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Nile Blue Staining of the Prostate Gland of Living Mice

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This paper is submitted to the faculty of Ursinus College in partial fulfillment of requirements for Departmental Honors in Biology.

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Work has been done fairly recently in trying to determine exactly what effect Nile blue BB has on the tissue of living animals. Lewis, Sloviter, and Goland have done a great deal in regard to the effect of blue staining, both vital and supravital, of malignant cells in the thymus, lymph nodes, and testicles of mice, while Cramer and Horning, and later Tobin and Brinbaum, in respect to the effect of Nile blue dyestuff on the adrenal glands of mice receiving it in their diet. It was found that the malignancies (sarcoma, carcinoma, and lymphoma) when stained are characterized by diffuse coloration of the entire cell rather than by staining of concrete granules within the cells. There are, however, a few exceptions to this for certain granules in the adrenal glands, the intestinal mucosa, the kidney tubules, the prostate gland, and in some macropages, do become stained blue, although the granules that become colored following administration of Nile blue are not the same as those that

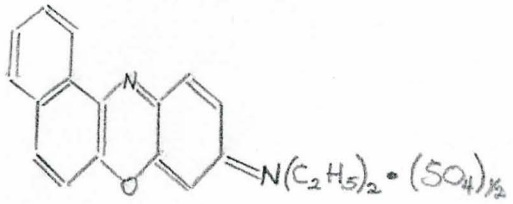
stain with vital dyes such as janus green, neutral red, methylene blue, and trypan blue. For example, granules in cells of the islands of Langerhans become deeply colored with neutral red, but not with Nile blue.

My particular project deals with the effect of Nile blue on the prostate of the mouse. As was stated before, the prostate is vitally stained by this dye. However, as yet, no one is believed to have determined exactly what these blue prostatic granules are. The ease of solubility of Nile blue has prevented this determination, and so fixation of the dye as such in the protoplasmic blue granule and its chemical analysis has not been accomplished. Rather than attempt to fix the dye, I have tried chemically to analyse the prostatic granules by using reagents which are specific in their effect on cellular constituents and making comparative photomicrographs. By photographing fresh, stained, excised tissue, and comparing such a picture with one of a tissue fixed with a reagent of known testing properties, I hope to find out what is the nature of these prostatic granules.

The Chemistry of Nile Blue Sulfate

Nile blue is a naphthophenoxazine. This class of compound contains one naphthalene nucleus attached to the heterocyclic ring.

a-Naphthylamine reacts with nitrosodiethyl-m-aminophenol sulfate in acetic acid solution to yield Nile Blue A -

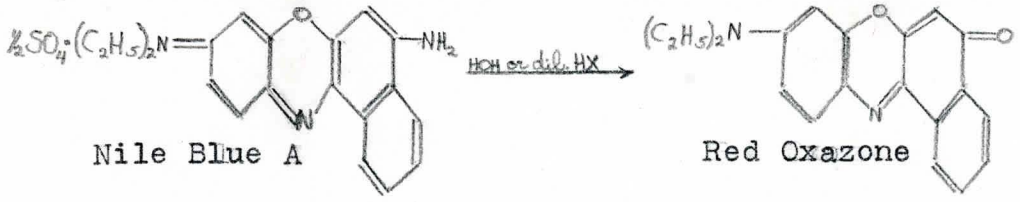


which gives a pure blue shade, little used today. Nile Blue 2B is made similarly using benzyl-a-naphthylamine in place of a-naphthylamine. This is stated to be so sensitive to alkalies that it may be used for testing the resisting power of glass ("Color Index," p. 225).

An important discovery respecting the behaviour of certain coloring matters of this series was made by Lorrain Smith. This pathologist found that when microscope sections containing protein matter mixed with neutral fat globules are stained by, for example, Nile Blue A, the protein matter is colored in the ordinary way blue, but the fat globules become at the same time deep red. This valuable property of causing differential staining was found to be possessed by other coloring matters of the series, such as New Methylene Blue GG, Nile Blue 2B, and Meldola's Blue, although these colors did not exhibit this property in so marked a manner as Nile Blue A. The cause of this phenomenon has been shown to be as follows:

The action of water or of very dilute mineral acid on Nile Blue A causes hydrolysis of the amino-group in

position (6) and yields the corresponding oxazone:



Nile Blue A Red Oxazone

The extent to which this change takes place depends on the length of time during which the acid is allowed to act and on the temperature, but a condition of equilibrium between the salts of the two bases appears to be reached after boiling with water or dilute acid for fifteen minutes.

