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RECENT STUDIES OF THE FEULGEN REACTION FOR DEOXYRIBONUCLEIC ACID*

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Résumé—Environ trois douzaines de nouveaux réactifs du type Schiff, spécifiques pour les polyaldéhydes tissulaires, ont fait l'objet de cette étude. Les noyaux peuvent se colorer par ces réactifs lors d'une réaction type Feulgen, dans une large gamme de teintes. Différents tests montrent la spécificité de ces réactifs pour les polyaldéhydes tissulaires. Beaucoup de ces réactifs sont fluorescents et peuvent être utilisés pour déceler de faibles concentrations d'ADN. Les réactifs sont préparés en saturant les solutions de colorants par SO_2. Les colorants prêts à réagir sont basiques et ont au moins un groupe amine primaire. On peut réaliser des colorations multiples d'aldéhydes en utilisant des réactifs appropriés de couleurs contrastantes. Certains colorants, contenant des impuretés colorées possèdant un radical amine primaire agissent comme des réactifs du type Schiff. Des réactions de remplacement se déroulent souvent quand des coupes sont colorées pour l'ADN dans un réactif du type-Schiff, lavées et ensuite colorées dans un réactif différent de couleur contrastante. Un phénomène semblable s'observe dans des réactions aldéhydiques doubles et avec des agents incolores bloquant les aldéhydes. L'analyse de quelques courbes d'absorption réalisées à partir de noyaux colorés est présentée en relation avec l'emploi de réactifs du type Schiff. Certains aspects de la réaction de Feulgen sont discutés.

THE popularity of the Feulgen reaction should not obscure the fact that there are gaps in our fundamental knowledge of the reaction. Additional studies may provide a firmer basis for cytochemical explorations and conclusions. Our own studies in this area have been concerned with the following:

(1) The development of analogues of the Schiff reagent, which are termed Schiff-type reagents.

(2) The use of cytochemical techniques to analyse reaction kinetics of the Feulgen reaction.

(3) Absorption-curve analyses of tissue polyaldehydes stained by Schiff and Schiff-type reagents.

In 1954, we began a purely empirical study to find other dyes which may readily substitute for basic fuchsin as Schiff-type reagents (KASTEN, 1956a, 1958a, 1959).

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This study involved thorough cytochemical tests of almost 450 different dye batches and over 200 different dyes. The results were gratifying and may be summarized briefly as follows:

(1) About forty different dyes were found to be specific for tissue polyaldehydes following saturation with SO_2 and oxidation of tissue sections or smears with periodic acid (PAS) or hydrolysis with hydrochloric acid (Feulgen).

(2) These reagents include dyes from all major dye classes. By choosing the proper reagent, one may stain nuclei selectively for colour transmission in any part of the visible spectrum. The colours include yellow, orange, brown, red, violet, blue, and green. A partial list of some of the more favourable reagents is shown in Table 1. Nuclei are stained very intensely by many of the Schiff-type

TABLE 1. PARTIAL LIST OF SELECTED DYES FOR USE AS SCHIFF-TYPE REAGENTS IN FEULGEN REACTION

Dye	C.I. no.	No. of reactive amine groups	Colour of stained nuclei
Acridine brown	—	1, 2	Brown
Acridine yellow	46025	2	Yellow
Bismarck brown R	21010	3*	Brown
Chrysoidine R	11320	1	Orange
Cresyl violet	—	1	Violet
Flavophosphine N	46065	2	Yellow
Rhodamine 3GO	45210	1	Red
Toluidine blue O	52040	1	Blue to blue-green

* If dye is present as dihydrochloride, there are two reactive groups.

reagents. Most reagents are not decoloured as is the conventional Schiff reagent after treatment with thionyl chloride, sulphur dioxide or sodium metabisulphite plus hydrochloric acid. The only dye to decolour completely is cresyl violet after reduction with sodium hydrosulphite. Many of the reagents are fluorescent and provide unique opportunities to detect low concentrations of polyaldehyde moieties in smears and tissue sections and to study the distribution of such moieties in biological material (KASTEN *et al.*, 1959). Certain dyes of contrasting colours may be used in multiple aldehyde-staining reactions.

(3) Various tests which verified the aldehyde specificity of these reagents are summarized in Table 2.

