THE CHEMISTRY OF THE FEULGEN REACTION AND RELATED HISTO- AND CYTOCHEMICAL METHODS*

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I. INTRODUCTION

The purpose of the present paper is to compare the chemical reactions concerned in two well-defined cytochemical methods and one in which the specificity of the reaction at present is obscure. These three methods are: The Feulgen Nucleal Stain, The Periodic Acid-Schiff Stain, and The Aldehyde-Mordanted Basic Fuchsin Stain. It is also intended to compare certain features common to these three methods.

As an integral part of the development of the subject, it is also intended to draw attention to the important problem of the action of formaldehyde on various tissue elements as demonstrated by two of the three methods to be considered. Because of the wide usage of formaldehyde as a fixing agent in the handling of tissues it is of importance to understand what its specific actions may be under various circumstances. At present the action of formaldehyde on intracellular structures is obscure. Explanations of its exact action are based for the most part upon speculation or analogy.

II. METHODS

Feulgen nucleal reaction

Pure pararosaniline (National Aniline Division of the Allied Chemical & Dye Co.), Lot # 11499, was used in the manufacture of Schiff's reagent. The dye in a concentration of 0.25% was decolorized according to the method of Barger and DeLamater (1) in which thionyl chloride is used as a source of SO_2.

Hydrolysis of the material to be stained was accomplished in a hot water bath at 60°C. The method differed from the classical procedure in the following ways: The initial and terminal exposure of the material to cold 1.0 N. HCl was dispensed with. The 1.0 N. HCl heated to 60°C., was applied directly to the cells and tissues. These were placed immediately in the hot water bath. The time of hydrolysis was measured from the moment the warm acid was applied. The hydrolytic action was stopped by placing the containers (test tubes) in an acetone-

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dry ice mush in order to cool the tubes and contents as rapidly as possible. The time of hydrolysis was considered ended at the moment the chilled acid was removed and replaced with distilled water. About one minute was required for the cooling and decanting procedures. In this way fairly well controlled hydrolysis periods could be set up.

Aldehyde-mordanted basic fuchsin stain

The procedure used was as previously described (2, 3). Pure pararosaniline (see Feulgen reaction above) was used in 0.25% concentration in $\frac{1}{25}$ N. HCl. The formaldehyde used as a "mordant" was made up by diluting commercial formalin (38–40% formaldehyde) to 2%, or by diluting 10% stock solutions 1:5 with distilled water. The hydrolysis procedures were carried out as described for the Feulgen reaction.

Periodic acid-Schiff reagent stain was not utilized in these studies and is presented here only for theoretical comparative purposes (4).

Spectrophotometric measurements of the solutions referred to were done with a Beckman spectrophotometer and a Coleman Senior spectrophotometer, and were checked on both instruments. Hydrogen ion concentrations were measured with a Beckman, Model G, pH meter.

Methods of reading the degree of reaction obtained

In the absence of an instrument for microscopic spectrophotometric analysis the grades of reaction were estimated visually by two of the authors (E. D. DeL. and J. D. B.) and the results obtained by each were compared. In all cases the readings so obtained were practically identical and never differed by more than one unit. Tissues were graded from 0 to 8, the highest number representing the most intense staining reaction observed. Fungus cells were graded from 0 to 4 in a similar manner. The minuteness and delicacy of the fungus nuclei made such a differential reading between the tissues and the fungi necessary.

The following species of fungi were used as test organisms: Blastomyces dermatitidis, Saccharomyces cerevisiae, Histoplasma capsulatum, Schizosaccharomyces octosporus, Zygosaccharomyces sp., Endomyces sp. All were fixed in Schaudinn's solution at 60°C. Comparison of the effects of fixation on these stains will be embodied in another paper. Detailed cytologic studies of certain of these fungi have been presented elsewhere (5–7). Mouse tissues obtained from the same animal were used in these studies. Fixation was also done with Schaudinn's fixative. Sectioning was performed with a microtome set at uniform thickness.

III. GENERAL CHEMISTRY OF THE PARAROSANILINE DYES

Michealis (8) in his extensive study of the basic dyes, states that the rosaniline dyes (triaminotriphenylmethane dyes) differ from other basic dyes and must be considered separately. He does not consider them further in his article nor does he indicate in what manner these dyes differ from other groups of basic dyes. The triphenylmethane dyes require serious study in the same manner and from

the same point of view used by Michealis on other basic dyes. The following discussion will present some of the characteristics of this group of dye substances. Many of these characteristics have been pointed out elsewhere in both the same and other connections (2, 9–14).

The triphenylmethane dyes are characterized by three phenyl rings attached to a central carbon atom. One of these rings is attached with a double bond, as seen in Figure I, 1, with three amine groups, one attached to each of the phenyl

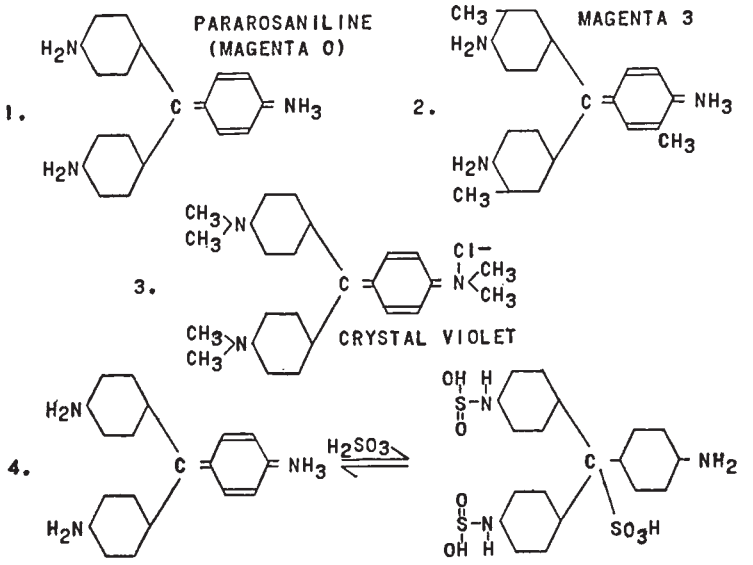

FIG. I

1. Pararosaniline (Magenta O), showing simplest form of rosaniline dye with amine groups attached to the phenyl rings in the para position, with three phenyl rings attached to a central carbon atom one of which is attached with a double bond and has corresponding double bond changes within the ring. This double bond linkage constitutes the chromophore group and is responsible for the presence of color in the compound.