Both salts are blue, and there is consequently no apparent change in the color of the dye solution, but the salt of the weaker ozazone base is readily dissociated, and the free red base can be completely extracted by shaking the solution with, for example, xylene, leaving the unchanged Bile Blue salt in solution. It therefore follows that when a section is stained in the boiled solution, the protein matter is colored blue by the unchanged Nile Blue present, whereas the neutral fat globules extract the oxazone base from the solution of its salt, and are consequently stained red.

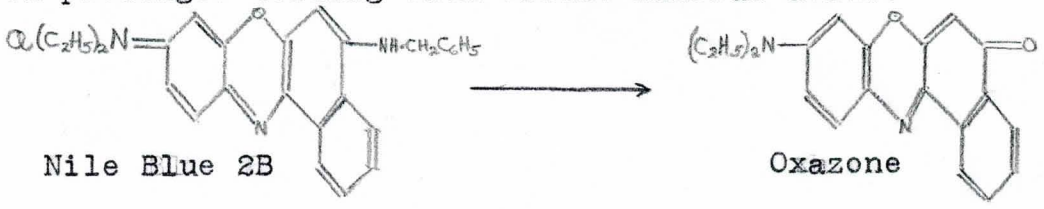
This property can be further illustrated by dyeing wool in the boiled solution of the coloring matter, for the blue-dyed fiber will become markedly purple when washed with water, owing to the formation of the red oxazone on the fiber.

The reason why Nile Blue A exhibits this property to a greater degree than any other member of the series

is because of the presence of the free amino-group in position (6). When the hydrogen atoms of this group are substituted, the formation of the oxazone depends on the nature of the substituting groups. Thus New Methylene Blue GG is transformed into the oxazone almost as readily as Nile Blue A -

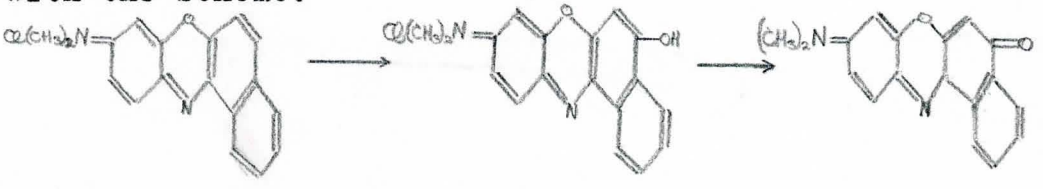
New Methylene Blue GG Oxazone

and this coloring matter can be used for differential staining. On the other hand, Nile Blue 2B is unaffected by boiling water, but is converted into the oxazone on prolonged boiling with dilute mineral acids:



Nile Blue 2B Oxazone

The remarkable reactive character of the position (6) in compounds of this type is also illustrated by the behavior of Meldola's blue, for this coloring matter also produces differential staining after having been boiled with dilute mineral acid. Experiment shows that in this case the oxazone is produced by the oxidation of the hydrogen atom in position (6) in accordance with the scheme:



Meldola's Blue (a) Oxazone

From the fact that the salts of the oxazones are blue,

it is probable that they possess a structure represented by formula "a", and that rearrangement ensues when the salt is transformed into the red oxazone. This assumption is strongly supported by the fact that wool is dyed blue in a bath of the oxazone containing sufficient acid to prevent dissociation, and that when the fiber thus dyed is washed with water it becomes red.

Method

Eight different techniques were used, each in duplicate, in the fixation of dyed prostatic tissue. Various supra-vital stains were employed when it was deemed advisable. The methods used were:

1. To try to bring out ascorbic acid granules with acetic acid - silver nitrate preparations.

Szent-Györgyi ('28) found that the hexuronic acid (ascorbic acid) had reducing properties stronger than other intracellular agents and was capable of reducing silver nitrate in the dark. Since then, Barnett and Bourne, and Dean and Morse have developed a cytological method for the demonstration of ascorbic acid by means of fixation of tissue in an acid solution of silver nitrate. These investigators claim that while other compounds can reduce silver nitrate at neutral and alkaline hydrogen-ion concentrations, ascorbic acid is the only substance in protoplasm that is known to reduce acidified silver nitrate. This behavior is due to the presence of an ene-diol system in the molecule. Since it is a lactone, it may be opened by treatment with an alkali.