TABLE 2. EVIDENCE FOR SPECIFICITY OF SCHIFF-TYPE REAGENTS FOR TISSUE POLYALDEHYDES

(1) Specific staining of structures known to contain potential aldehyde groups.
(2) Non-specific staining after omission of essential aldehyde-producing step.
(3) Identical optimal hydrolysis times for all reagents in Feulgen technique.
(4) Reaction with aldehyde-blocking reagents (hydroxylamine thiosemicarbazide, phenylhydrazine).
(5) Reactivity and blockage with formaldehyde.
(6) Chemical-extraction procedures (TCA, PCA).
(7) Use of enzymes to check staining specificity (DNase, diastase, RNase, pepsin).
(8) Positive film tests.

(4) An analysis of the chemical structures of potentially-reactive dyes indicates that these are all basic dyes and each contains at least one primary amine group. The non-reactive dyes, which number approximately 160, all lack primary amine groups or are acid dyes. The structural formulae of some of the reactive dyes and closely related but non-reactive dyes are shown in Fig. 1. The Feulgen staining test

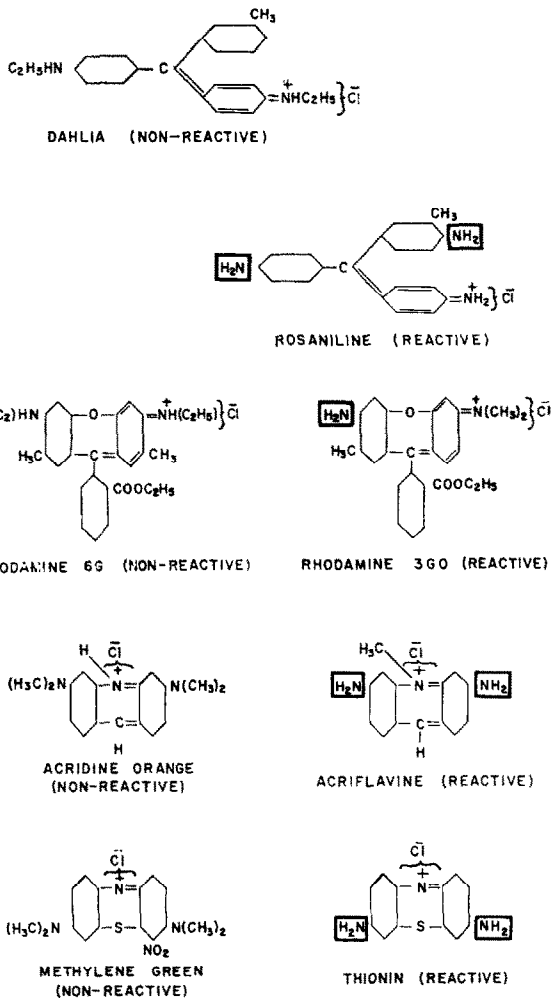

FIG. 1. Chemical formulae of several potentially-reactive dyes and closely-related but non-reactive dyes. Note presence of at least one primary amine group on each reactive dye.

is sensitive enough to detect primary amine dye impurities. Such impurities have been detected in batches of azure B, crystal violet, methylene blue, methylene violet, pyronin B, and pyronin Y, and are under investigation.

The WIELAND and SCHEUING (1921) scheme interpreting the formation of Schiff's reagent first involves the production of the leucosulphonic derivative from basic

fuchsin and then the stepwise addition of sulphinic acid to each of the primary amines. From our studies partly summarized above, we can say that participation of at least one primary amine group is certainly essential. Whether or not other primary amines necessarily are involved cannot be answered with certainty. In any event the Feulgen reaction may be carried out using dyes with one, two, or more reactive sites, depending on the Schiff-type reagent used. In view of this fact, it seems difficult to explain even partial reactivity between deoxyribonucleic acid (DNA) polyaldehyde and Schiff's reagent on the basis of stereochemical relationships as was suggested some years ago (LESSLER, 1951). The fact that the sulphur to sulphur distance in Schiff's reagent bears any numerical relationship to distances between nucleotides on DNA is a coincidence. This suggestion had been based on a sequence of purines on alternate nucleotides. The evidence now is that the purines on a given helix have a more random distribution. Reaction between single amine Schiff-type reagents like neutral red–SO_2 or toluidine blue O–SO_2 with DNA polyaldehyde could not be explained on the same stereochemical basis as polyamino reagents.