2. Magenta III differs from Magenta O only in the presence of methyl groups attached to each of the phenyl rings.

3. Crystal violet in which the para-amine groups are methylated.

4. The reaction between pararosaniline and H_2SO_3 to form Schiff reagent in which the quinonoid linkage is reversibly destroyed by the addition of a sulfinic radical to the central carbon and by the addition of two sulfonic radicals to two of the para-amine groups.

rings in the para position from the central carbon. This double bond with its associated bond changes in the attached phenyl ring constitutes the so-called quinoid linkage. Because of this double bond structure at the center of the molecule the triphenylmethane dyes as a group are relatively unstable compounds, and are easily oxidized by nascent oxygen, ultraviolet light, etc., with the destruction of the molecule at this point. This acts for their rapid fading in sunlight, and has proven a disadvantage in the dye industry. Stowell (15) does not seem to have recognized this fact.

The quinonoid linkage may be affected in other ways. In the presence of a strong alkali, such as sodium hydroxide, a carbinol is produced with the reversible disappearance of the chromophore group (See Figure II). The carbinol is colorless. In the presence of reducing substances the quinonoid linkage may be reduced with the addition of hydrogen to form a triaminotriphenylmethane or "leuco fuchsin" (9). This modification of the quinonoid linkage is also reversible and the compound produced is colorless (Figure II). Thus, in the presence of acid or alkali the dye may act as an indicator system, changing or losing color in a low acid or in a high alkaline range, and reversing as the pH rises or falls with reconstitution of the color. The particular pH at which the color loss occurs appears to depend upon the length of the chains attached to the para-amine groups of the dye molecule.

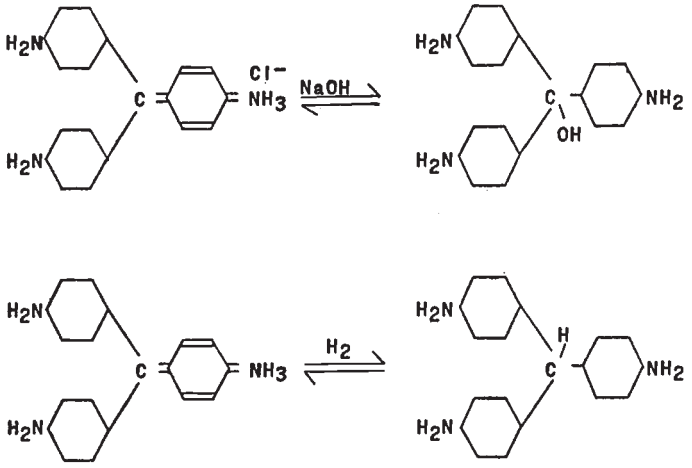

FIG. II

5. Reversible loss of quinonoid linkage with hydroxide to form colorless carbinol.
6. Reversible loss of quinonoid linkage by reduction to form triaminotriphenylmethane (leuco base).

In the presence of H_2SO_3 a special reaction occurs which differs from a comparable reaction with other inorganic acids. The so-called Schiff reagent is formed by the addition of a sulfonic radical to two of the para-amine groups, and of a sulfinic acid radical to the central carbon, replacing the quinonoid double bond. The substance so produced is colorless and very unstable. The H_2SO_3 is rapidly released with the recoloration of the dye substance by the reconstitution of the quinonoid linkage (Figure I, 4). The Schiff reagent (also designated as "leuco fuchsin" by some authors (15), has been found to have a special usefulness because of the specificity of its reaction with aldehydes. This reaction will be considered more completely when the Feulgen nucleal reaction is taken up (Section V).

Other characteristics of the pararosaniline dyes should also be considered. Variations in color occur with the addition of modifying groups to the molecule.

Pure pararosaniline, as indicated in Figure I, 1, contains no modifying groups. Its color is bright red and its maximal spectral absorption is about 539 mμ. The dye molecule may be modified by the addition of one methyl group to each phenyl ring (Figure I, 2). As the number of methyl groups added is increased, the color changes from bright red to magenta, and the maximal spectral absorption becomes about 542 mμ with one methyl group added; 548 mμ with three methyl groups added, one to each ring. Basic fuchsin, as sold commercially, is usually a mixture of the rosaniline dyes with varying degrees of methylation of the phenyl rings. One dye component, however, is usually in preponderance. The use of a pure dye becomes of paramount importance when spectrophotometric analyses are to be done on stained material.

In addition to the color changes produced by methylation of the phenyl rings, color changes may also be produced in the same general direction of color change (toward the longer wave lengths) by methylation of the para-amine groups. Methyl violet is a triphenylmethane dye of such a type (Figure I, 3). Methylation of the para-amine groups from the chemical point of view is of importance because it blocks the reactivity of the para-amine groups and prevents certain types of linkages from occurring. This will be considered in more detail when the aldehyde-mordanted basic fuchsin stain is considered. It should be pointed out in passing, however, that such methylation prevents permanent linkages with nuclear components from occurring; prevents the formation of a true Schiff reagent; and prevents the so-called mordanting effect produced with formaldehyde in the aldehyde-mordanted basic fuchsin stain. However, function of the dye as an acid-base indicator is not affected.

As with other basic dyes (8) the color of the rosanilines is affected by the pH of the solution and by the type of solvent used. The specific changes in spectral absorptions in different solvents have not yet been clarified nor have the influences of such substances as DNA, mucoprotein, etc. Readily apparent changes in color from bright red to magenta are, however, seen in different solvents.

Figure III demonstrates both the change in color and the change in maximal absorption with a change in pH in the dye compound produced by the reaction between Schiff's reagent and formaldehyde. There appears to be a straight line relationship between maximal absorption (color) and pH.