The actual technique is as follows:

a. Place tissue in fixing solution in thr refrigerator for one-half ($\frac{1}{2}$) hour.

Fixing solution:

95% alcohol.	50 cc.
Distilled water.	40 cc.
Glacial acetic acid.	10 cc.
Solid silver nitrate	5 gms.

b. Wash with hypo solution for three (3) hours in the refrigerator with several changes of hypo solution.

Hypo solution:

Sodium thiosulfate	20 gms.
Sodium bisulfite.	4 gms.
Distilled water.	400 cc.

c. Wash off excess hypo solution in distilled water and leave overnight in running tap water.

d. Dehydrate starting with 70% alcohol.

e. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

f. Imbed in paraffin.

2. Try to fix Nile blue BB with ammonium molybdate solution in the same way that methylene blue is fixed.

The preservation of the stained tissues involves several problems. Fixation must be rapid to prevent fading,

and such that the dye will not dissolve out during dehydration and clearing. Eight per cent ammonium molybdate (a commonly used reagent) produces a fairly insoluble precipitate of methylene blue, but is a poor tissue fixative. Whether any reaction will occur with Nile blue remains to be seen. Methylene blue is tetramethyl thionin, while Nile blue is a naphthophenoxazine. The chemistry of the two are not alike, but some work being done in England at the present time with molybdate solutions has given me some hopes. Poljak (1941) has developed a number of improved fixing fluids, by combining formalin or dichromate and either acetic or trichloracetic acid with the molybdate. I have used a modification of his molybdate, dichromate formula without acid. One per cent potassium dichromate was found to be compatible with 8% ammonium molybdate for at least twenty-four hours and gave better fixation than molybdate alone (Heller, Thomas, and Davenport). After twenty-four hours, this mixture may precipitate, but tissues that can be stained by immersion would not require more time than this. Obviously, a fresh solution is required for fixation. After a twenty-four hour fixation, tissues were washed for a similar length of time in several changes of distilled water.

Molybdate-precipitated methylene blue is partly soluble in the usual alcohols used for dehydration. Tertiary butyl alcohol (recommended by Levine, 1939) has only a very slight solvent action and was a satisfactory dehydrating agent. It has the disadvantage of

penetrating about half as fast as ethyl alcohol so that dehydration takes longer. Another disadvantage is that it has a melting point of 25.5° C., and must be kept above this temperature to prevent crystallization.

Specimens were transferred from the wash water to full strength tertiary butyl alcohol after the excess water was removed with blotting paper. The alcohol was changed twice during the course of dehydration. Clearing with xylene and embedding in paraffin were handled in routine fashion.

The actual technique is as follows:

a. Place tissue in fixing solution for 2-4 hours.

   Fixing solution: (use a fresh solution)

   Ammonium molybdate. . . . . 8.0 gms.

   Potassium dichromate. . . . . 1.0 gms.

   Distilled water. . . . . 100.0 cc.

b. Wash with several changes of distilled water for twenty-four hours.

c. Dehydrate in pure tertiary butyl alcohol (at 26-28° C.) for three to five days and change the alcohol twice. Since tertiary butyl alcohol has a melting point of 25.5° C., it must be kept above this temperature to prevent crystallization.

d. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

e. Imbed in paraffin.

3. Use of the plasmal reaction to demonstrate acetal lipids - fixation with mercuric chloride and the use of Schiff's reagent.

E. Russell Hayes has redefined the plasmal reaction. It is here modified so that it is made strictly specific for acetal lipids alone. The two essential points in making such specificity possible are: (1) limiting the duration of the action of $HgCl_2$ to 2-10 minutes, thus insuring that neither acid hydrolysis nor oxidation participates in the unmasking; (2) obtaining negative controls upon sections of the same block of tissue, these being treated identically in all respects except that immersion in $HgCl_2$ is omitted. Lipids positive under these conditions are acetals but not necessarily acetal phospholipids. In order to satisfy the second criterion above, material must be either unfixed or fixed for only 1-6 hours in formol. Longer fixation in formol both destroys the acetals and unmasks some non-acetal lipids.