The availability of diverse Schiff-type reagents provides unique opportunities to begin studies on reaction kinetics involved in Feulgen staining. The DNA–dye complex is extremely stable under ordinary conditions. Cells washed in water and in alcohol retain all their chemically-bound dye. However, if washed cells are placed in a solution of a second Schiff-type reagent, in many cases the first dye is replaced by the second dye. For example, acriflavine–SO_2 replaces basic fuchsin–SO_2 in the sequence described above. If cell nuclei are first stained with the acriflavine reagent, then basic fuchsin–SO_2 does not replace it. The replacement phenomenon is easily seen with these two reagents since one stains nuclei yellow and the other stains them violet. One might consider that the presence of yellow nuclei instead of violet nuclei means that more aldehyde sites are opened up by hydrolysis in the second Schiff-type reagent. This possibility was checked by using suitable controls and cannot account for the results. Feulgen staining for lengthy periods of time in one reagent, twenty-four hours in many cases, show the same staining intensity that is obtained with 1 hr staining. Also, these Schiff-type reagents lack hydrochloric acid and do not lead to any production of aldehyde sites during staining.

TABLE 3. FACTORS STUDIED IN FEULGEN REPLACEMENT REACTIONS

Cells or tissues	Schiff-type reagents used	Staining time Dye 1 Dye 2
Ehrlich–Lettré ascites cells	Acriflavine (yellow)	1 hr→1 hr
	Azure A (blue)	3 hr→1 hr
Bull sperm	Basic fuchsin (violet)	16 hr→1 hr
	Bismarck brown R (brown)	16 hr→10 min
Mouse duodenum	Chrysoidine Y (orange)	
	Neutral red (brown)	
	Safranin O (red)	
	Toluidine blue (blue)	

Experiments like those described above were carried out on a variety of tissues using different Schiff-type reagents of contrasting colours. Another factor that was studied here was the effect of staining time in dye 1 and in dye 2. Sixteen different staining combinations were studied. These factors are summarized in Table 3.

The detailed results of this particular experiment will not be discussed here for lack of space. Some general statements can be made:

(1) There are marked differences in displacing ability. Certain dye reagents replace others but are not themselves replaced.

(2) There seem to be few differences in displacement phenomena among the three cell-types and tissues used.

The results are summarized in Table 4. Bismarck brown R–SO_2 exhibits the greatest displacing ability in the Feulgen reaction, whereas, basic fuchsin–SO_2 (Schiff) is the easiest to displace in the group studied.

TABLE 4. DISPLACING ABILITY OF SCHIFF-TYPE REAGENTS IN FEULGEN REACTION*—PRELIMINARY RESULTS

(1) Bismarck brown R–SO_2
(2) Neutral red–SO_2
(3) Acriflavine–SO_2
(4) Safranin O–SO_2
(5) Azure A–SO_2
(6) Toluidine blue O–SO_2
(7) Basic fuchsin–SO_2 (Schiff's reagent)

* The first dye listed has the greatest displacing ability. Numbers (5) and (6) are equal in displacing ability.

A logical interpretation of these results leads us into the area of reaction kinetics. Although the reaction between dye reagent and aldehyde is probably not carried out in a single step (WIELAND and SCHEUING, 1921; HORMAN *et al.*, 1958), one could make a general statement that dye A–SO_2, reacts with DNA polyaldehyde under certain equilibrium conditions finally to form a stable dye complex that resists water and alcohol treatment. Dye B–SO_2 likewise may achieve a stable union with DNA polyaldehyde. Because of differences in physical-chemical properties between these dyes, the average equilibrium constants differ. This scheme is visualized in Fig. 2. If either one of these two-dye–DNA-polyaldehyde

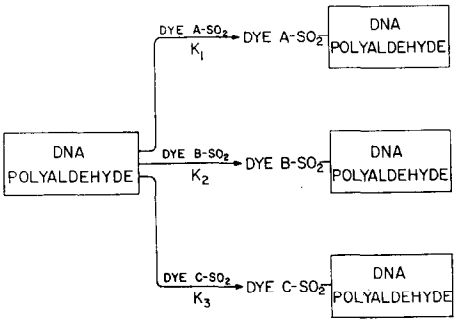

FIG. 2. Scheme showing reactivity between DNA–polyaldehyde and different Schiff-type reagents. Final reaction product results from different equilibrium constants in each case. Intermediate products are probably present in each case although not shown here.

complexes is treated with dye C–SO_2, the new equilibrium constants may force dye A or dye B to be displaced. The result is the partial or complete replacement summarized in Fig. 3.