IV. HYDROLYSIS

The necessity for hydrolysis of tissues with acid in order to obtain the specific reaction with Schiff reagent was demonstrated by Feulgen and Rossenbeck (10) in their original description of the Feulgen stain in 1924. Subsequently, Robinow (16) (working with bacteria) and DeLamater (1–7) (working with fungi) demonstrated that hydrolysis of these cells was necessary in order to obtain specific nuclear reactions with other stains. Extensive studies (23) on the effects of acid hydrolysis of nucleic acids indicate that there is a combined depolymerization and splitting of the contained nucleic acids of the cell with a specific release in the case of desoxyribosenucleic acid of an aldehyde linkage in the contained desoxy-

pentose, which in the case of the Feulgen stain reacts specifically with the Schiff reagent.

DiStefano (18, 19) (Figure IV) in two recent papers has critically demonstrated what occurs during acid hydrolysis of the desoxyribosenucleic acid on the cartilage nuclei of Rana pipiens tadpoles. (See also #28) DiStefano carried out his hydrolysis over a period of twenty-four minutes. He ascertained the purine and pyrimidine content of the nucleus at four minute intervals during this period. He used the methyl green stain to indicate the presence of desoxyribosenucleic

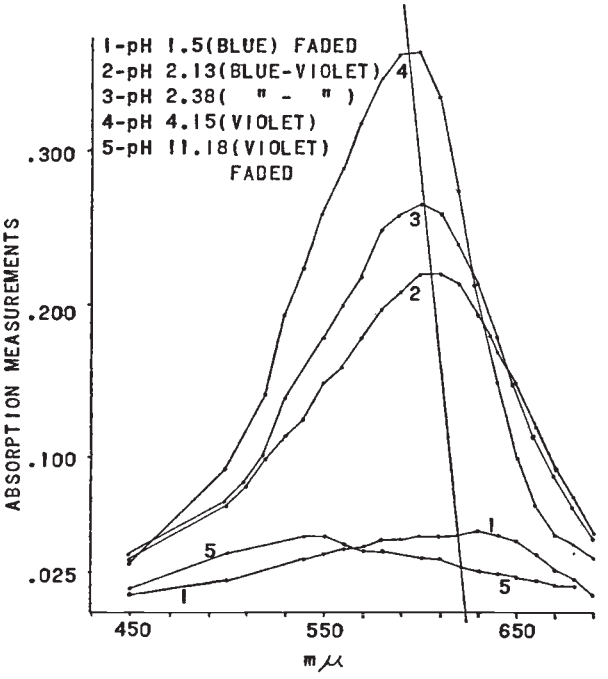

FIG. III

Effect of pH on absorption spectrum of the blue compound produced by the interaction of Schiff reagent and formaldehyde. There appears to be a straight-line relationship between the absorption maxima obtained at each pH.

acid, and he used the Feulgen reaction to indicate the presence of reactive aldehyde groups freed by the hydrolysis. He found that the ultraviolet absorption spectrum for purines and pyrimidines decreased in a straight line curve to zero in twenty-four minutes. The methyl green stain gave a consistent reaction up to twelve minutes after which time there was a rapid decline during the next eight minutes. The Feulgen nucleal reaction rose to a maximum at twelve minutes and fell off to an approximately zero reading at twenty-four minutes. DiStefano's work represents the most critical study of these phenomena to date and indicates clearly the effects of acid hydrolysis upon the desoxyribosenucleic acid of the cell.

Hydrolysis requirements for the Feulgen reaction and for the aldehyde-mor-

danted basic fuchsin stain are similar and routinely carried out in 1 N. hydrochloric acid at 60°C. Comparable results may be obtained with 1 N. sulphuric acid (half molar), 1 N. nitric acid, etc. Nitrous acid does not produce these effects. Hydrolysis may be modified, as will be seen in a later section, by using a more dilute acid for longer periods, and it may prove that such modifications under controlled conditions may have certain advantages in the analysis of differences between intracellular constituents. A stoichiometric relationship between the concentration of acid and hydrolytic effect has so far not been established.

For special purposes other methods of hydrolysis, which appear to be specific in their activity, have been developed. Periodic acid (HIO_4) has been found to

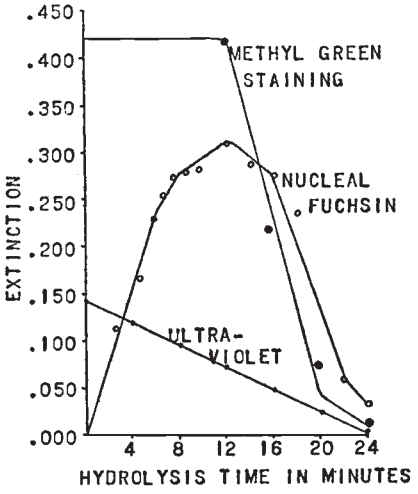

FIG. IV

The effect of hydrolysis on the cartilage nuclei of tadpoles as demonstrated by DiStefano, 1948. Ultraviolet absorption demonstrates the loss of purines and pyrimidines during the hydrolysis period. Methyl Green Stain demonstrates the rapid destruction of desoxyribose-nucleic acid during the second twelve-minute period. The nucleal fuchsin stain demonstrates the increase of aldehyde freed by hydrolysis up to twelve minutes with a rapid loss of aldehyde after this maximum.

hydrolyze certain of the lipoidal mucoproteins with the release of two aldehyde groups which also will give specific reactions with the Schiff reagent. This will be considered more fully in Section V, Periodic Acid Schiff Stain (4). It is considered to be specific by McManus (20, 21) for this group of mucoproteins.

McManus also suggests that various polysaccharide substances may be successfully hydrolyzed by such agents as formaldehyde, chromate, chromic acid, permanganate, bichloride of mercury, etc. The specificity of these substances as hydrolytic agents of polysaccharides has not yet been clarified from a cytochemical point of view.