There has been much discussion concerning the histochemical demonstration of carbonyl lipids and fundamentally different opinions have been expressed as to the chemical significance of the methods used. The principal contrasing points of view of American workers are adequately expressed in two recent papers, those of Albert and Leblond (1946) and Wislocki and Wimsatt (1947). Both agree with Gomori (1942) that the plasmal reaction of Feulgen and Voit (1924) is

equivalent to the phenylhydrazine reaction of Bennett (1940). They disagree as to whether the lipids visualized are the plasmalogens (acetal phospholipids) of Feulgen and Bersin (1939) or whether they are to an important extent ketosteroids.

As a necessary preliminary to the study of whether the plasmal reaction and Bennett's reaction are identical, a more rigorously defined plasmal reaction was devised and used. The specificity of this reaction is strictly limited to acetal lipids alone.

Carbonyl reagents do not react directly with untreated sections or smears, meaning that tissues contain no free carbonyl compounds in histochemically detectable amounts. Carbonyl groups must first be freed from some masking linkage before they will react. Two classic examples of this are the nucleal reaction of Feulgen and Rosenbeck (1924), in which short acid hydrolysis releases carbonyl groups from desoxyribose nucleotides, and the Bauer (1933) reaction, in which oxidation makes glycogen reactive to carbonyl reagents. The ultimate reagent used to visualize the unmasked carbonyl compounds has no influence upon the histochemical picture obtained and thus, presumable, none upon the chemical specificity of the reactions. It follows, then, that the specificity of any reaction for carbonyl lipids derives solely from the method used for the unmasking of the reactive products and does not depend upon whether Schiff's reagent, phenylhydrazine or 2, 4-dinitrophenylhydrazine is used.

There are three methods for the unmasking of carbonyl

lipids: (1) brief action of $HgCl_2$; (2) more prolonged acid hydrolysis (or the action of the acidic Schiff reagent itself); and (3) formol fixation. The first two methods are included in the original definition of the plasmal reaction by Feulgen and Voit (1924). The third method derives from the work of Bennett (1940); Gomori (1942) suggests that the mechanism in this case is oxidative.

The striking action of $HgCl_2$ upon the acetal bond, splitting it with the formation of a free carbonyl compound, was fundamental to the discovery of the acetal lipids. This lability of plasmalogen toward $HgCl_2$ enabled Feulgen and Bersin (1939) to recognize that the fatty aldehyde and glycerol of plasmalogen were joined in an acetal linkage. Riesch and Kilpatrick (1935) have described the catalysis of the hydrolysis of the acetal bond by neutral salts, and Meerwein (1927) explains this on the basis of complex formation. Oster and Schlossman (1942) say that $HgCl_2$ acts "more specifically and quicker" than hydrolysis by mineral acids. It is, of course, necessary to establish the lipid nature of the acetals by extraction of sections in organic solvents.

Acid hydrolysis and formol fixation are less likely to have a specific action upon the acetal bond. Hydrolysis by acid undoubtedly breaks acetal bonds to form free carbonyls but it is quite possible that such treatment may release carbonyls from substances other than acetals. Further, since this process takes an

appreciable length of time (1-2 hours), opportunity is offered for other unmasking processes to take place. Oxidation of an acetal bond will split it, but with the formation of an acid rather than a free carbonyl group. Therefore, if the unmasking of lipid substances can be restricted to the action of $HgCl_2$ alone, it is certain that the reactive lipid originally contained an acetal bond. We cannot, however, be certain that these were phospholipids, for the possibility remains that non-phospholipid acetals exist.

Insistence upon two points in the procedure affords assurance that the unmasking is so restricted: (1) very brief action of $HgCl_2$, 2-10 minutes in a 1% solution, to minimize the possibility of the slower actions of acid hydrolysis or oxidation; (2) obtaining negative control sections from the same block of sissue, these sections being treated identically except fro the imersion in $HgCl_2$. Any material that reacts positively in the control sections cannot be known to be derived from acetal lipids.

