STUDIES ON NUCLEIC ACID METACHROMASY

II. Metachromatic and Orthochromatic Staining by Toluidine Blue of Nucleic Acids in Tissue Sections

NED FEDER and MERRILL K. WOLF

From the Biological Laboratories, Harvard University, and the Laboratory of Cellular Neuropathology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Acrolein-fixed, polyester wax-embedded tissue sections showed excellent preservation of light microscopic architecture and, when stained with toluidine blue, intense color contrast between DNA, which stained orthochromatically, and RNA, which stained metachromatically. This method has practical value for differentiating DNA from RNA in the same section. The color contrast was impaired by substituting formaldehyde for acrolein or paraffin for polyester wax, and was negligible in tissues fixed in formaldehyde or Carnoy's fluid and embedded in paraffin. Quality of structural preservation paralleled degree of color contrast. Metachromatic staining can be analysed, by the quantitative parameters of Bradley and colleagues, to provide inferences regarding the conformation of biopolymers in tissue sections. Comparison of the nucleic acid color contrasts in toluidine blue–stained sections with titrations of fixative-treated nucleic acids against toluidine blue in solution indicated a greater difference in conformation between DNA- and RNA-protein in acrolein-polyester sections than between acrolein-treated free DNA and RNA in solution. This is supported by recent evidence that the conformation of ribosomal RNA is quite different in whole ribosomes from that assumed by the same RNA free in solution. The acrolein-polyester method may enhance color contrast by providing superior preservation of ordered nucleoprotein conformations.

INTRODUCTION

While studying the properties of a new fixative, acrolein, we noticed that tissue specimens fixed in this agent, embedded in polyester wax (20, 21), and stained with the metachromatic dye, toluidine blue, showed a striking color contrast between structures containing DNA,[1] which were stained orthochromatically (blue-green), and structures containing RNA, which were stained metachromatically (purple). Although similar color contrasts have been described elsewhere (8, 18), the consistency and magnitude of the color contrast in acrolein-polyester wax sections surpassed anything obtained with our toluidine blue staining procedure after other preparative methods. This method appeared to have considerable practical value for distinguishing DNA and RNA in tissue sections. Furthermore, the metachromasy of polyelectrolytes is known to reflect certain features of their molecular con-

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[1] Abbreviations employed: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; P/D, the ratio of the number of polymer dye-binding sites to the number of dye molecules in any given polymer-dye system; K, stacking coefficient.

formation in a quantitative fashion (3–5). A metachromatic color contrast between DNA and RNA might offer clues to their conformation in tissue sections. We have therefore investigated the relative contributions of the fixative and the embedding method to the color contrast in acrolein-polyester sections. The results are discussed in terms of the spectrophotometric titrations described in the previous paper (14).

MATERIALS AND METHODS

Small pieces (maximum size 5 mm thick) of brain, tongue, duodenum, liver, pancreas, spleen, testis, and epididymis of adult rat and ribs of newborn rat were fixed and embedded. Some specimens were fixed in acrolein; some in formaldehyde, the standard aldehyde fixative; and some in Carnoy's fluid, a simple denaturant fixative commonly employed in nucleic acid studies. Acrolein was used as a 10 per cent solution in 0.5 per cent aqueous calcium acetate (15). Similar results have been obtained with 10 per cent acrolein in $M/10$ phosphate buffer at pH 7.2, in Tyrode's balanced salt solution, in plain water, or in xylene. Formaldehyde was used as 10 per cent formalin (i.e., final concentration 3.8 per cent formaldehyde) in 0.5 per cent aqueous calcium acetate. Carnoy's fluid is 10 per cent (v/v) glacial acetic acid, 60 per cent ethanol, and 30 per cent chloroform. This formulation was chosen because of its previous use by Schümmelfeder et al. (19) in studies of acridine orange metachromasy. With acrolein, optimum fixation and color contrast were achieved after fixation for 1 hour at room temperature or overnight at 0°C. With formaldehyde, the optimum time was overnight at room temperature or several days at 0°C. Fixation in Carnoy's fluid was carried out at room temperature for 4 hours. A few specimens were first fixed in one and then postfixed in another of the solutions.

Paraffin wax was compared with polyester wax. Some specimens fixed in each way were dehydrated in graded ethanols, transferred to xylene, and embedded in paraffin wax. Other specimens were dehydrated in a 1:1 mixture of methanol and methoxyethanol (7), transferred to ethanol and then to n-propanol, and embedded in polyester wax (20, 21). Other methods of dehydration, preceding embedding in polyester wax, gave similar results.