Another experiment along this same line was carried out by first treating DNA polyaldehyde in tissue sections with an aldehyde-blocking reagent, thiosemicarbazide. The block is complete as checked by subsequent staining in Schiff's

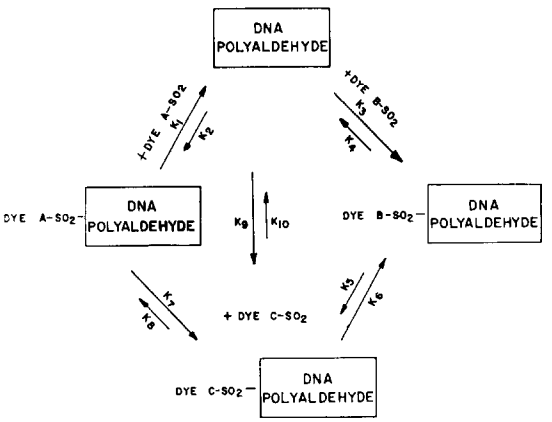

FIG. 3. Suggested mechanism to account for partial or complete replacement of Feulgen-dye complex by another Schiff-type reagent (dye C–SO_2).

reagent. However, if a more efficient displacing reagent than basic fuchsin is used nuclei are now stained. The results for Bismarck brown R are shown in Fig. 4. Differences in equilibrium reaction constants probably also account for these results.

Other experiments have been carried out using Schiff-type reagents in double-aldehyde staining techniques. The displacement phenomenon is also observed here. For example, sixteen different tissues were subjected to double staining in the Feulgen and periodic-acid–Schiff techniques using Schiff's reagent and

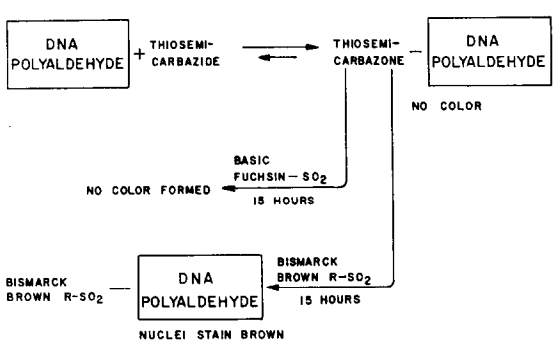

FIG. 4. Representation of blockage of DNA–polyaldehyde by thiosemicarbazide. Basic fuchsin–SO_2 does not overcome block, whereas Bismarck brown R–SO_2 does overcome block.

toluidine blue O–SO_2 as the reactive aldehyde reagents. Four different reaction sequences were followed for each tissue. An outline of the experiment is shown in Table 5. The results confirm data described earlier. Regardless of tissue or species origin of tissue, toluidine blue O–SO_2, replaces Schiff's reagent either partly or completely. This replacement occurs irrespective of whether Schiff stained structures

TABLE 5. OUTLINE OF DOUBLE-ALDEHYDE STAINING EXPERIMENT

Tissues	Reaction sequences	Aldehyde reagents
Mouse liver Mouse intestine Rat kidney Rat ovary Rat thyroid Rat salivary gland Rat pituitary Rat skeletal muscle Rat small intestine Rat stomach Rabbit liver Rabbit trachea Rabbit aorta Human thyroid	Feulgen (dye 1)→PAS (dye 2) Feulgen (dye 2)→PAS (dye 1) PAS (dye 1)——→Feulgen (dye 2) PAS (dye 2)——→Feulgen (dye 1)	Dye 1: Schiff's reagent (basic fuchsin–SO_2) Dye 2: toluidine blue O–SO_2

result from Feulgen or PAS treatments. Furthermore, Schiff's reagent never seems to replace toluidine blue O–SO_2. This is in agreement with previous observations that toluidine blue O–SO_2, is a more effective aldehyde replacing agent than Schiff's reagent. Other extensive experiments have been carried out using many other combinations of Schiff-type reagents. The results have not been evaluated fully.