Schiff's reagent or fuchsin-sulfurous acid is preferred as the ultimate demonstrating reagent. Phenylhydrazine (Bennett, 1940) or its 2, 4-dinitro derivative (Albert and Leblond, 1946) may be used. With these latter reagents, the preparations are not as distinct, although the results as regards distribution and relative intensity of reacting substances are identical to those with the Schiff reagent. The various ways of preparing the Schiff reagent do not influence the histochemical

pictures of the acetal lipids. It is necessary to use a basic fuchsin that is certified for the Feulgen reaction by the Biological Stain Commission, for not all batches of this dye are satisfactory. The best method of preparation is that of deTomasi (1936), using $K_2S_2O_5$. Decolorization is completed with activated charcoal as suggested by Coleman (1938). This results in a colorless solution in which the first hint of returning color can be detected and the solution discarded.

Care must be taken not to allow even traces of Schiff's reagent or $HgCl_2$ to be carried backward in the series, as this can lead to confusion.

Sections mounted in glychrogel do not keep. By taking advantage of the fact that the fuchsin-carbonyl lipid compound is practically insoluble in organic solvents, permanent preparations in Clarite may be obtained. The procedure is to float the section upon a slide and to apply successively 50% alcohol, 95% alcohol, absolute alcohol, and xylene. This is followed by mounting in Clarite. If a light nuclear counterstain is desired in such preparations, which I may not find necessary, methyl green is quite satisfactory.

Any fixation that neither destroys nor itself unmasks carbonyl lipids could theoretically be used. The only method of fixation known to the author of the article on the plasmal reaction that allows negative control sections to be obtained is brief (1-6 hours) fixation in 10% formol. This makes sectioning much more satisfactory but

destroys some of the acetal lipids. Longer fixation in formol will eventually destroy all the acetal lipids as well as unmask positive material in the control sections. The actual technique is as follows:

a. Use unfixed tissues. Sectioning of such material is aided if blocks are immersed in a dilute gum arabic solution for 5-10 minutes and sectioned in a drop of this solution. Most material can be cut at 15-20 u. by this method.

b. Wash in several changes of distilled water or physiological saline.

c. Place one section or group of sections in 1% aqueous mercuric chloride for 2-10 minutes. It seems necessary to allow only sufficient time for penetration to be complete.

d. Transfer these sections and a control group which has not been exposed to the mercuric chloride to separate closed dishes of Schiff's reagent. Sections may be left here as long as the controls remain negative, but 5-15 minutes is usually sufficient for a maximal reaction to be secured.

e. Wash both groups of sections in three changes of sulfurous acid, at least two minutes in each change.

f. Wash in water and dehydrate as outlined in a previous paragraph.

g. Mount in glychrogel or in Clarite.

4. Use of periodic acid and Schiff's reagent to demonstrate glycogen or other carbohydrates.

Periodic acid acts upon the 1, 2 glycol linkage (-CHOH -CHOH-) of carbohydrates in tissue sections to produce aldehyde (RCHO + RCHO) which can be colored with Schiff's reagent. The method can be used on frozen or paraffin sections and is useful as a reaction for carbohydrates of tissues.

In abnormal tissues, it colors many of the "hyaline" materials - amyloid infiltrations, arteriolosclerotic hyaline, colloid droplets, mitotic figures, etc.

The histochemical uses of the periodic-acid-Schiff's reagent (PAS) need careful control because of the possibility of attachment of iodate or periodate to tissue constituents, producing a recoloration of the Schiff's reagent. Whenever possible, the positive reacting material should be further identified by other methods since Lison showed other substances besides aldehydes can recolorize Schiff's reagent.

Carbohydrates and carbohydrate compounds can be demonstrated in microscopic sections of tissue by the action of a solution of periodic acid. The aldehyde formed from carbohydrates by periodic acid is colored with Schiff's reagent as in Feulgen's test, (Feulgen, 1924.) Malaprade (1934) found that periodic acid acted upon 1, 2 glycols (-CHOH-CHOH-) to form aldehydes, and used the method in quantitative analyses of alcoh-

ols. Nicolet and Shinn (1939) demonstrated that aldehyde is formed by periodic acid in 1, 2 glycols even if one hydroxyl group be substituted by an amino group as in the amino acids, serine, threonine, and hydroxylysine. The necessary linkage does not appear in the combined amino acids of tissue proteins while the free amino acids are water soluble with the possible exception of hydroxylysine.

