Differential Staining of Nucleic Acids. II. Thionin and other metachromatic stains

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Some of the thiazine and oxazine basic dyes such as thionin, toluidine blue or cresyl violet are known to be typically metachromatic. Aside from mucopolysaccharide and metaphosphate (Lison, '36; Terayama, '47; Wislocki *et al.* '47; Wiame, '49), also nucleic acids have been pointed out to give rise to metachromasia of these dyes under certain conditions (Kelly and Miller, '35; Michaelis, '48; Sibatani, '48b and c; Wislocki and Dempsey, '46; Yasuzumi *et al.*, '50). Out of these dyes, thionin was found to stain cellular nucleic acids metachromatically (Sibatani, '48a and b, '49b). This paper summarizes the results of the author's investigations on the nature of such metachromasia of thionin in comparison with the qualitative observations on the reaction of this dye with nucleic acids *in vitro* which is accompanied by a distinct metachromasia (Sibatani, '48a-d, '49a and b).

The preparations of nucleic acids and nucleoproteins and tissue sections used in this study were the same as those included in the preceding paper (Sibatani, '52). Histone was extracted and purified from desoxy-pentose nucleohistone of beef spleen by the method of Mirsky and Pollister ('46).

Results

By some preliminary observations it was established that thionin, cresyl violet, and toluidine blue were most suitable to study metachromasia caused by nucleic acid *in vitro*, though other dyes of thiazine and oxazine classes and also some of the azine or other dyes show slight metachromasia with nucleic acids as well as mucopolysaccharides (Sibatani, '48b). As already pointed out by Kelly and Miller ('35) and Michaelis ('48), the color of thionin or toluidine blue are changed according to the proportion in which they are mixed with nucleic acids. When dilute aqueous solution of nucleic acids (0.1 per cent) of whatever type and degree of polymerization is added dropwise with aqueous solution of thionin, the color of the solution undergoes a gradual change from light blue to purple, until precipitate of thionin nucleate appears finally.

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The mixture containing such precipitate is spread over a slide glass, dried at 37° and washed with alcohol to remove excess dye, and the slide is observed under microscope. Amorphous precipitate remaining on the slide is typically pink-purple. It is to be noted that this color is decidedly different from that produced by mucopolysaccharides, the latter being deep red or reddish brown (Sibatani, '48b and c).

Therefore, the colors taken by thionin in combination with nucleic acids and mucopolysaccharides may be classified into three grades. The first, called α -color according to Michaelis ('48), is blue and obtained by the addition of excess nucleic acids, the second, β -color, is purple or pink-purple and represented typically by the precipitate or dye nucleate, and the last, γ -color, is deep red or reddish brown, produced invariably with mucopolysaccharides. With cresyl violet the corresponding colors are more or less similar to those with thionin: namely, α is blue, β pink-purple, and γ reddish pink. With toluidine blue the over-all coloration is somewhat shifted towards the longer wave lengths in the absorption spectrum. Thus α is bluish green, β bluish violet, and γ pink-purple.

As by all of the other metachromatic dyes, the difference of β - and γ -colorations exhibited by these dyes is always unmistakable, whereas that of α and β is less conspicuous in most of other than the three dyes mentioned.

In this connexion it may be added that phosphotungstic acid behaves just like nucleic acids against thionin, but against toluidine blue or cresyl violet no typical color change can be observed.

The peculiar situation of thionin, which is represented by the last experiment, is now strikingly reflected by tissue staining. Here, the chromatin stains blue (α) with thionin whereas nucleolar and cytoplasmic basophilia appears in purple or pink-purple (β) (Sibatani, '48 a-c, '49b). The ribonuclease test has shown that the β -coloration of nucleolus and cytoplasm is actually due to the presence of PNA, thus chromatin in α -color and mucopolysaccharides in γ -color remain in digested sections.