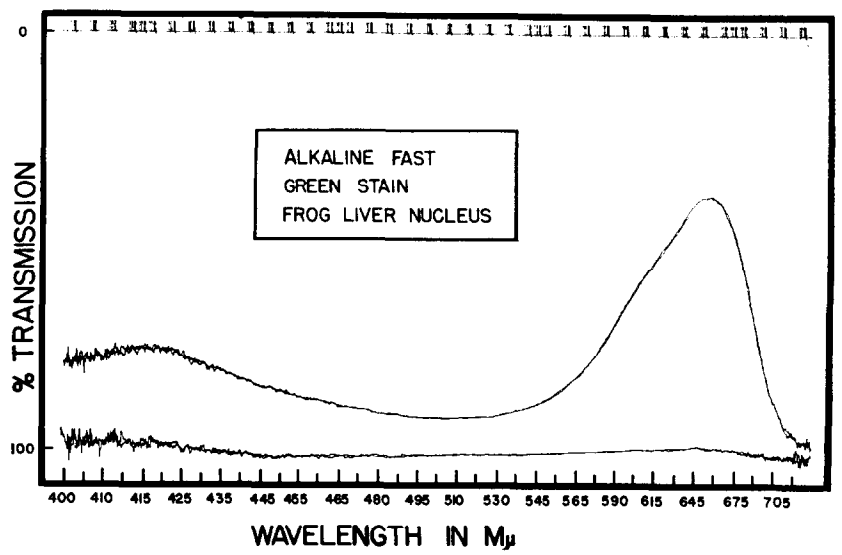

FIG. 5. Example of precision of double-beam ratio-recording microspectrophotometer. Two background curves and two transmission curves obtained successively. Time to obtain all four curves was 45 min.

Some absorption curve analyses of Schiff-type reagents and of the replacement phenomena were carried out. One of the instruments used was the double-beam ratio-recording microspectrophotometer at Columbia University (POLLISTER and ORNSTEIN, 1955). Fig. 5 illustrates the precision attainable with this instrument. A trace through a clear area of the stained section was obtained and was followed by another trace. A nucleus stained for histones by alkaline-fast green was placed in the field. Two transmission curves were obtained from the same nuclear area. Fig. 6 shows the transmission curves through six different frog-liver nuclei previously stained by thionin–SO_2 in the Feulgen reaction. Although the concentration

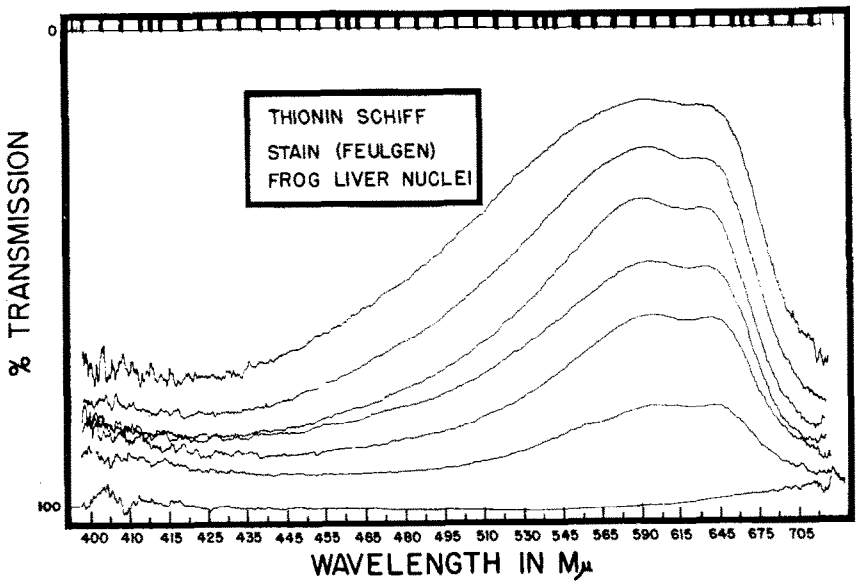

FIG. 6. Transmission curves of six different nuclei stained in Feulgen technique by thionin–SO_2.

of thionin–SO_2–DNA polyaldehyde varies in all nuclei, curve shape appears to be the same. Further analyses of these curves are in progress. Previous reports (KASTEN, 1956b, 1957, 1958b) confirm the marked stability of the conventional Feulgen DNA–dye complex in various normal and abnormal tissues.

Chrysoidine Y–SO_2 ordinarily does not displace Schiff's reagent in the Feulgen reaction. This is shown in Fig. 7. The initially-rapid reaction between Schiff's reagent and DNA polyaldehyde is demonstrated in Fig. 8. In this case, hydrolysed frog-liver nuclei were exposed to Schiff's reagent at 6 °C for 2 min. The section was washed in the usual way and then immersed in chrysoidine Y–SO_2 at room temperature for 45 min. Transmission curves from different nuclei clearly show the presence of the Schiff peak at approximately 580 mμ in this case in addition to the broad peak at 465 mμ of the chrysoidine Y–dye complex. Details of experiments partly summarized here will be published elsewhere.

It might be profitable to consider briefly the present state of affairs of the Feulgen reaction, to relate it to the Watson–Crick model of DNA, and to mention some potential sources of variation in dye–SO_2 binding to DNA polyaldehyde.