Sections were cut at 5 μ, mounted on glass, dewaxed, hydrated, stained for 8 minutes in aqueous toluidine blue in 0.02 M benzoate buffer, pH 4.2 (21), dehydrated in tertiary butyl alcohol for 5 minutes, as recommended by Michaelis (17 a) and Flax and Himes (8), transferred to xylene, and mounted in Permount. A toluidine blue staining solution buffered with phosphate gave similar results. Toluidine blue

0, Color Index 52040, was used as purchased. In addition, a few slides were stained with a sample of toluidine blue purified and supplied by Lamm, Childers, and Wolf (14). The same results were obtained with both samples of dye.

Flax and Himes (8) recommend azure B over other metachromatic dyes for differential staining of nucleic acids. In our hands, toluidine blue 0 proved equal or slightly superior to azure B for this purpose, whether staining was performed at room temperature (22°C) or at 40°C as done by Flax and Himes (8) and Pelling (18). Staining at the higher temperature gave a greater color contrast, particularly with Carnoy's-fixed material; but the results of staining sections at room temperature can be directly correlated with the spectrophotometric titrations described in the companion paper, since these were carried out at room temperature of 22°C. The description and discussion that follow are therefore based exclusively on staining at room temperature.

The color of DNA was studied in vegetative nuclei, in sperm heads, and in the chromosomes of dividing cells. The color of RNA was studied in nucleoli, in the zone between separating telophase chromosomes of dividing cells (12), and in the ergastoplasm of cells such as neurons, liver parenchymal cells, and pancreatic acinar cells. The color contrast between DNA- and RNA-rich structures was visually estimated as 0 to + + + + in terms of a rough scale of increasingly metachromatic color: blue-green through blue to purple. Thus, 0 indicates identical colors; +, the smallest contrast that could be positively identified by both authors; + +, a larger contrast such as between blue and purplish blue or between blue-green and blue; and + + + +, the largest contrast observed, that between blue-green and purple.

The acrolein-polyester method, in addition to its effect on nucleic acid hues, strikingly enhanced the metachromasy of structures containing acid mucopolysaccharides, such as cartilage matrix, the granules of blood and tissue basophils, and mucous droplets in goblet cells. This effect will be the subject of a separate report.

The color contrasts proved difficult to record in color photographs. Satisfactory results were finally obtained with Ektachrome Type B 4 × 5-inch sheet film through the use of exposure times and color compensating filters individually selected by trial and error for each new batch of film.

Evaluations of structural preservation were based on lifelike contiguity of cells and extracellular components, without artificial shrinkage spaces; on predominantly smooth and continuous outlines of cells and nuclei; and on delicacy of detail in nucleoplasm and cytoplasm. Parallel sections were stained with acid fuchsin to assess the structural integrity and stainability of mitochondria.

RESULTS

Sections of specimens fixed and embedded in different ways differed markedly in structural integrity and in color contrast on staining with toluidine blue. In both respects, the acrolein-polyester specimens were best (maximum color contrast being considered best), the Carnoy's-paraffin specimens worst, and other specimens intermediate. Examples of the best and worst specimens are shown in color in Figs. 1 and 2, and results are summarized in Table I.

The acrolein-polyester specimens showed an obvious contrast between the colors of stained chromatin, which was blue-green, and of nucleoli and ergastoplasm, which were purple. Preservation of cell and tissue structure was judged to be very good, and companion sections stained with acid fuchsin showed intact mitochondria. The colors of DNA- and RNA-rich structures were most readily demonstrated in cells with open vesicular nuclei and abundant ergastoplasm, such as pancreatic acinar cells, which were chosen on this account for the color plate. However, RNA had the same purple color whether in the nucleolus, the ergastoplasm of pancreatic acinar cells, hepatic parenchymal cells, or neurons, or in association with the telophase plates of dividing cells. DNA stained more darkly in compact nuclei than in open ones, and appeared almost black in sperm heads or in the chromosomes of dividing cells; but when examined with intense light, these darker staining DNA-rich structures were seen to have a blue-green hue, quite different from the hue of the same structures in specimens processed by other methods.