It has been found by DeLamater that optimal hydrolysis of different organisms (i.e. Blastomyces and Saccharomyces) requires different hydrolysis periods and that it is necessary in dealing with such microorganisms to define the optimal

period for reaction for each organism. This is demonstrated in the accompanying figures (V, VI, VII) in which the observed cytologic reaction is plotted against the hydrolysis period for Blastomyces, Saccharomyces, and Zygosaccharomyces for both the Feulgen reaction and the aldehyde-mordanted basic fuchsin stain. It will be seen that although these optima lie relatively close together, two to four minutes apart, the reactions, due to the minute size of the organisms and their included nuclei, are so definite that precise readings of this nature can be made. Comparable observations have been made on Histoplasma, Endomyces, and others. It would appear that the optimal hydrolysis period specifically characterizes a particular organism for both these stains.

Comparable studies have been done under two sets of conditions with liver,

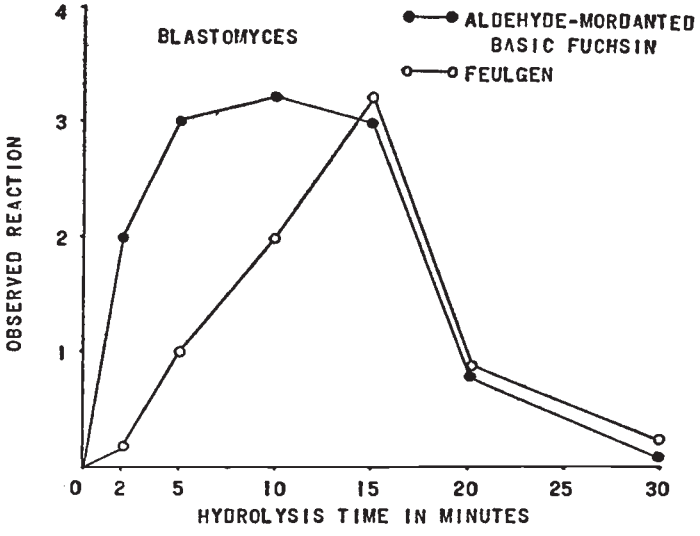

FIG. V

This demonstrates the hydrolysis period for each of the two stains indicated when Blastomyces dermatitidis is used as a test organism.

spleen, thymus, and kidney tissues obtained from the same mouse. It will be seen in the accompanying figures (VIII, IX) that hydrolysis with 1 N. hydrochloric acid at 60°C. produces curves comparable to those demonstrated for the nucleal reaction by DiStefano, the curves lying relatively close together. However, it will be seen that the speed of the reaction differs per tissue. The thymus is distinctive in that the optimal reaction is approached very rapidly and is maintained, contrary to what occurs in the other tissues, for a long period. If the tissues are hydrolyzed at 60°C. in N/10 hydrochloric acid the differences in the speed of development of the optimal reaction for each tissue become more manifest. Again the thymus rises rapidly to an optimal reaction which it maintains for a long period. The kidney, spleen and liver each attain their optima at different hydrolysis periods. It is suggested that the use of more dilute acid hydrolysis may be useful in demonstrating differences (chemical or polymeric?) in the

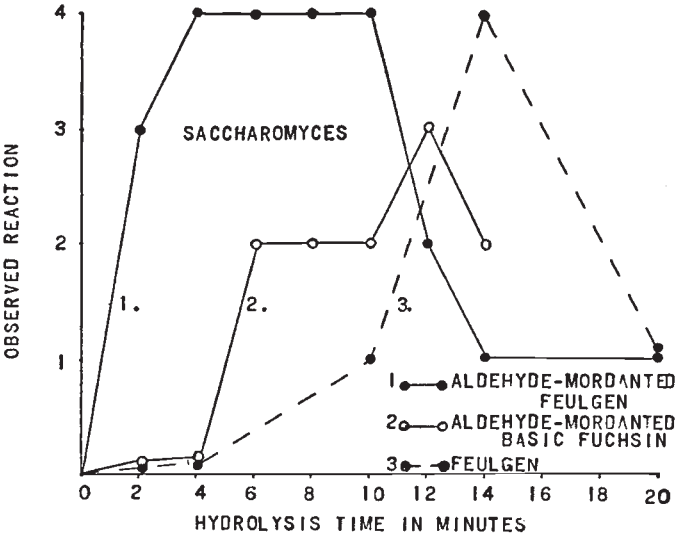

FIG. VI

This figure demonstrates the hydrolysis period required for each of the three stains indicated when Saccharomyces cerevisiae is used as the test organism.

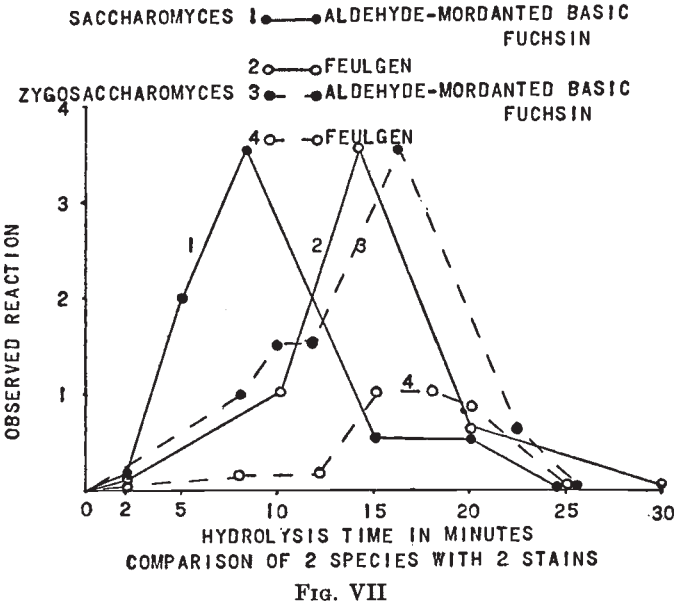

FIG. VII

This demonstrates the hydrolysis periods required for optimal staining for Saccharomyces and Zygosaccharomyces for each of the stains indicated. This chart clearly demonstrates the differences in optima not only for each of these organisms but for each of the stains.

