necessary to consider the thickness of the materials studied. If the Beer-Lambert law is followed, the modal extinctions would be expected to increase with increasing thickness, unless differences in nucleic acid content just happened to cancel out this effect. Actually, as can be seen from Table 1, the value $E = 0.3$ tends to be limiting despite rather large variations in thickness of the materials studied. The four sets of data yielding maxima at 0.3 were obtained from material 5 μ, 5 μ, 5–10 μ, and 10–16 μ respectively. The measurements made on the thinnest sections (nuclear structures of 2 μ thickness) give the only modal E value which is less than 0.3. These observations conform with the relationships expected from the orientation effect. In oriented material E can vary with N only when the number of molecules contained in the object is so low as to give extinctions less than about 0.3; with increasing values of N (e.g., due to increasing thickness of samples) E remains at the limit of 0.3 imposed by the orientation phenomenon.

Consideration of the measurements made on plasma cells, which show a modal E value of 0.5, provides further evidence on the orientation effect, and suggests that the optical properties of the nucleic acids in undifferentiated cytoplasm are largely dependent on whether or not the cell studied is living or dead.

The plasmacytes (Fig. 4A) represent the only group of material comprised of cells which were apparently alive at the time of measurement. The cells

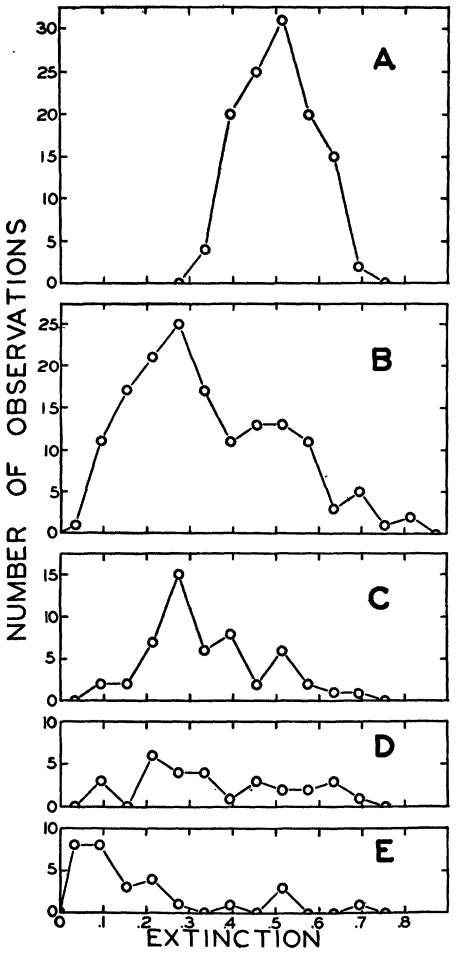

FIG. 4. Distribution frequencies for the extinctions at 257–260 mμ given by various cytological structures. See Table 1 for details of the conditions for measurements. A—Living rabbit plasmacytes (data taken from reference *33*); B—an apparently mixed population of living and dead rabbit erythroblasts (data taken from reference *32*); C—rabbit neurones, fixed (data taken from reference *17*); D—various tumor cells, fixed (data taken from reference *9*); E—various nuclear structures, fixed (data taken from reference *6*).

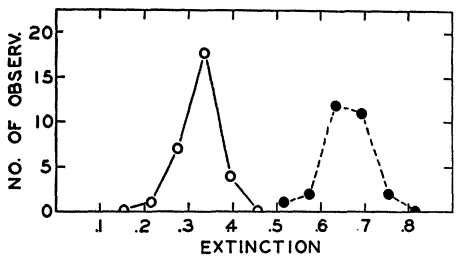

FIG. 5. Distribution frequencies for the extinctions at 257–260 mμ given by neurones from normal rabbits (solid line) and rabbits treated with malononitrile (broken line). (Data taken from reference *18*.) See Table 1 for further details.

used were taken from rabbit blood and mounted in saline, and it is common experience that such cells readily survive in this medium. Furthermore, the authors indicate that special care was taken to avoid the killing effect of long exposures to ultraviolet light during the measurements.

The distribution yielded by these surviving cells is a relatively simple curve with a single peak at about 0.5. Since this value is greater than 0.3, and since the predominance of values in the range of 0.2 to 0.3 expected from the orientation effect is lacking, these data suggest that *the cytoplasmic nucleic acids of living plasma cells are largely unoriented.*

The significance of this observation is amplified by the comparison between the ultraviolet absorption of living and dead cells reported by Larionov and Brumberg (*20*). Using a new reflecting objective which made it possible to focus with visible light, these workers avoided the ultraviolet damage incident to most measurements made with conventional ultraviolet objectives. They found that the cytoplasm of living fibroblasts shows a relatively high absorption at 260 mμ, but that it drops sharply when the cells are killed by exposure to ultraviolet light, or by other means. The absorption of living nuclei, however, was low and increased significantly when the cell died. They suggest that the low absorption usually reported for cytoplasm stems from the fact that most measurements have been made on cells killed by fixation or by exposure to ultraviolet light during the experiment.