From this experiment it is now to be concluded that with thionin DNA stains in α -color, whereas PNA stains in β . Curiously enough, such a differential staining can hardly be observed with toluidine blue and cresyl violet, although in rare instances the presence of a similar tendency is suggested. It should be mentioned, however, that the results of thionin staining are subject to variation not infrequently, sometimes sections showing an over-all deep blue staining probably due to strong adsorption by proteins of the dye, sometimes DNA as well as PNA staining heavily in violet. Under what conditions such unsuccessful stainings take place is not clear at the present time. However, such unfavorable situations can usually be overcome by using several batches

of staining solution of thionin. An over-all heavy staining was experienced also with toluidine blue or cresyl violet.

From experiments with purified nucleic acids it may be postulated that α -color is the results of combination of the dye with excess nucleic acids, dye molecules being distributed among nucleic acid molecules more or less apart from each other and leaving much of phosphoric acid groups of the nucleic acid molecules available for combination vacant; if the amount of the dye is increased, phosphoric acid groups would more and more enter into combination with dye molecules; at the end all available positions would be occupied by the latter and the complex resulted would precipitate by losing most of their electrostatic charges. This situation seems to correspond to the full development of the β -color. Michaelis ('48) explained the appearance of α -, β -, and γ -colors by assuming occurrence of monomer, dimer, and tri- or polymer of the dye molecules in solution as well as in the state bound to different acidic compounds usually of relatively high molecular weight, whilst Terayama ('47) postulated that modifications of the resonating structure of the dye molecule, which is to be resulted from its combination with such acidic compounds or from other various causes, are responsible to its color change. Whatever the nature of this phenomenon may be, stoichiometry of dye-nucleic acid complex may be different in α - and β -colored materials.

From such a consideration it can be expected that conditions affecting the combination of metachromatic dyes with nucleic acids to some extent would introduce α -coloration of PNA in the cell, which otherwise stains in β -color. Experiments with sodium chloride in relatively high concentration have proved that this is the case (Sibatani '48d).

As is expected, the combination of nucleic acids with basic dyes is of electrostatic nature. This is evidenced by experiments as follow: β -colored precipitate of thionin-DNA complex was collected and washed with water, suspended in saturated solution of sodium chloride and left to stand overnight; the suspension was centrifuged, supernatant poured into two volumes of alcohol; fibrous precipitate formed was dissolved in water and reprecipitated with alcohol. On repeating this procedure white fibrous sodium desoxypentosenucleate was obtained. Thus, nucleic acid could be liberated at neutral pH from thionin-nucleic acid complex with high concentration of neutral salt, indicating that the base and the acid were in salt-like combination.

Also tissue staining with thionin appears to be realized by electrostatic combination. To prove this, a slide stained with thionin was left overnight in saturated sodium chloride solution. A control slide received similar treatment with distilled water. The test slide lost all of the stain attached to nucleic acids on the next morning while the

control showed no appreciable diminution thereof.¹

From such experiments it is evident that the presence of sodium chloride is effective in dissociating thionin nucleate suspended in water as well as produced on tissue preparations. The next step was taken by staining tissue sections in the presence of sodium chloride. Thionin was very readily salted out, so that toluidine blue was to be used in this experiment. Sodium chloride was dissolved in 0.1 per cent toluidine blue so as to give various final concentrations. On staining tissue sections with these solutions DNA and PNA exhibited various degrees of metachromasia from α to β : the higher the concentration of sodium chloride, the more shifts the color towards the genuine α -coloration, and with solutions with the highest concentration of the salt a typical α -color of blue green could be observed at the site of both nucleic acids. Here, the partial inhibition of the staining reaction of nucleic acids resulted not only in a reduction of the color taken by the cell, but also in a change of the coloration.² The appearance of α -color in PNA staining was experienced also as the result of partial inhibition of the staining reaction caused by different agents. As α - β -metachromasia is characteristic to nucleic acids,³ such a test would reveal the nucleic acid nature of the stained substance in the cell on the one hand, and suggest electrostatic nature of the staining reaction on the other hand.