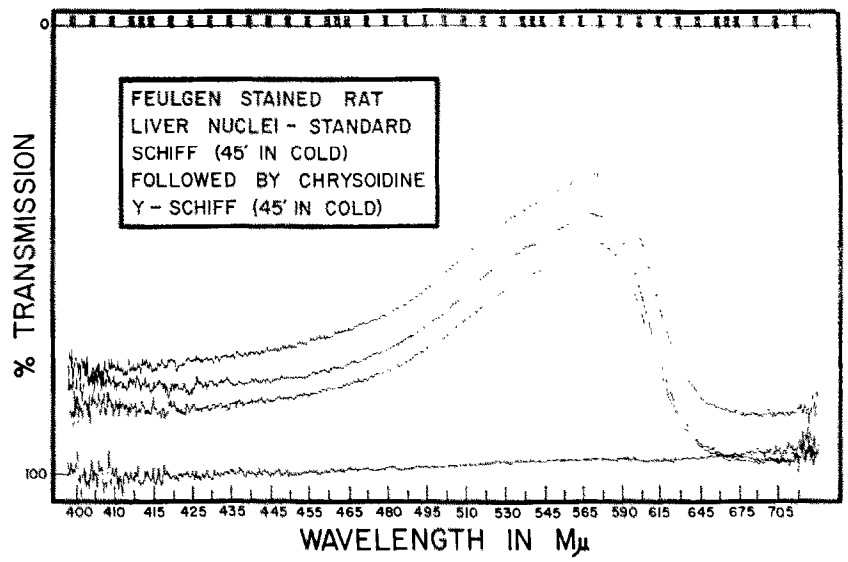

FIG. 7. Transmission curves demonstrating non-replacement of Schiff reagent by chrysoidine Y–SO_2. Schiff peak is unaltered.

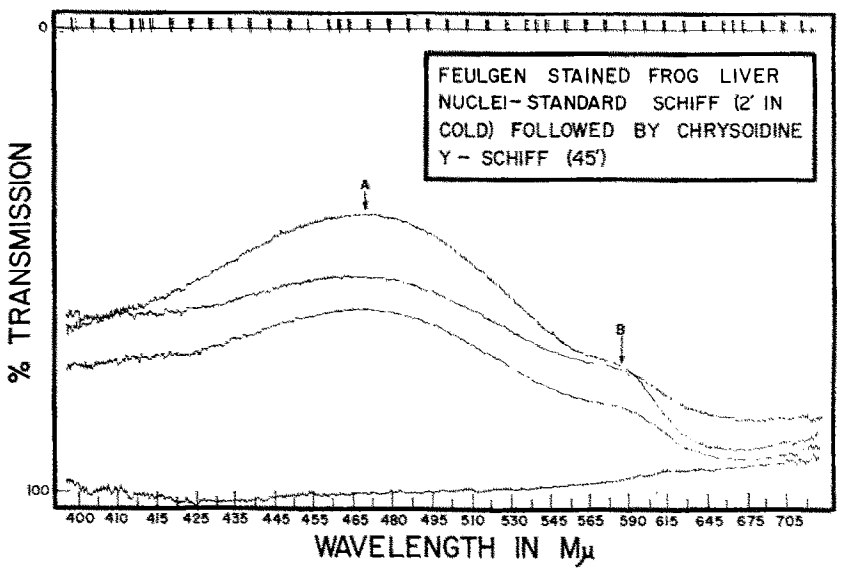

FIG. 8. Rapid reaction of Schiff's reagent with DNA–polyaldehyde detected in transmission curves by presence of dye peak at point B. Broad chrysoidine Y–DNA peak shown at point A.

The production of Schiff's reagent and of Schiff-type reagents for use in cytochemistry requires a basic dye with one or more primary amine groups to serve as reactive sites for aldehyde binding in the presence of sulphur dioxide. According to WIELAND and SCHEUING (1921), each of these fully-reacted sites has the structure, R—NH—SO_2—CHOHR', where R is the attachment to the dye molecule and R'

is the attachment to the sugar moiety of DNA. Recently, HÖRMAN *et al.* (1958) advanced the view that the fully reacted site has the following structure, R—NH—CH_2—SO_3H, where formaldehyde is the reacting aldehyde shown. The evidence for both points of view cannot be given here. Conceivably, both structures may be correct and may be present under different conditions of SO_2 concentration and other variables.