These results have been confirmed by Ris and Mirsky (*26*), who found that photographs of living chick fibroblasts taken at 2537 A showed strong absorption in the cytoplasm, which decreased markedly when the same cells were killed by exposure to ultraviolet light. These authors suggest that the loss of ultraviolet absorption on death is a result of "ribose-nucleic acid leaking out of the disintegrating cell." This explanation seems doubtful, however, for the very large nucleic acid molecules would diffuse slowly, and as pointed out both by Larionov and Brumberg and by Ris and Mirsky, a corresponding loss of nucleic acid from dead nuclei does not occur.

On the basis of the orientation phenomenon an alternative explanation can be offered for this effect—namely, that the loss in absorption at 260 mμ results from the orientation of cytoplasmic nucleic acids which occurs at death of the cell. This would cause the extinctions to fall to the limit of 0.3 despite the fact that the cytoplasm might contain sufficient nucleic acid to give higher extinctions in the unoriented state. This view is supported by observations (*27*) that sharp increases in the birefringence of cytoplasm may be induced by fixing agents.

The bimodality of the distribution curve for erythroblasts (Fig. 4B) may be explained similarly. The predominance of values in the range 0.2 to 0.3 suggests that a large fraction of the measured cells contained oriented cytoplasmic nucleic acids. But the appearance of the secondary maximum at 0.5 would indicate that at least some of the cells contained unoriented cytoplasmic nucleic acids. It would be expected, therefore, that the population of erythroblasts consisted of a mixture of living and dead cells at the time of measurement (cf. Fig. 3C).

The nature and treatment of the erythroblasts is in accord with this expectation. Thorell reports that the erythroblasts were obtained from living bone marrow, teased out in saline, and mounted on slides under cover glasses sealed with vaseline to prevent drying. Although care was taken to avoid killing by undue exposure to ultraviolet light, the survival time of teased-out bone marrow cells in a thin sealed film is probably limited; and one would anticipate that some fraction of the cells were dead when measured. Furthermore, some of Thorell's preparations were treated with lanthanum acetate to precipitate the nucleic acids and provide a "more stable preparation" (page 46, reference *32*). Since such cells were dead, this treatment apparently contributed to the size of the nonliving fraction of the population. The bimodal character of the frequency distribution given by the erythroblasts may therefore reflect the fact that some of the cells measured were living (unoriented cytoplasmic nucleic acids, modal $E = 0.5$) and others dead (oriented cytoplasmic nucleic acids, modal $E = 0.3$).

The data are thus consistent with the idea that the nucleic acids in the undifferentiated cytoplasm of living erythroblasts and plasmocytes are unoriented, therefore yielding extinction values which are relatively high and probably proportional to the actual nucleic acid content. On the other hand, the cytoplasmic nucleic acids of dead cells tend to become oriented, thus yielding extinction values which fail to exceed 0.3 and so are independent of nucleic acid contents which would, if unoriented, give higher E values.

It is possible that the changes in the ultraviolet absorption of nuclei on death which have been noted (*20, 24, 26*) may be due to similar variations in the degree of orientation imposed on the nucleic acids. However, the available data are too incomplete to provide an adequate basis for an analysis similar to the one outlined, and the question must remain open at present.

The increase in extinction at 260 mμ of neurones from rabbits treated with malononitrile (Fig. 5) is interpreted by Hydèn and Hartelius (*18*) as representing a proportional rise in the nucleic acid content

of these cells. However, the ultraviolet photographs of the treated cells show that malononitrile causes a marked change in the configuration of the structures, which are apparently rich in nucleic acid (the Nissl bodies). In the untreated cells, the Nissl substance has the "tigroid" appearance of more or less clumped material. Cells from malononitrile-treated animals, on the other hand, show a structureless dense mass diffusely spread through the cytoplasm. It seems likely, therefore, that the treatment induces a significant change in the degree of aggregation of the Nissl substance. Since the aggregated (normal) form of Nissl substance gives modal E values of about 0.3, there is reason to believe that it may contain oriented nucleic acids. It seems possible that the increase in the modal extinctions exhibited by the treated material may be a result of disaggregation (and the concomitant disorientation) of nucleic acids caused by malononitrile. The rise in extinction induced by the treatment would not reflect an actual increase in nucleic acid content.

This analysis suggests that differences between the extinction at 260 mμ of cellular structures may frequently arise from variations in the degree of orientation of their constituent nucleic acids, rather than from real differences in nucleic acid content. Thus, where structures differ considerably in the degree of orientation of their nucleic acids, failure to consider this factor may lead to large errors in interpretation.