The fact that PNA of the cell stains with thionin and its allies in β -color suggests that most or all of the phosphoric acid groups of PNA which are available for combination with dyes in dissolved state are also available for it when PNA of the fixed cell is subjected to the reaction with them. On the contrary, the finding that DNA of the cell takes usually α -color when stained with thionin may be assumed as the result of competition of histone or other proteins with the dye for combination with DNA. This would be examined with isolated nucleoproteins. However, pentosenucleoprotein and desoxypentosenucleohistone equally produced precipitates of β -color with thionin. Moreover, DNA did give β -colored precipitate even when it was added to aqueous solution of thionin in which histone had been dissolved prior to the addition of DNA. However, when DNA was first precipitated by the addition of neutral aqueous solution of histone and then added further with an excess of the latter, the fibrous precipitate of DNA-histone complex took an α -color on addition of a large volume of thionin

¹ Crystal violet staining of chromatin failed to give a comparable result by resisting to the treatment of saturated sodium chloride solution.

² However, Kurnick and Mirsky's experiment ('50) on the combination of methyl green with DNA showed only slight reduction of methyl green bound to DNA in the presence of concentrated sodium chloride.

³ Various proteins tested failed to show typical β -color with thionin, producing α -color if they took the stain.

solution, and this α -color was retained for some minutes. It was an impressive experience that we could make an observation that a further addition of DNA to such a mixture produced immediately a β -colored precipitate, thus α - and β -colored fibrous precipitate of DNA did exist in this instance side by side in one and the same medium (Sibatani, '49a). It has thus been established that at least under favorable conditions histone does actually compete with thionin for combination with DNA. Similar experiments with PNA gave no clear-cut result because it produced fine flocculent precipitates with protein as well as thionin.

It remains, however, to be explained why out of several metachromatic basic dyes thionin alone undergoes partial inhibition so readily, presumably by some basic proteins, in combining with DNA of the fixed cell nuclei so as to produce α -color, whereas toluidine blue and cresyl violet do react with DNA of the cell under comparable conditions just as PNA of the fixed tissues, giving unquestionable β -color.¹ Experiments with isolated chromosomes may throw some light upon the question indicated. It is to be mentioned here that on staining non-fixed, fresh tissue homogenate with thionin nuclei take readily α -color whereas cytoplasmic constituents, when they take any dye, stain usually in β -coloration.

Discussion

Metachromosia of some basic dyes caused by nucleic acid was already reported by Kelly and Miller ('35), Terayama ('47), Wislocki and Dempsey ('46), Michaelis ('48), Kurnick ('50 a and b), Kurnick and Foster ('50), and Yasuzumi *et al.* ('50). Most of their data seem to be consistent with the results obtained by the present author. We could not confirm Michaelis' statement ('48) that treatment with alcohol or dehydration and mounting through alcohol and xylol of the slides stained with metachromatic stains resulted in α -color of all nucleic acids. However, this may be true for DNA of the nuclei. It may be that on staining with thionin DNA takes first β -color and it is only after differentiation with alcohol that DNA, but not PNA, does exhibit α -color. If this is true, then Michaelis' statement is to be confirmed at least with DNA stained with thionin. But even this can not hold in the case of toluidine blue or cresyl violet staining of DNA.

Observation of Yasuzumi *et al.* ('50) that DNA of the giant chromosomes of larval salivary gland of *Drosophila* reacts metachromatically against toluidine blue, if chromosomes are first digested with trypsin, is worth being mentioned. Judging from the description given by

¹ As noted above, cellular DNA may stain occasionally in β -color even with thionin. The recognized difference between thionin and toluidine blue or cresyl violet resides, therefore in whether or not the tendency of these dyes to give α -color is strong enough when they stain cellular DNA.

Yasuzumi *et al.*, metachromasia in this case seems rather to correspond to γ -color. Somewhat similar situation could be created in my experiments in which beef spleen nuclei isolated by Mirsky and Pollister's method ('46) and frozen sections of rat submaxillary gland were stained with toluidine blue. No explanation has thus far been obtained about such phenomena. But if Yasuzumi's experiment had given DNA stained in β -color, this result would readily be understood by postulating that the elimination of proteins which acted in this case competitively with toluidine blue gave rise to DNA stainable in β -color.