In Carnoy's-paraffin sections the chromatin and ergastoplasm stained the same shade of blue or blue-violet in any given region of the section. There was some variation in hue from region to region within a section of the same tissue, but the extreme greenish-blue and purple colors were only seen in regions where the quality of fixation or of sectioning was very poor. Carnoy's paraffin was the only preparative method producing a difference in color between the ergastoplasm and the nucleolus: the latter structure was distinctly pink in several cell types in Carnoy's-paraffin sections. This observation is of unknown significance. Preservation of architecture was poor, and mitochondria could not be identified in companion sections stained with acid fuchsin.

When acrolein-polyester and formaldehyde-polyester specimens were compared, structural preservation in the formaldehyde specimens was good, although inferior to that in acrolein specimens, and color contrast was uniform and substantial; however, ergastoplasm and nucleoli were distinctly less metachromatic than in acrolein specimens, chromatin slightly more metachromatic. Comparison of acrolein-polyester and acrolein-paraffin specimens showed the best areas of the paraffin specimens to be almost as good as representative areas of the polyester specimens; however, the paraffin specimens were much less uniform in color contrast and structural integrity. Formaldehyde-paraffin and Carnoy's-polyester specimens had barely perceptible color contrast or none at all; their structural preservation was only slightly superior to that seen in Carnoy's-paraffin sections.

In specimens exposed successively to two fixatives, structural integrity and color contrast were determined largely by the first fixative. It was particularly striking that the quality of acrolein-fixed specimens was little affected by postfixation in Carnoy's fluid.

DISCUSSION

Histochemical Applications of Nucleic Acid Metachromasy

Most investigators using thiazine dyes have not reported any difference between the colors of DNA and RNA in stained tissue sections, and some have stated explicitly that no difference exists. This subject has been reviewed elsewhere (2, 8, 16). It is clear, however, that under some conditions DNA and RNA are stained differently. For example, Flax and Himes (8) have described a method of staining with azure B at elevated temperatures by which cellular structures rich in RNA, namely nucleoli and ergastoplasm, are stained metachromatically (purple), while chromatin, rich in DNA, is stained orthochromatically (blue-green). Similar observations have been made by others (15, 17, 18, 23, 24). It appears from these reports that production of a color contrast between DNA and RNA by thiazine dyes depends on the choice of fixative, dehydrating and embedding agents; on dye concentration, pH, ionic strength, temperature, and subsequent differentiation and dehydration; and on

the particular thiazine dye chosen. This helps to explain the previous dispute concerning the existence of a color contrast between DNA and RNA in thiazine-stained tissue: the contrast may be subtle, and it is sensitive to the conditions of processing and staining the tissue.

Of the preparative methods we tested, fixation in acrolein and embedding in polyester wax gave the most striking and consistent color contrast between DNA and RNA on staining with toluidine blue. In our opinion this simple procedure has considerable merit as a practical means of distinguishing DNA from RNA in the same tissue section. Most of the available staining methods developed for this purpose are notorious for their variability; the large literature on modifications of the methyl green–pyronin method attests to this. Many of these methods require fixation with coagulant mixtures, such as Carnoy's fluid, which badly distort the finer features of cell structure. The modified May-Grunwald-Giemsa stain of Jacobson and Webb (12) can only be used on methanol-fixed cell smears or tissue culture monolayers. Acridine orange yields large color contrasts between DNA and RNA after many preparative methods, but specimens stained with this dye must be mounted in water and examined by fluorescence microscopy. Aside from their inconvenience and lack of permanence, such preparations deny to the microscopist the use of the highest resolving power of his instrument. The method described here provides permanent preparations with excellent structural preservation which can be usefully examined with oil immersion objectives of high

numerical aperture. For example, in Fig. 1, structural preservation and color contrast are sufficiently good to show apparent continuity between the RNAs of nucleolus and cytoplasm in a pancreatic exocrine cell. This phenomenon, which was described by Huber (10) and which is seen fairly often in acrolein-polyester sections, is interesting in the light of modern concepts concerning the synthesis and transport of cytoplasmic RNA. The present method might be useful for the study of this and other functionally significant relationships between RNA and DNA. Positive identification of an unknown basophilic structure as DNA- or RNA-rich would, of course, require more specific histochemical methods such as the Feulgen stain or treatment of sections with nucleases.

Stacking Behavior of Cationic Dyes on Nucleic Acids. A Review

The stacking concept is described at length in the preceding paper (14), and is reviewed briefly here as a necessary introduction of our discussion of the significance of polychromatic staining (following section).