For example, a study of the extinction of nuclei in various stages of spermatogenesis in the locust has led to the conclusion (*4*) that a "considerable amount of nucleic acid disappears from the nucleus" as the sperm ripens. The reduction in extinction is evident from ultraviolet photographs (reference *4*, Figs. 79, 80) in which spermatocytes show considerably darker nuclei than do neighboring sperm cells. However, the two cell types also differ greatly in the degree of orientation of their nuclear material. During sperm formation, the spherical nucleus of the spermatocyte stretches into the elongated spindle form which characterizes the mature sperm. Optical studies (*23, 27*) show that the birefringence of spermatid nuclei increases in parallel with elongation, and that the nucleic acid molecules thereby become markedly oriented. This orientation has been directly demonstrated (*7*) in photographs of sperm bundles taken with ultraviolet light (260 mμ) of varying planes of polarization. The material is highly dichroic, yielding a maximum extinction when the plane of polarization is perpendicular to the long axis of the sperm nuclei. It seems likely, therefore, that the reduction in ultraviolet extinction accompanying the elongation and maturation of the sperm nuclei is not due to "loss" of nucleic acid, but is rather a consequence of the orientation which is imposed on the nuclear material during spermatogenesis.

A similar case is the determination of the extinction of muscle fibers (*10*). The E values for isotropic segments of striated muscle fibers are significantly higher than those given by neighboring anisotropic segments. Since the absorption at 257 mμ is probably related to the adenylic acid (and its phosphorylated compounds) of the muscle, this observation led to the conclusion that most of the adenine compounds are localized in the isotropic segments.

An alternative interpretation of these data may be offered. The anisotropy of the muscle fibers seems to be due to oriented micelles of myosin. The myosin fibers are also the locus of the adenosine triphosphatase activity of the muscle, and the formation of a complex between myosin and adenosine triphosphate has been demonstrated. It seems possible that a significant part of the nucleotide in muscle fibers will be oriented by attachment to myosin wherever this protein is itself oriented (i.e., in the anisotropic bands). Hence the isotropic bands' showing greater extinctions than the anisotropic bands may result from the fact that the adenine compounds tend to be more strongly oriented in the latter, rather than from any difference in concentration.

The foregoing analysis suggests that the orientation effect probably is important in determining the extinctions given by cytological structures at 257–260 mμ. The ultraviolet absorption of such structures cannot be evaluated by the simple application of the Beer-Lambert laws. The interpretation of intracellular extinction values at 257–260 mμ seems to be subject to the following reservations:

1) Where part or all of the nucleic acid in a structure is oriented in three dimensions, E is not proportional to the nucleic acid content unless E is smaller than about 0.1–0.2.

2) In general, the E values yielded by oriented aggregates may be considerably smaller than the E value which the same number of nucleic acid molecules would yield if not oriented; but this relationship is reversed when very low nucleic acid contents are encountered. For the case of complete orientation, E cannot rise above 0.3, regardless of the number of nucleic acid molecules scanned.

3) If the orientation effect occurs, it is not possible to calculate the nucleic acid content of a cell structure by comparing its extinction with the extinction given by a known number of nucleic acid molecules in solution.

4) In comparing two cytological structures, it would appear impossible to obtain a ratio of their nu-

cleic acid contents from the ratio of their extinction values (for equal thicknesses) unless it can be shown that the same degree of orientation occurs in both structures.

These reservations apply to the interpretation of qualitative observations, such as the darkness of ultraviolet photographs, as well as to the treatment of quantitative photoelectrical measurements.

The analysis presented is suggestive rather than conclusive. It does, however, indicate that the entire subject needs to be more closely scrutinized before it is possible to accept the assumptions on which many studies of ultraviolet absorption are based, or to look upon the resulting conclusions (*8*) as a sound foundation for theories of protein metabolism, gene action, and the special roles of the nucleic acids.

The need for new data is apparent. The location of the absorption axes of the nitrogen bases must be known if the optical behavior of nucleic acids is to be fully understood. Determination of dichroism at 260 mμ could yield precise information on the orientation of nucleic acids. The validity of Lambert's law could be determined by studying the extinctions of various thicknesses of the same or similar structures. Some way needs to be found for testing Beer's law within cells. The optical changes which occur on the death of the cell seem to be sufficiently important to warrant much attention.

Structural considerations other than orientation proper may be related to the light-absorbing powers of cellular substances. The work of Sheppard, Scheibe (see reference *12* for references), and others shows that polymerization and simple aggregation may cause serious changes in the absorption characteristics of certain substances. Such effects may also induce anomalous behavior in nucleic acid solutions and may account for the recent observation (*19*) that polymerization of nucleic acid causes a significant change in extinction coefficient.

The optical consequences of orientation of cellular materials may play an important role in determining the properties of photochemical systems in living cells. There is evidence (*29*) that the degree of aggregation of some substances may determine the appearance of new absorption maxima which result from intermolecular electron transfers. The fundamental importance of such molecular behavior in living systems has been suggested by the work of Szent-Györgyi (*31*).

The value of the application of optical methods to cellular biology is apparent from the important results already achieved. It may not be amiss to suggest, however, that the full use of this tool depends largely on an understanding that we are dealing with a complicated and dynamic state of matter, in which relationships inconsequential in simpler systems become of decisive importance. Close attention to the special and uncommon optical properties of living cells may be more revealing than their similarities to the states of matter which are dead.

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