The recently reported azure-phthalate staining of Flax and Himes ('50) should be mentioned here with great evaluation. As noted by these authors, azure A is a sort of metachromatic basic dyes. According to these authors, an addition of potassium acid phthalate to azure A effected an astonishing ability of differential staining of DNA and PNA. DNA stains blue whereas PNA red (at pH 4). If pH was lowered, also PNA tended to stain blue. Color shifting of the dye which they reported to emerge from combining with different nucleic acids of the cell under different pH's is altogether consistent with the findings of the author using thionin. No data are given as to the question of whether such differentiating effects of azure-phthalate is demonstrable with isolated nucleic acids. From its similarity to thionin staining, it would be natural to suspect whether the reported blue staining of cellular DNA with azure phthalate is due to the presence of competing proteins. Addition of potassium acid phthalate to thionin and cresyl violet readily causes precipitation of the dyes, so that phthalate ion may compete with nucleic acid for binding the dye in azure phthalate staining. Addition of phthalate to toluidine blue was found to be slightly effective in producing chromatin stained in α -color (bright blue-green) in contrast to cytoplasmic basophilia which stains in β -color (blue-violet) as usual.

In this connexion Ohno's experiment ('51) offers an interesting example. He stained virus-infected tissues containing many lymphocytes with pycnotic nuclei with toluidine blue at pH 4.2. The pycnotic nuclei DNA of which is pyroninophilic with methyl green-pyronin and thus seems to be partially degraded (Leuchtenberger, '50; Sibatani, '52), stain in β -color, while normal nuclei of various cells stain in α -color under these experimental conditions. Staining of normal nuclei in α -color may be due to somewhat acidic pH of the staining solution employed. According to Leuchtenberger ('50) proteolysis of the nucleus precedes to degradation of DNA into lower molecular fragments when it becomes pycnotic. Therefore it seems probable that α -color of normal nuclei is conditioned by the competition of nuclear proteins with toluidine blue under the pH employed, while in pycnotic nuclei the autolysis

of such proteins allows further combination of DNA with toluidine blue, resulting in β -colored dye nucleate. Under such conditions, one could expect cresyl violet to be much more favorable to contradistinction of pycnotic nuclei and normal ones, because the difference of α - and β -color is by the far pronounced in this dye (β is pink-purple and α is blue). Mr. Ohno has kindly conducted for me the experiment with cresyl violet, in which the result expected was actually obtained (*cf.* Ohno, '51).

From the experiments with thionin, the interference of proteins in staining reaction of cellular DNA with basic dyes would be expected to be highly important. This was first recognized by an experiment *in vitro* using histone, DNA and thionin (Sibatani, '49a). Kurnick and Mirsky ('50) and Mirsky and Ris ('51) have also demonstrated that proteins may compete with basic dyes for combination with nucleic acids. This was true for desoxypentosenucleoprotein of the isolated chromosomes and cell nuclei, while pentosenucleoprotein failed to show any significant difference from PNA in the dye-combining capacity, suggesting that association with protein of PNA is not through its phosphoric acid groups (Kurnick and Mirsky, '50). This fact is consistent with my experiments in which PNA of the cell was found to stain in β -color with thionin in contrast to DNA staining in α -color.

However, data presented by Kaufmann *et al.* ('48 and '50), or by Himes and Flax ('50) show that a part of cellular PNA is occluded by proteins attached to it and that its presence can be demonstrated by basic stains only after destroying proteins by proteolytic enzymes or, curiously enough, after subjecting slides to prolonged action of desoxyribonuclease. This seems to be especially true for PNA of chromatin or chromosomes. Interference of proteins in staining nucleic acids was also shown by Herrmann *et al.* ('50) with toluidine blue.