Both DNA and RNA, whether native or denatured by physical or chemical agents have nearly the same metachromatic absorption spectrum when each polymer binding site is occupied by a dye molecule (P/D = 1), that is, when the polymer is saturated with dye; and they have the same orthochromatic absorption spectrum when the dye binding sites greatly outnumber bound dye molecules (P/D → ∞), that is, when a very small proportion of the avail-

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FIGURE 1   Color photomicrograph of 5μ section of rat pancreas fixed in acrolein, embedded in polyester wax, and stained with toluidine blue. Quality of structural preservation is very good, as judged by the criteria described in the text. Note the similarity in color between nucleolus and cytoplasmic basophilic substance (ergastoplasm), and the difference in color between these two and the chromatin. This color contrast was even more striking in the original microscope slide. The round unstained areas are zymogen granules. Parallel sections (not shown) stained with acid fuchsin show good preservation of mitochondria and of zymogen granules, both of which are intensely stained. × 2300.

FIGURE 2   Specimen similar to that in Fig. 1, but fixed in Carnoy's fixative and embedded in paraffin wax. Chromatin and ergastoplasm are stained the same color. Quality of structural preservation is considerably poorer here than in Fig. 1. Cells and nuclei are smaller than in the specimen shown in Fig. 1, and there are large intercellular spaces. Cytoplasm and nucleoplasm are coarsely coagulated, and in parallel sections stained with acid fuchsin (not shown) mitochondria are not visible and have presumably been disrupted during fixation or embedding. × 2300.
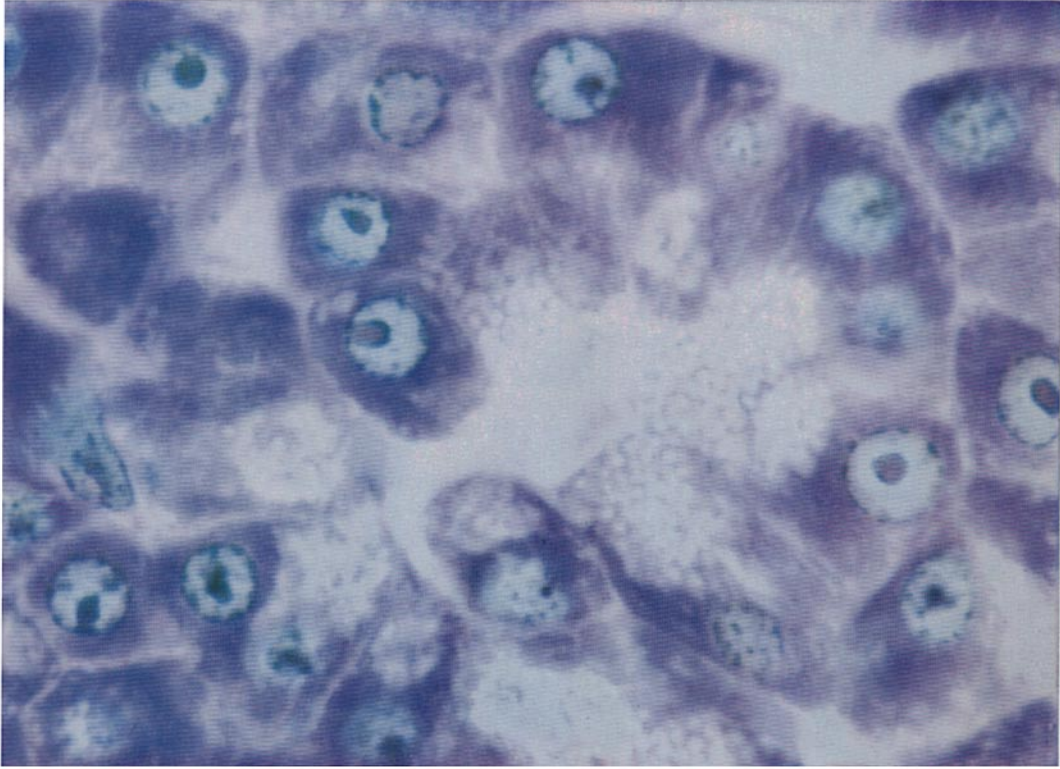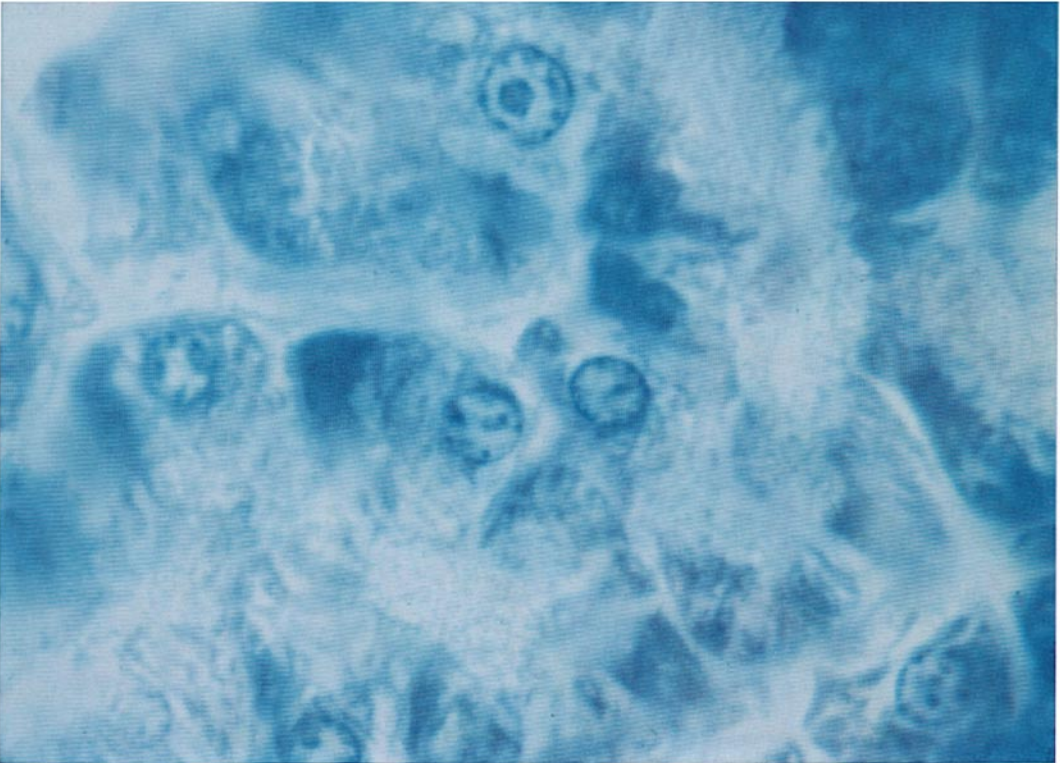
TABLE I