Hotchkiss appears to have been the first to use periodic acid on tissues for carbohydrates. He has made the most complete analysis of the chemistry involved. (Hotchkiss, 1948). The first publication describing the use of periodic acid (McManus, 1946) dealt with the demonstration of mucin. It was mentioned that the reaction colored also "certain cells in the pituitary, colloid of the pituitary stalk and thyroid, granules in some nerve cells in the medulla of the rat and the human intestine and the basement membranes of the tubular epithelium and of the glomerulus." More recently, Lillie has used periodate and nitric acid (periodic acid) as a basis of a similar reaction for reticulum and basement membranes and for glycogen.

The major advantage of periodic acid for use in histochemistry is the known specificity of the reaction upon the 1, 2 glycol linkage. The use of chromic acid in Bauer's (1933) "test" for glycogen was developed in an empirical fashion and the exact reaction involved is unknown, as Hotchkess points out. Similarly the use of potassium permanganate by Casella (1942) and by Lillie

(1947) and of sodium persulfate by Bignardi (1946) to produce substances colorable with Schiff's reagent appear to produce interesting results, the full significance of which is not now apparent.

The demonstration of carbohydrates in tissues depends primarily upon the carbohydrates normally present and their fate in fixation and dehydration. There has been a reversal of feeling on the effect of water on tissue carbohydrates. The studies of Lison (1936) have shown that aqueous solutions need not be avoided in the preservation of glycogen, for example, into sections.

The explanation of the chemical basis for the use of periodic acid in histochemistry has been done most thoroughly by Hotchkiss (1948). Since the 1, 2 glycol linkage (R-CHOH-CHOH-R) is split by periodic acid to form aldehyde (R-CHO ≠ R-CHO) there must be the following requirements to give a positive coloration: (1) preservable material with the proper linkage must be present; (2) the RCHO groups must not be diffusable, that is the aldehyde must be attached to a non-diffusable substance; and (3) concentration of the final aldehyde-fuchsin compound must reach a certain level of concentration in section.

Hotchkiss (1948) introduces a reducing rinse "to remove entrapped or combined periodate or iodate" since either salt will re-colorize Schiff's reagent.

As a final point, I would like to mention that the use of periodic acid in histology and histochemistry is

not yet two years old. The obvious use is for glycogen (Marchese, 1947), and for that reason, its use as been employed here.

The actual technique is as follows:

a. Any fixation may be used.

Fixing solution:

Neutral formalin. . . . . 1 part

Absolute alcohol. . . . . 9 parts

Fix in this solution for 12-24 hours, then put in absolute alcohol for 2-24 hours with at least two changes.

b. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

c. Imbed in paraffin.

d. Paraffin sections to water.

e. 0.5% periodic acid in water for five minutes.

f. Rinse in distilled water.

g. Schiff's reagent for 15 minutes.

h. Rinse in three changes of sulfurous acid, each two minutes.

i. Wash in running water three to five minutes.

j. Stain in Harris' hematoxylin 20 to 30 seconds and wash in running water for 5 minutes. This step may be omitted.

k. Dehydrate in two changes of 95% alcohol.

l. Two changes of absolute alcohol.

m. Clear in xylene and mount in balsam.

5. Try to demonstrate acid phosphatase by Gomori's

method. Gomori uses formalin fixation.

Since the histochemical method for exhibiting acid phosphatase in bodily tissues is said to depend upon the enzyme acting on suitable substrates, it is possible to test it stability by various tests when it is present in tissues. It has been found that the background element or elements, whatever they may be, concerned with the "staining" properties of the reaction are very stable and somewhat resistant to destruction by Lassek (1947).

Since Gomori (1941) introduced his histochemical method for detecting acid phosphatase in bodily tissues, an increasing number of investigators have utilized this approach on normal or pathological tissues of different animals. A number of speculations have been made concerning its possible functional and pathological significance. This method is said to depend on the action of the enzyme, when present in tissues, to split the phosphate radical from a suitable substrate (sodium glycerophosphate and others) contained in a solution having an acid buffer mechanism and lead nitrate. The hydrolyzed phosphate ion is supposed to combine with lead to form lead phosphate at the exact site of enzymatic activity. Subsequent treatment with ammonium sulfide converts any lead phosphate formed to lead sulfide which has a distinct brown coloration. Acid phosphatase, like other enzymes, is thought to be very sensitive to temperature and pH changes and certain other inactivating substances.