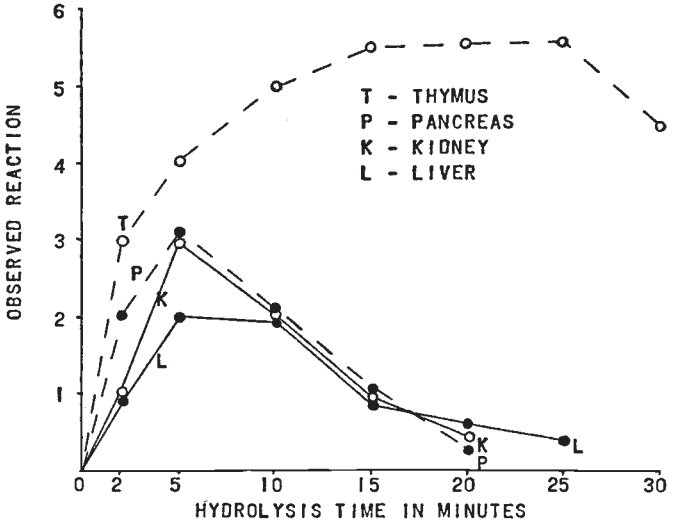

FIG. VIII

This demonstrates the relatively minimal differences between the optimal required hydrolysis periods of different tissues when the hydrolysis is done with 1 N. HCl at 60°C.

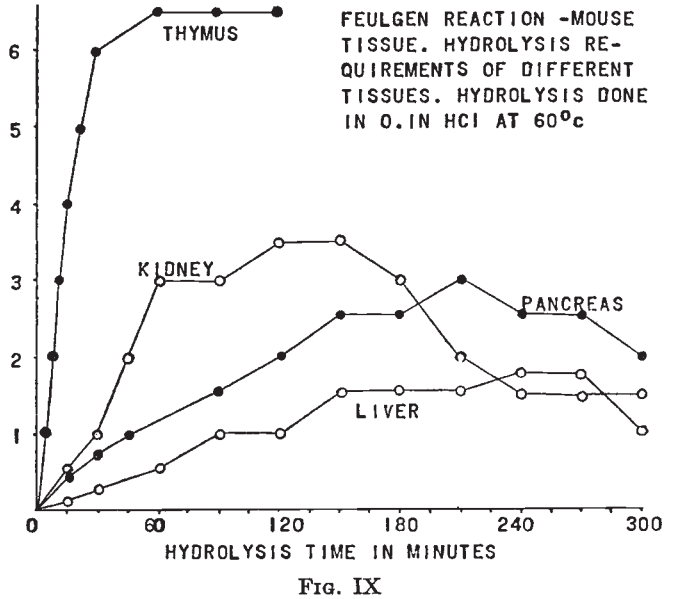

FIG. IX

This demonstrates the marked differences in optimal required hydrolysis periods between the same tissues as Figure VIII when hydrolysis is carried out in 1/10 N. HCl at 60°C.

desoxyribosenucleic acid present in the nuclei of different tissue cells. It has been shown recently that there are probably different nucleic acids in the different tissues of a single organism and in different types of the same organism, and ob-

served phenomena presented here may well be related to the observed specific differences demonstrated by others (24, 25).

It becomes necessary on the basis of such studies to ascertain for each tissue and/or each organism the optimal hydrolysis period if one is to obtain the most satisfactory cytologic preparations, and to produce standards for critical comparative or quantitative studies.

V. THE FEULGEN NUCLEAL REACTION

The basic requirements for this staining method are (a) hydrolysis of the tissues as already described, and (b) the use of Schiff reagent produced by the action of H_2SO_3 on basic fuchsin. This reaction is supposedly specific for the

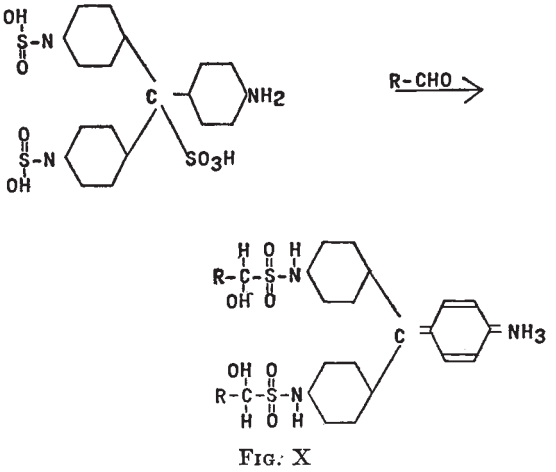

FIG. X

The chemical reaction which occurs between Schiff reagent and an aldehyde as formulated by Wieland and Scheuing, 1932.

presence of an aldehyde freed by the acid hydrolysis of the desoxyribosenucleic acid of the cell. However, it is known that a delayed reaction will occur between the Schiff reagent and acetone with the production of a blue compound.

The chemical reactions which occur in the nucleal stain, according to the work of Wieland and Scheuing (12), are seen in Figure X. The aldehyde in some manner removes the sulphinic radical from the central carbon of the dye molecule and reconstitutes the quinonoid linkage with a concurrent redevelopment of color. In addition there is a reaction between sulfonic linkages at the para-amine groups of the dye molecule in the manner indicated, and the color of the compound produced is magenta, with maximal absorption spectrum of about 580 mμ. The magenta color so produced is probably related to the length of the linkages at the para-amine groups.

If pure desoxyribosenucleic acid is hydrolyzed in a test tube and Schiff reagent added, this reaction occurs in the same manner as in an intact cell. It is possible under these conditions to analyze the stable compound produced and demon-

strate the presence of sulphur in the molecule. The absorption maximum of this compound can be measured. It is likewise possible to do a comparable experiment in the test tube in which formaldehyde is added to the Schiff reagent (Figure III). Sulphur is also easily demonstrated in the substance found, indicating that the sulfonic group persists between the para-amine group and the aldehyde in this molecule (12).

It is also possible under these conditions, by studying the spectral absorptions of different rosaniline dyes, to determine the specific influence upon the maximal absorption of the product of methylation of the phenyl rings, etc. It has been observed that the addition of these methyl groups tends to push the maxima absorption bands toward the longer wave lengths.