Color Contrast with Toluidine Blue Staining after Different Fixatives and Waxes

	Waxes	
Fixative	Polyester	Paraffin
Acrolein	+++++	++
Formaldehyde	+++	+
Carnoy's fluid	+	0 to +
Acrolein, then Carnoy's fluid	+++++	not done
Carnoy's fluid, then acrolein	++	not done

After fixation, embedding and staining the color gap between DNA- and RNA-rich structures was evaluated as described in the text. In this table, "++++" indicates the largest color contrast observed between DNA- and RNA-rich structures, and "+" indicates that the two types of structures were barely different in color. In Carnoy's-paraffin sections, chromatin and ergastoplasm were stained nearly or exactly the same shade of blue. However, nucleoli were stained pink. Pink-stained nucleoli were not encountered with any other combinations of fixative and wax.

able binding sites are occupied by dye. These observations can be explained by the aggregation or "stacking" of dye molecules bound to neighboring sites, which causes a change in the absorption spectrum of bound dye. When $P/D = 1$. each bound dye molecule can interact with the dye molecule bound to the nearest neighboring site, so that all the dye is "stacked." Stacked dye has the metachromatic color. As P/D approaches ∞, dye molecules distribute themselves among the increasing excess of binding sites, and the number of dye molecules having nearest neighbors with which they can interact approaches zero; that is, the dye approaches complete "unstacking." Unstacked dye has the orthochromatic color.

At intermediate values of P/D (*i.e.*, polymer only partly saturated with dye) a new phenomenon appears, one not predicted by the aggregation concept alone: a given dye may show different absorption spectra when bound to DNA and RNA. This phenomenon can be explained quantitatively by differences in the so called stacking coefficients of DNA and RNA. At intermediate values of P/D the observed

absorption spectrum is the sum of the spectra of a mixture of stacked and unstacked dye molecules, the proportions being determined by the distribution of dye molecules among available binding sites. This distribution is not strictly random, but is weighted in favor of the stacked state. The amount of weighting, that is, the preference of dye molecules for stacked rather than random distribution, varies widely from one polymer and dye to another, so that for different polymer-dye systems the same intermediate value of P/D will produce different ratios of stacked to unstacked dye, and corresponding differences in the amount of metachromasy. The amount of weighting for a particular polymer-dye system is quantitatively expressed as the stacking coefficient, K, which is a measure of the tendency toward stacking. K is high when the tendency toward stacking is great. It has been found that K of nucleic acids increases with increasing randomness of secondary structure. Native DNA has the lowest K yet observed for any polymer; native RNA has a higher K; denaturation of either nucleic acid raises its K.