The generation of DNA polyaldehyde from DNA *in situ* requires the splitting of the glycosidic linkage between deoxyribose and purine bases by optimal acid hydrolysis. Purine bases are not restricted to alternate nucleotides as was thought formerly. According to the Watson–Crick model of DNA, a purine is found on each nucleotide, on either one or the other helix of the double-helical chain. This means that when hydrochloric acid splits the purine linkages to deoxyribose at the optimal hydrolysis time (TAYLOR, 1958; SAVAGE and PLAUT, 1958), and when the deoxyribofuranose components are converted into aldehydes (OVEREND and STACEY, 1949; BROWN and LYTHGOE, 1950) there may be from 3000 to 15,000 aldehyde sites per DNA molecule (molecular weight of from one to five million). This also assumes that each aldehyde site is freely available to the amine complex of the dye reagent. Results of certain test-tube studies suggest that proteins may interfere with the Feulgen reaction (CASPERSSON, 1932; SIBATANI, 1953) but these systems bear little resemblance to the true situation *in situ*. Feulgen absorption curves are identical from nuclei with or without native protein present (KASTEN, 1956b). In another experiment, Feulgen DNA per nucleus in mouse kidney was unaffected by previous enzymatic removal of nuclear protein (KASTEN, 1958b).

The multiplicity of reactive sites in hydrolysed DNA could account for the initially-rapid colour development usually seen in cytochemical preparations. The fact that the rate of reaction between Schiff's reagent and DNA polyaldehyde levels off and achieves maximal staining in approximately 45 min might be explained by the repulsion of cationic-dye structures. Since each nucleotide is only 3·4 Å

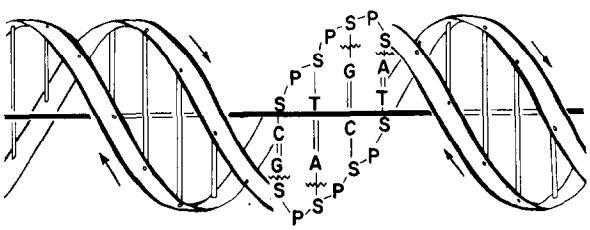

FIG. 9. Watson–Crick model of DNA with breaks shown at linkages between purine bases and deoxyribose molecules as a result of optimal acid hydrolysis in Feulgen reaction.

apart, the free aldehyde sites will also be a short distance apart in those adjacent nucleotides containing purine components on the same helix (Fig. 9). The multiplicity of aldehyde sites favours an initially rapid reaction in the presence of dye reagent. As the number of aldehyde sites become chemically bound to dye cations, one could envisage unreacted dye cations being replused, especially where aldehyde

sites happen to be adjacent on the same helix. Another explanation is that the rate of reaction slows down due to changes likely in concentration of reacting groups.

There are reports in the literature of mature oocyte nuclei which are unstained in a Feulgen reaction (MARSHAK and MARSHAK, 1955; IMMERS, 1957). Various explanations have been postulated:

(1) There is no DNA present.

(2) There is DNA present but the concentration is so low that no colour is seen.

(3) Interfering substances, such as lipids or proteins prevent proper reactivity and colour formation.

Another possible explanation may be added to this list. Is it conceivable that the distribution of purine and pyrimidine bases in DNA is altered in the mature oocyte so as to inhibit the production of aldehyde sites during hydrolysis or the subsequent reactivity between dye reagent and aldehyde? This might operate through the absence of labile purine N-glycosidic linkages or through the presence of a large number of purines lying adjacent to one another on the same helix. These considerations are presented in a speculative way, but may serve to suggest other experimental approaches to the problem. It may be feasible and desirable to run chemical analyses and also obtain X-ray diffraction data on mature oocytes.

The use of the Feulgen reaction to obtain quantitative data by cytophotometry has provided solutions to many problems. In other cases, conflicting results or interpretations have arisen from this approach. It seems well established that the DNA content per chromosome set is constant for a given species. A pressing problem seems to be whether the measured range of DNA values in a diploid cell population is a real biological variation in terms of numbers of DNA molecules, whether this is due to instrumental and operational errors, or whether slight variations in the Feulgen reaction account for this distribution pattern. In addition, there is the problem of interpreting DNA changes in endocrine organs. There are various approaches to this problem but we will deal here specifically with the Feulgen reaction as the potential source of trouble.