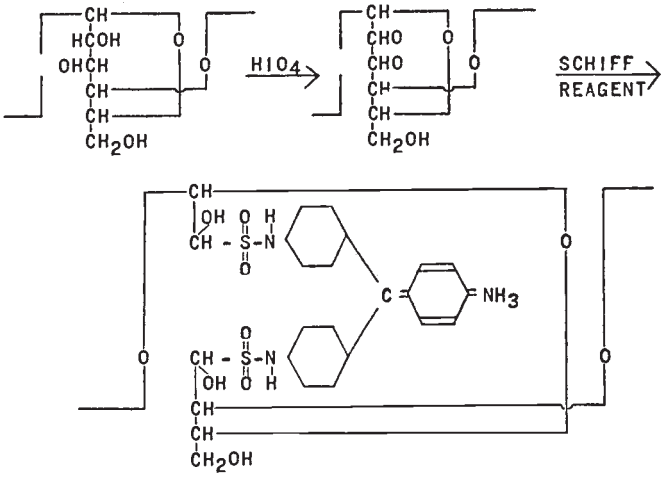

FIG. XI

This demonstrates the chemical reactions which occur when polysaccharides are exposed to periodic acid with the formation of aldehyde radicals at carbon atoms 2 and 3, and the subsequent reaction which occurs between these aldehyde radicals and Schiff reagent as formulated by Hotchkiss, 1948.

VI. PERIODIC ACID—SCHIFF STAIN

The requirements for this reaction, as worked out by Hotchkiss (4) are the hydrolysis of tissue with periodic acid, HIO_4, and the staining of tissues so hydrolyzed with the Schiff reagent. McManus considers that the stain produced under these controlled conditions is specific for the lipoidal mucoproteins. The chemical reactions involved, according to Hotchkiss, are presented in Figure XI. These consist of the hydrolysis of the CHOH radicals, on carbon atoms Nos. 2 and 3, to two adjoining aldehyde radicals, as indicated. In the presence of the Schiff reagent there is a reaction between each sulfonic group attached to the para-amine groups of the dye molecule with each of the two aldehyde groups produced, with a rupture of the linkage between the two carbon atoms involved in the sugar molecule.

The parallelism between this reaction and that defined by Wieland and Scheuing (12) for the Feulgen reaction is immediately apparent upon comparison of their reactions (see Figures X & XI). The only essential difference between these two reactions is the condition for hydrolysis.

VII. THE ALDEHYDE-MORDANTED BASIC FUCHSIN STAIN

The aldehyde-mordanted basic fuchsin stain, as developed by DeLamater (2, 3) for use cytologically on microorganisms, is not yet sufficiently understood so that a complete comparison from a chemical point of view can be made between it, the Feulgen nucleal reaction, and the periodic acid-Schiff reaction. However, there are certain similarities between these methods which deserve consideration and comparision in an effort to define what is taking place in this less well-known reaction.

It was first observed by Choudhuri (22) that a stain could be produced by the addition of formaldehyde to Schiff reagent which was highly selective for nuclear substance. Choudhuri considered that this stain was the same as the Feulgen stain because it was essentially the same color (magenta). Choudhuri carried his observations no further. However, had he tested the relative tenacity of this stain and the Feulgen stain in such menstrua as acid alcohol or H_2SO_3, the ready solubility and the, therefore, impermanent linkage of his stain with the nuclear components would have been demonstrated, whereas the Feulgen stain would have been shown to be permanent (2). Danielli (23) confirmed Choudhuri in his observations that the Schiff reagent colorized with aldehyde acted as a nuclear stain. These studies have been more extensively confirmed and analyzed by DeLamater (2). Carr (27) considered the stain produced by acidification of basic fuchsin with HCl to be identical with the Feulgen reaction. From a chemical point of view this is probably not true. Carr's stain compares most closely with the aldehyde-mordanted basic fuchsin stain.

The aldehyde-mordanted basic fuchsin stain may be defined as one in which acid hydrolysis of the cells, identical with that used in the Feulgen reaction, is used, but in which the hydrolyzed tissues are exposed to a weak solution (2%) of formaldehyde prior to their exposure to the dye used. The dye used is *not* the Schiff reagent but is a solution of pure pararosaniline in a concentration of 0.25% in N/25 hydrochloric acid solution. This stain, therefore, differs in two respects from the two stains just considered.

The use of formaldehyde in this procedure prior to the exposure of the tissue to the stain was deliberately developed because it was demonstrated that the stain became more tightly bound after this treatment than without it, and permanent stained preparations of the delicate nuclear structures of fungi could be obtained in permanent form only by this means (2, 3, 5–7).

It has been demonstrated that if aldehyde is added to a pure solution of pararosaniline a magenta compound precipitate occurs which has an absorption maximum at approximately 560 mμ, half way between pure pararosaniline and the Schiff reagent. It is apparent that there is a reaction between the formaldehyde and the pare-amine groups of the dye molecule, and it is apparent (Figure XII)

that the length of the chains at the para-amine is shorter than that formed with aldehyde and Schiff reagent. Theoretically the maximal absorption should fall about where it does. This reaction is not a stable one and if the compound is isolated and continually washed or dialized, pure pararosaniline is reconstituted. It is also apparent that the effect of the aldehyde upon the stain is to block the reaction so that in the presence of an excess of formaldehyde adequate staining of the tissues is prevented and no permanence of the stain is obtained. It is considered that the reaction which occurs at the para-amine groups with formaldehyde is equivalent to the effects of methylation of these groups as seen in crystal violet and that blocking of these para-amine groups prevents a reaction from occurring between the dye and whatever component within the cell it ordinarily reacts with.