Mechanism of Differential Staining of Nucleic Acids in Tissue Sections

In the preceding paper, the spectra of solutions containing toluidine blue and pure nucleic acids were shown to be determined, even after fixative treatment or in the presence of fixative, by only two variables: P/D and K. The difference in P/D between two nucleic acid-dye solutions necessary to produce a metachromatic color contrast between them without a difference in K was shown to be so large that differences in P/D are inadequate as a sole explanation for metachromatic color contrasts in tissue sections. Differences in K between DNA and RNA (stacking differentials) were therefore considered to be important in the production of such color contrasts.

If the color contrast between DNA and RNA in stained tissue sections is determined by the P/D and K of each polymer, it is necessary to assume that P/D in sections is almost always greater than 1, since at $P/D = 1$ both polymers are completely metachromatic regardless of K. In solution, P/D is easily manipulated by varying the concentrations of polymer and dye, but no independent method has been described for measuring P/D in a stained tissue element. P/D in sections can be influenced only by inhibiting dye binding to, or removing bound dye from, a

fixed but unknown concentration of nucleoprotein. Many staining methods with basic dyes incorporate features which tend to oppose binding of dye and might well raise P/D to the high values we postulate. The production of color contrast with acridine orange (1, 6, 19, 22) requires either staining at low pH or subsequent differentiation with solutions of very high ionic strength such as M/10 CaCl$_2$. In the present study the staining solution of toluidine blue had a buffer with ionic strength ten times higher, and pH 2.3 units lower, than the buffer system chosen in the companion study to promote complete binding of dye. Other procedures for thiazine staining (8, 15, 17, 23, 24) specify low pH, and one (8) depends upon elevated temperature.

In solution, both formaldehyde and acrolein increase the stacking differential between DNA and RNA with toluidine blue; the K of RNA is greatly increased, the K of DNA unaltered. Carnoy's fluid, on the other hand, reduces the stacking differential by raising the K of DNA more than that of RNA. This is consistent with the present observation that formaldehyde-fixed and acrolein-fixed tissues show good color contrast between DNA and RNA when stained, while Carnoy's-fixed tissues show little or none. The strong color contrast obtained in Carnoy's-fixed tissues by Flax and Himes (8), using azure B, and by Pelling (18), using toluidine blue, depended on staining at elevated temperatures, followed by prolonged differentiation. Thus, the relevant parameters of binding and stacking were different from those applying at room temperature. Acridine orange, for example, gives a larger stacking differential at room temperature between Carnoy's-treated DNA and RNA than toluidine blue does, and acridine orange can be used at room temperature to produce a color contrast after Carnoy's fixation (19).

The stacking differential in solution is raised somewhat more by formaldehyde than by acrolein. In the sections, on the other hand, acrolein produces more color contrast than formaldehyde, which suggests that the color contrast is influenced by properties of the two fixatives other than their action on the nucleic acids alone. The importance of other properties of the fixatives is further shown by the specific colors seen in the sections. In the acrolein-polyester sections, DNA stained a greener color than the blue of the free toluidine blue, while RNA stained purple. Solu-

tions of DNA with toluidine blue become more green than solutions of free dye only at very high P/D: 20 or 30 or even more. The purple color, shown by RNA in the sections, is seen with all nucleic acids in solution at P/D close to 1, but is rapidly lost as P/D is raised by addition of excess nucleic acid. RNA in solution in the presence of acrolein loses the purple hue when P/D is increased beyond 2.2. Thus, even in the presence of acrolein, the color contrast seen in the section can be reproduced in solutions of pure nucleic acids only by a great difference between P/D on the two nucleic acids: 2 or less on RNA, 30 or more on DNA. Such a large difference between the P/D of two stained tissue elements within the same section is not only unlikely *a priori* but appears inconsistent with the following additional observations. In the first place, although nucleic acids were more variable in color in sections showing less color contrast, comparison of other specimens with acrolein-polyester ones always showed that RNA was less metachromatic (except for the pink nucleoli in Carnoy's-paraffin sections) while DNA was more metachromatic. As color contrast decreased, the two nucleic acids approached the same intermediate blue or blue-violet color. If DNA is orthochromatic only because very little dye is bound to it, and RNA metachromatic only because it is completely saturated with dye, we must then suppose that any alteration of tissue processing which diminishes the color contrast causes more dye to be bound to DNA while less dye is bound to RNA. It seems very unlikely that a variety of non-specific procedures would simultaneously enhance dye binding to DNA and inhibit dye binding to RNA. In the second place, if the color contrast is to be explained almost entirely by differences in P/D, then the degree of metachromasy should increase with the intensity of staining. In fact, the degree of metachromasy was to a large extent independent of intensity of staining, both within the same section and in corresponding tissue elements of differently processed sections.