Recently, SRINIVASACHAR and PATAU (1959) analysed the effects of different sequences of washing Feulgen-stained cells, especially the effects of SO_2 water and alcohol dehydration. They found that if stained cells are carried through the conventional washing, dehydration and mounting procedures, there is no differential loss of dye–aldehyde complex. However, if cells have been once mounted, then demounted and brought back to SO_2 water, there is a differential loss of dye or aldehyde moiety from cells, even in cells from different stages of the mitotic cycle. Another interesting approach involves measurements of Feulgen-dye absorption in the same nucleus at the visible dye peak and at the natural ultra-violet peak of 260 mμ (WALKER and RICHARDS, 1957). The recent report by CERRONI and NEFF (1959) that uranyl and ferric ions inhibit Feulgen staining of *Acanthamoeba* and *Tetrahymena* nuclei may have some general significance, especially in regard to negative Feulgen reactions of mature oocytes.

It is conceivable that the acid hydrolysis procedure could produce a differential production or loss of aldehyde moieties among cells in a diploid population, but there seem to have been no decisive experiments to prove this.

Another factor must be considered here which undoubtedly has an important bearing on the stoichiometry of the Feulgen reaction. This concerns the fact that there are three amine groups on the basic fuchsin molecule. One of these is in pentavalent form serving as the site for salt linkage and probably does not enter into linkage with SO_2 and aldehyde. The fact that there are two other potentially-reactive binding sites makes very likely the possibility that SO_2 and aldehyde each react in sequential steps with both amine groups. We have no assurance that complete reactivity or retention occurs at both amine sites. A variety of intermediate compounds are undoubtedly present in the reaction solution. The availability of many Schiff-type reagents having only one reactive amine group, such as azure A, chrysoidine R, chrysoidine Y, coriphosphine O, cresyl violet, neutral red, neutral violet, rheonin, rhodamine 3GO, toluidine blue O and typogen brown may provide additional evidence about this potential source of DNA variability. It seems logical that a reactive dye having only one binding site will have simpler stoichiometric relations than the conventional Schiff reagent and provide a firmer basis for quantitative applications.

SUMMARY

About three dozen new Schiff-type reagents specific for tissue polyaldehydes are reported. Nuclei may be stained by these reagents in the Feulgen reaction in a wide range of colours. Various tests demonstrate the specificity of these reagents for tissue polyaldehydes. Many of the reagents are fluorescent and may be used to detect low concentrations of DNA. Reagents are prepared by saturating dye solutions with SO_2. Potentially-reactive dyes are basic and have at least one primary amine group. Multiple-aldehyde staining may be accomplished by using appropriate reagents of contrasting colours. Certain dyes containing primary amine dye impurities work as Schiff-type reagents. Replacement reactions often result when tissue sections are stained for DNA in one Schiff-type reagent, washed and then stained in a different reagent of a contrasting colour. Similar phenomena are observed with certain double-aldehyde reactions and with colourless aldehyde blocking agents. Some absorption curve analyses from stained nuclei are illustrated in relation to the use of Schiff-type reagents. A discussion of certain aspects of the Feulgen reaction is given.

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DISCUSSION

T. CASPERSSON: Does any one of the dyes you describe look especially promising and could be better than fuchsin?

F. KASTEN: Some of the dyes give a more intense colour reaction than fuchsin. This includes some of the blue dyes like thionin or toluidine blue O and certain brown dyes like Bismarck brown or typogen brown. I believe that many of these dyes may be used in multiple-aldehyde techniques or in fluorescent microscopy. At present, we are studying the suitability of some of these dyes for quantitative purposes and would not recommend their use in Feulgen cytophotometry as yet.

J. DANIELLI: It has clearly become important to improve DNA detection by a factor of 10 or 100. There must be many ways of doing this, of which I will mention two.

(1) Use as primary agent NH_2—  —polysaccharide which will combine with the aldehyde group of hydrolysed DNA through the NH_2 group. Then use periodate oxidation of the polysaccharide followed by reduced fuchsin. This can give many dye groups per aldehyde group released by Feulgen hydrolysis.

(2) Use as primary agent NH_2—  —antigen, and follow by fluorescent antibody to the antigen.

F. KASTEN: Some of the fluorescent Schiff-type reagents may provide the sensibility needed to detect low concentrations of DNA. Your suggestions also may prove very useful.

W. PLAUT: Do you know of any applicable fluorescent dyes which are stable during u.v. illumination?

F. KASTEN: Many of the fluorescent Schiff-type reagents are stable when bound to normal nuclear DNA. I do not know as yet what will happen in those cases where the DNA concentration is very low.