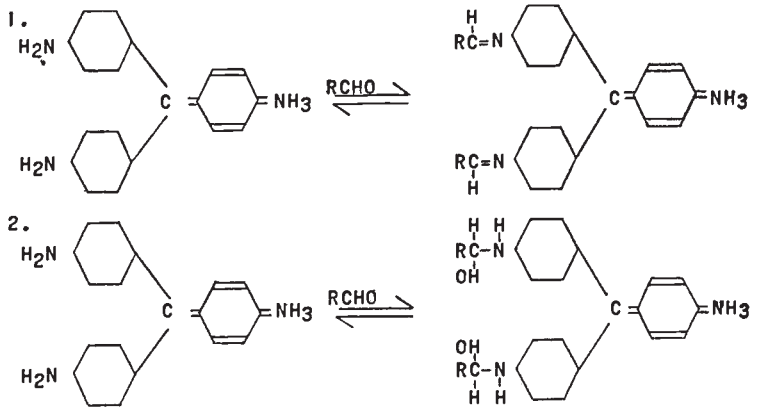

FIG. XII

This demonstrates the postulated reaction which can occur between pararosaniline and aldehyde directly.

On the other hand, the question as to what occurs when formaldehyde is added to tissue, either before or after hydrolysis, must be considered in detail in order to interpret its effects. The importance of this problem extends far beyond its immediate importance in attempting to understand its action in this particular staining reaction.

The use of formaldehyde in the binding of amine groups has long been utilized as a method for the titration of acid radicals in amino acids, polypeptides, etc. If it acts to bind the amine groups of the nucleoprotein or nucleohistone these substances will then become more acid and react more effectively and tenaciously in binding the para-amine groups of the basic dye molecule.

If a section of tissue is treated with formaldehyde and subsequently stained with pararosaniline the staining reaction is greatly intensified over a controlled section untreated with formaldehyde, and is stained magenta. However, if another section is hydrolyzed for an optimal period in 1 N. hydrochloric acid at 60°C. and subsequently is stained with pure pararosaniline, but not treated with

formaldehyde, there is also a marked intensification of the staining reaction produced and the stain produced is also magenta. If a fourth section is hydrolyzed in the same manner, exposed to formalin, and then stained with pararosaniline, this reaction is also comparable to the two just described but is more tenacious and less easily removed by such substances as acid-alcohol, H_2SO_3, etc. It is obbiously a question whether the effects of formalin can be considered comparable to the effects of hydrolysis even though hydrolysis of the tissues frees an aldehyde radical. Whether the aldehyde released operates to bind the rosaniline has yet to be demonstrated in the intact cell. It should be possible, using proper methods, to analyze and evaluate these effects specifically chemically. Careful photometric analysis will be necessary.

Figures VI, VII, and XIII demonstrate the effects of formaldehyde upon the Feulgen and the aldehyde-mordanted basic fuchsin stains. In the test tube a

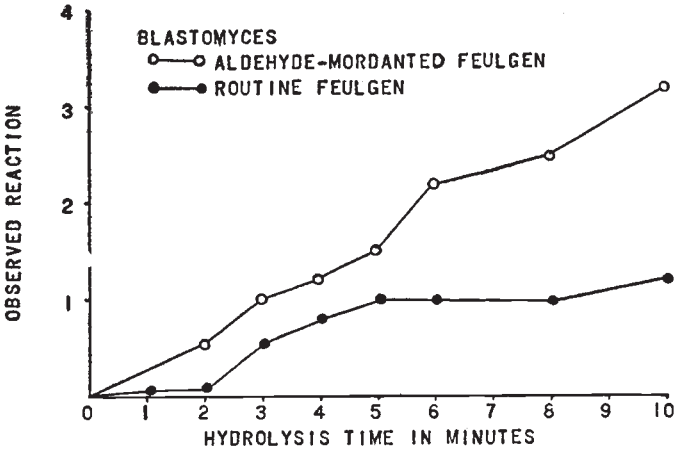

FIG. XIII

This demonstrates the effect of formaldehyde mordanting of tissues stained with the Feulgen nucleal stain. There is a marked intensification of the staining reaction.

compound is formed between hydrolyzed DNA and pararosaniline and the compound can be precipitated at about pH 11.5. A similar compound probably forms in the intact cell. By using proper methods of specifically blocking such reactions the formation of such compounds can be demonstrated from a negative point of view. Such studies are planned. As stated above, it is perfectly clear that in a test tube a reaction between both a cellular aldehyde as well as between a noncellular aldehyde and the para-amine radicals of the dye can occur.

McManus (20, 21) has suggested that formaldehyde is one of the substances which can act to free aldehyde linkages by reduction of sugar molecules. Does formaldehyde under the conditions of these experiments operate to increase the number of aldehyde or carbonyl linkages present within the cell? The only suggestion at present that such may be the case is indicated by the increased staining reaction obtained in polysaccharide walls of fungi following treatment with

formaldehyde. Whether this may be utilized as an indication that such an action occurs is still problematical.

In most of the studies done to date on fungus cells by the authors the organisms have been killed and fixed in Schaudinn's Solution. This fixative contains mercuric bichloride. McManus also suggests that this compound, in addition to binding the acid radicals in protein, may also, like formaldehyde, act to free aldehyde and carbonyl linkages in the cell. This also needs clarification.

VIII. FIXATION

It is clear that the type of fixation modifies markedly the type of reaction obtained both with the aldehyde-mordanted basic fuchsin stain and with the Feulgen reaction. This has been readily apparent on numerous instances both in the experiments upon which this report is based and in more extended studies to be reported, and has been clearly demonstrated also by DiStefano (18, 19). The method of fixation must be taken into consideration in any analysis of cytochemical methods, and probably must be considered a part of the definition of the method, if repeatable experiments are to be performed.