The analogy between the staining of nucleic acids in solution and in the tissue sections is imperfect, since the nucleic acids in tissue sections are bound to proteins. If bound proteins in the sections blocked nucleic acid dye-binding sites in a strictly random manner, this would correspond to an alteration of P/D in solution.

Therefore, the inconsistencies discussed in the previous paragraph cannot be resolved by assuming random blockage of dye-binding sites by protein.[2] It is much more likely that proteins bound to nucleic acids *in vivo* form structures with a high degree of molecular order. If this order were preserved in sections, dye-binding sites might be blocked in a manner such that the remaining exposed sites would form a regular array, quite different in its stacking properties from the array of all sites potentially available on the naked nucleic acid. It is also possible that in the native nucleoprotein complex the secondary structure of the nucleic acid itself might be different from the structure assumed by the same nucleic acid free in solution. In either case, the K of an ordered nucleoprotein macromolecule might differ from the K of the isolated nucleic acid component of that molecule.

We propose that the stacking differential between DNA- and RNA-protein in acrolein-polyester sections is greater than that between acrolein treated free DNA and RNA in solution, for either or both of the following reasons: K of DNA-protein is lower than K of free DNA, and K of RNA-protein is higher than K of free RNA. The K values in the sections are assumed to reflect preexisting ordered structures of nucleoprotein molecules, which are better preserved by the acrolein-polyester procedure than by the procedures with which it has been compared. Under this assumption, the close correlation between degree of color contrast and preservation

FIGURE 3 This is a representation, in the style of Fig. 5 in the companion paper (14), of the K values (with acridine orange) of native nucleoproteins compared to the K values of the corresponding free nucleic acids. The points shown for ribosomes and those shown for ribosomal RNAs are the limits of the range of values observed by Furano and Bradley. All native two-stranded DNAs have K values within the narrow range 1.2 to 1.4. The "?" next to the point for native chromatin indicates that this particular K has not yet been measured; our observations on toluidine blue–stained sections suggest that the K of native chromatin may be even lower than the K of native DNA (see text). The metachromatic color contrast between stained ribosomes and chromatin in tissue sections is enhanced by superior fixation of the native nucleoproteins, which preserves the stacking differential, or difference between their K values. With increasing denaturation of proteins, the K values will more closely approach those of the free nucleic acids and the stacking differential between ribosomes and chromatin will be reduced.

[2] The role of protein binding in modifying nucleic acid metachromasy has often been investigated by using reagents, such as acetic anhydride or nitrous acid, which block or remove amino groups. Such blockade or removal presumably eliminates the cationic sites on protein molecules that permit proteins to bind to nucleic acid phosphates. However, these reagents also block or remove amino groups on nucleic acid bases, leading to disruption of hydrogen-bonded base pairs and disorganization of helical secondary structure. Dilute solutions of nitrous acid, in fact, are commonly used (9, 11, and references cited therein) to produce controlled alterations of DNA molecules. Thus, experiments with these reagents will not decide the relative importance of protein binding and nucleic acid secondary structure in determining the staining of nucleoproteins. Doubt has also been cast, for other reasons, on the significance of experiments with reconstituted nucleoproteins (13).
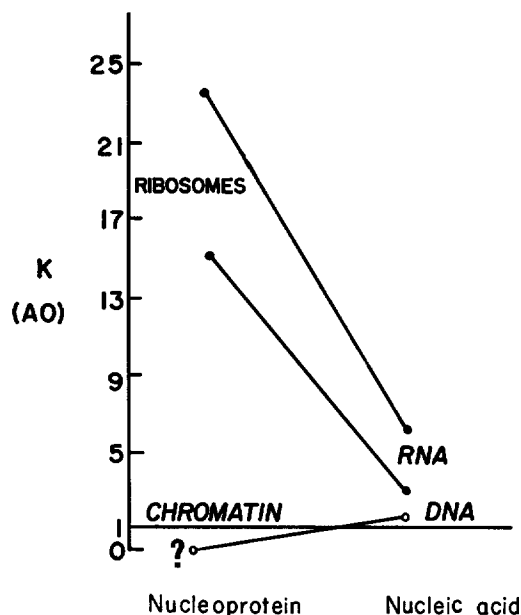
of cytoarchitecture is easily understood, since both reflect the fixation of proteins in life-like tridimensional order. Acrolein, though similar to formaldehyde in many of its properties, introduces more chemical cross-linkages (W. G. Aldridge, University of Rochester, personal communication) which stabilize cytoarchitecture and molecular architecture against subsequent denaturation. Polyester wax has a lower melting point and is a more polar compound than paraffin