The interference of proteins to the nucleic acid staining implies a serious difficulty in photometric estimation of the amount of nucleic acids present within the cell by way of staining with basic dyes. At this point the work of Kurnick ('50b) may be cited, which deals with photometric measurements of methyl green stain in order to estimate DNA content of the nucleus. Owing to the recognized competition of histone with methyl green, Kurnick subjected test slides of formalin-fixed cells to a treatment with 0.1 N HCl for 5 minutes in order to remove histone, and showed evidence that under such conditions DNA of the nucleus may be determined quantitatively by methyl green staining. However, it is questionable that histone can be removed from *formalin-fixed* material by such a mild treatment with dilute acid. Di Stefano ('48) has shown, on the other hand, also photometrically, that treatment of the slides with 1 N HCl at 60° for more than 12 minutes is necessary to detach histone from *Carnoy-fixed* cell nuclei. The reason

why here again (*cf.* Sibatani, '52) the results of Kurnick and of Di Stefano contradict each other is not known. However, if Di Stefano's result be wrong, as the Stedmans' experiment ('50) suggests, another difficulty would remain in photometric measurement of methyl green stain. This stems from experiment of Mirsky and Ris ('51) showing the fact that also non-histone protein of the chromosomes competes with crystal violet for combination with DNA.

With ordinary basic stain it would be almost impossible to detect, by a simple microscopic observation, whether competition of proteins with dyes for combination with nucleic acids is involved in the staining reaction. Thionin stain is profitable in this respect, as it stains PNA as well as DNA intensely, and furthermore it differentiates them; and this differentiation in turn suggests that some partial inhibition may have occurred in the staining reaction of nucleic acids, presumably as the result of competence of proteins.

Metachromasia of thionin and under suitable circumstances also that of some allied dyes will also be useful in evaluating the specificity of staining reaction of nucleic acids. Furthermore, it may be emphasized that these thiazine and oxazine dyes stain cellular nucleic acids with remarkable intensity, by the far surpassing methyl green-pyronin in this respect. This fact appears also to have been recognized by Brachet ('48). As PNA stains with thionin in β -color, and proteins which may take occasionally the dye stain in α , simple visual inspection would be sufficient to detect extraneous staining due to proteins by examining whether β -color of PNA is not contaminated with α -color. On the other hand, possible inhibition of the staining reaction would be recognized by the lack of β -color at the site of PNA in the cell. Successful staining would then easily be discriminated. This affords obviously a reliable criterion of selective staining for nucleic acids. Experiments *in vitro* on the interaction of nucleic acids with basic dyes under various conditions would be facilitated by the occurrence of the characteristic color change if metachromatic basic dyes are used. Quantitative estimation of the color shift from β to α would further offer valuable information as to the degree of protein competition in microscopic preparations, and probably also to the study of the structure of nucleic acids.

Summary

1. Thionin, being a basic dye of thiazine group, stains DNA and PNA of the cell differentially in blue (α) and purple (β) respectively.
2. Metachromasia of thionin can be observed *in vitro* by mixing solution of the dye with various amounts of nucleic acids of whatever type. When large excess of nucleic acids is added with small amount of thionin, the color of the solution is blue (α); increase in the amount

of the dye in comparison with that of nucleic acids causes gradual change of the color taken by the solution from blue to purple (β) and finally precipitate in the latter coloration appears.

3. Marked metachromasia caused by nucleic acids *in vitro* is shared by toluidine blue and cresyl violet, but metachromatic staining of nucleic acids *in situ* with these dyes can not so readily be observed as with thionin.

4. Either of the α - and β -colors taken by these dyes can readily be distinguished from the metachromasia caused by mucopolysaccharides (γ -color).

5. Staining of nucleic acids with toluidine blue is partially inhibited by the presence of sodium chloride in high concentration. Thionin-nucleic acid complex can be dissociated by saturated sodium chloride solution *in vitro* as well as *in situ*. These facts suggest that nucleic acid stainings with thionin and toluidine blue are of electrostatic nature.

6. Partial inhibition of the staining reaction of nucleic acids with thionin, toluidine blue, or cresyl violet by different means gives rise to PNA stained in α -color, which otherwise stains in β -color.

7. On the basis of an experiment using histone and DNA, the α -color of thionin taken by cellular DNA is assumed to be resulted from competition of proteins for binding DNA. No such evidence was obtained with cellular PNA.

8. Implications of the metachromasia of some basic dyes caused by nucleic acids and of the interference of proteins in nucleic acid staining with basic dyes are discussed.

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