SUMMARY AND COMMENT

1. A consideration of the general chemical reactions and characteristics of the pararosaniline dyes has been presented.
2. The influence of the characteristics of this group of dyes in a consideration of the Feulgen reaction, the periodic acid-Schiff reaction, and the aldehyde-mordanted basic fuchsin stain, all of which can be produced with certain members of this group of dyes, has been considered.
3. The chemical reactions have been presented and compared so far as they are known, and an effort made to evaluate the aldehyde-mordanted basic fuchsin stain in the light of these known reactions.
4. It has been demonstrated that the following factors must be considered in interpreting biologic reactions with the rosaniline dyes:
   A. Hydrolysis:
      a. The necessity for adequate hydrolysis in order to obtain optimal staining reactions.
      b. Tissues and microorganisms may differ in the optimal periods of hydrolysis they require.
      c. These differences are more apparent with dilute (0.1 N. HCl) acid hydrolysis than when more concentrated acid is used.
      d. Hydrolytic agents are being evaluated which permit selective hydrolysis of different intracellular and extracellular substances.
         (1) HCl for DNA
         (2) HIO_4 for lipoidal mucoproteins
         (3) The possible selective action of other hydrolytic agents.
   B. Chemistry of Dyes:

a. The presence of color depends upon the presence of the quinonoid linkage.
b. The chromophore group is easily oxidized and irreversibly destroyed.
c. The color and absorption of the dyes depend upon:
(1) The degree of methylation of the phenyl rings.
(2) Methylation of the para-amine groups.
(3) The solvent used.
(4) The pH of the solution; these dyes are indicator systems.

C. Action of Formaldehyde:
a. Aldehyde may act as a "mordanting" agent in the aldehyde-mordanted basic fuchsin stain.
b. The reaction between aldehyde and Schiff's reagent and the possibility of a similar or comparable reaction between pararosaniline and aldehyde directly is demonstrated in vitro both with formaldehyde and with hydrolyzed DNA. Whether this reaction occurs in the intact cell has yet to be proved.
c. The action of aldehyde in binding the amine groups of the nucleoproteins is also considered as a possible action of formaldehyde on tissue.

D. Fixation:
a. Fixation must be considered an integral part of a cytochemical method, as it may markedly influence the results obtained.

At present it is possible to accept the Feulgen nucleal reaction and the periodic acid-Schiff stain as specific cytochemical methods, provided the conditions of fixation and hydrolysis are constant and controlled. The aldehyde-mordanted basic fuchsin stain cannot yet be considered as a specific cytochemical method, but must wait upon the further analysis of the reaction of formaldehyde upon tissues and a clarification of what radicals within the cell can react directly with the para-amine groups of the dye molecules. Even in the absence of more specific information on the chemistry of this latter method, it still constitutes a useful cytologic procedure, especially for the study of certain pathogenic and other microorganisms.

The complexities of the problems involved in such studies are apparent. It is obvious that highly specialized knowledge of the chemistry of the dyes used in biological and medical problems is necessary. Knowledge of the reactions of which they are capable, of the influence of solvents, of the influence of pH, and the influence of various intracellular components or systems is all necessary to the development of modern cytologic and cytochemical studies of a critical nature. The development of spectrophotometric methods, both macroscopic and microscopic, makes approach to these problems more direct. However, the necessity for a high order of critical judgment and the need for proper and adequate controls remains.

It is apparent that these methods are all useful in the study of various diseases of the skin. The studies of Pollister (26) and his associates demonstrate nicely how such methods can be developed for critical quantitative determinations of specific intracellular substances in both health and disease.

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DISCUSSION

DR. THEODORE CORNBLEET: The Feulgen reaction which is a stain for desoxyribonucleic acid, may turn out to be an important tool in studying the evolution and changes in the epidermis. With exceptions in pathological states, only the nuclei are stained, whereas related material ribonucleic acid present in nucleoli, cytoplasm and richly in keratohylin is Feulgen negative. Ultraviolet microscopy shows the different nucleic acids and thus by subtracting what is Feulgen positive from the total shown by ultraviolet light, there is thus obtained an indication of the ribonucleic acid.

Curiously, this subject has been developing by accident with investigations of polysaccharides found in connective tissue ground substance and in the epidermis. The McManus and Hotchkiss reactions could be conveniently discussed in conjunction because they, too, depend on the presence of aldehyde groups as does the Feulgen. The former are nonspecific because they can be obtained at different sites. However, for the dermatologist they may come to have much significance in the study of fibrinoid degeneration states as seen in lupus erythematosus. There is present here probably, a depolymerization of the polysaccharides of the collagen. We are attempting to show this by the dye substance, Evans Blue. The polysaccharides take up the dye in proportion to their degree of depolymerization. We are testing this in the dermatological so-called collagen diseases.

DR. R. L. MAYER: The Feulgen method for staining nucleic acids has many applications in dermatology. Since studies on nucleic acids and their significance in genetics, virology etc. have become more and more important, the comparison of different specific staining technics for nucleic acids and derivatives are valuable. I have developed a new nucleic acid stain using oxidized p-phenylenediamine, especially quinone diimine, and have been able, as shown in the following slides, to selectively stain the bands of chromosomes. Like quinone diimine, other quinone compounds give characteristic pictures.

DR. STEPHEN ROTHMAN: Dr. DeLamater has clearly pointed out that the demonstration of polysaccharides by the McManus method is based on oxidation of carbohydrate groups to aldehydes and on the staining of these latter groups by the Feulgen-Schiff aldehyde reagent. Of course, such a reaction is not specific for any polysaccharide. However, Dr. Stoughton in his presentation tomorrow will show you that it is possible to differentiate with this stain such polysaccharides as glycogen, hyaluronic acid, and other mucoid sugstances by preliminary treatment of sections with specific enzymes.

Closing discussion by DR. EDWARD D. DELAMATER: I wish to thank the discussors for their comments.

I did not have sufficient time to emphasize that the specificity of the reactions depend largely upon the type of hydrolysis utilized. If we were to look for other compounds to be utilized as hydrolytic agents it would be found, I am sure, that these too would be specific for specific types of compounds.

As I intimated in the beginning, this is a developing subject. We are really just in the beginning of experimentation, not only in its development, but also in its applications, so that we now have opportunities to develop and direct it for specific purposes. In connection with Dr. Cornbleet's comment, the recent work of Pollister in New York, lupus erythematosus shows that the basophilic substances in this disease are apparently of desoxynucleic acid.

In reply to Dr. Mayer, I would like to say that probably in these very beautiful slides which he showed the banding is mainly due to the presence of concentrations of this oxyribosenucleic acid. Whether the bands themselves are genes or only the loci of genes is not yet clear. It may well be significant that Dr. Mayer's dyes, which are carcenogens, have affinity for the Chromosomal bands.