wax; for both reasons, infiltration with polyester wax might have less tendency to denature proteins. It is also possible that molten paraffin wax might denature DNA, raising its K and thus reducing the stacking differential. It is noteworthy that formaldehyde-fixed brains embedded in celloidin, a procedure carried out entirely at room temperature and in which the final embedding matrix is a relatively polar compound, may show substantial color contrasts between DNA- and RNA-protein when stained with thiazine dyes (23). Finally, Carnoy's fluid, in addition to reducing the stacking differential between pure nucleic acids, will denature and partially strip away the associated proteins.

It is easy to construct plausible models of DNA-protein in which those phosphate groups which remain free to bind dye are so far apart that stacking of bound dye is hindered or even completely prevented. (If stacking is hindered, K is lower than K of free DNA; if stacking is prevented, K has no physical meaning but can be considered formally equal to 0. According to the stacking equations, unstacking can become complete at finite P/D only in the limiting case K = 0.) For example, if every nth site (n being some small integer) is free to bind dye, but all the intervening sites are blocked, the DNA-protein may be able to bind an amount of dye such that P/D = n, and yet the bound dye may remain completely unstacked, although there would be appreciable stacking at the same P/D on free DNA.

It might seem harder to imagine how bound protein could enhance stacking of dye on RNA. However, experiments are now in progress which demonstrate enhancement of stacking by protein in at least one RNA-protein–dye system. Furano and Bradley (personal communication) have studied the binding of acridine orange to preparations of whole ribosomes and of the corresponding ribosomal RNA freed from protein. The amount of dye bound per nucleotide phosphate is the same for whole ribosomes as for free ribosomal RNA. However, the K of various ribosome preparations ranged from 15 to 23, or even higher, depending on the buffer system employed, whereas when the RNAs are stripped away from their associated proteins the values of K are lowered to the range of 4 to 7. These findings are summarized in Fig. 3, which also represents our speculation concerning the K of DNA-protein, and is drawn

to conform in style to Fig. 5 of the preceding paper (14). Although these experiments have not yet been extended to other dyes such as toluidine blue, or to all the fixatives compared in this paper, they already provide evidence that the conformation of ribosomal RNA is radically altered by isolation from the specific ribosomal proteins. Furano and Bradley speculate that RNA in the ribosome is constrained to the shape of a straight chain, whereas when free in solution it acquires regions of helical secondary structure which lower the value of K. A straight chain structure, in which all the nucleotide bases are free, is more consistent than a partially hydrogen-bonded helical structure with the requirements of proposed mechanisms for the read-out of the genetic code.

The metachromatic dyes have been considered unsuitable for quantitative histochemistry because of their failure to conform to Beer's Law. However, we have seen that the very property which is responsible for non-conformity to Beer's Law, namely, the variation of molar extinction and spectral shape of metachromatic dyes according to their state of binding and aggregation, is amenable to quantitative treatment and can be used to study the structure of the molecules to which the dyes are bound. In the present study, the metachromatic color contrast between chromatin and ergastoplasm in toluidine blue–stained sections of appropriately prepared tissues has been used to show that DNA-protein and RNA-protein in tissues differ more in their conformation than do free DNA and RNA in solution; the color contrast has also provided at least a partial clue to the nature of the respective conformations. This approach can be expected to gain in versatility from the use of other metachromatic dyes with distinctive binding and stacking properties, and to gain in precision from the substitution of microspectrophotometry for subjective visual judgments of color. The quantitative study of altered dye spectra in stained tissues has great promise as a source of new information about the structure of the components of living matter.

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