The actual technique is as follows:

a. Fix tissue in 40% formalin (commercial) for twenty-four hours.

b. Incubate at 37° C. for one to ninety-six hours in Gomori's solution.

Gomori's solution:

Molar acetate buffer pH 5. . . 30 ml.
Aqueous lead nitrate.(5%) . . 10 ml.
Distilled water. . . . . 60 ml.
Alpha sodium glycerophosphate (52%) in a 2% aqueous solution. 30 ml.

6. Demonstration of nucleoli and plastids with Carnoy fixation and Tannin-iron III staining.

Tannin-iron III, as a technique, differs very little from Salazar's tannin-iron I, and must be considered as a new modification of the latter. But the results obtained are very different and its use in plant cytology will be found very advantageous due to its specificity for certain cellular organs according to Rezende-Pinto (1947). Whether or not this holds for animal cytology remains to be seen.

This specificty arises from the previous hydrolysis in normal hydrochloric acid. The time of hydrolysis depends on the composition of the fixing fluids. In this way, the nucleoli and the "grana" of the plastids acquire a specific secondary tannophyly although they are not at first tannophylic.

The actual technique is as follows:

a. Fixation in Carnoy solution (about 24 hours).

Fixing solution:

Absolute alcohol (ethyl). . . 2 parts

Glacial acetic acid . . . . . 1 part

b. Place in absolute alcohol for 2-24 hours with at least two changes.

c. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

d. Imbed in paraffin.

e. Hydrolysis

Paraffin sections to water.

Rinse in cold N HCl.

Hydrolyze in hot N HCl (58° - 60° C.) for four minutes.

Rinse in distilled water.

f. Place sections in Salazar's acetic tannin in which they must remain for five minutes.

Salazar's acetic tannin:

Dissolve six (6) grams of tannic acid in 10 ml. of glacial acetic acid.

Add 30 ml. of distilled water.

g. Wash in distilled water.

h. Stain sections in 4% iron alum solution for about five minutes.

i. Wash thoroughly in running water.

j. Dehydrate and mount in Canada balsam.

7. Hydroxybenzene compounds as cytoplasmic fixatives. Mitochondria, secretion granules, and other specific types of granulation are well preserved in tissues fixed by this method.

This particular technique (Huseby, 1946) was presented in an abstract, the original paper of which I was unable to secure. However, because of the fact that secretion antecedants and other types of granulation are demonstrated by this method, I thought it would be worthwhile to try it on the prostate gland.

Polyhydroxybenzene compounds are oxidized in an alkaline medium and consequently reduce cellular inclusions, particularly secretion granules which are in a highly active state. In this respect, hydroquinone and catechol mixtures are less effective than pyrogallol or resorcinol. The increased solubility of the latter two may be the reason.

The actual technique is as follows:

a. Keep tissue in fixation solution 6-24 hrs.

Fixing solution:

Pyrogallol is added to 10% neutral formalin in 3.5 to 7% concentrations.

or

Resorcinol is added to 10% neutral formalin in 4.5 to 9% concentrations.

Both mixtures are alkalinized with 1% by volume of 1N NaOH.

b. Dehydrate starting with 70% alcohol.

c. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

d. Imbed in paraffin.

e. Use any stain specific for cytoplasmic granules.

8. Try to demonstrate water-stable mucoproteins by the use of Bismarck Brown.

Bismarck brown has been used for many years as a stain for mucin. But some types of mucoprotein are so water-labile that they cannot be demonstrated by the aqueous or weak alcoholic solutions usually employed. It has been found that Bismarck brown in slightly acidified, strong alcoholic, solution stains mucin. A simple method will be given for using this solution for staining water-stable mucoproteins. Other methods must be used for the more water-labile mucoproteins. By the use of these methods, Leach (1947) has been able to demonstrate a wide range of mucoproteins (mast cells of Hardie, zona pellucida of the graafian follicle, etc.).

Bismarck brown (C. I. 331), also known as vesuvin or phenylene brown, was one of the first azo dyes to be prepared. It is formed by a combination of two molecules of m-phenylenediamine with one molecule of tetrazo-m-phenylenediamine. It was used by Weigert (1878) as a nuclear stain. List (1885) employed it in aqueous or absolute alcohol solution for staining mucin; nuclear counterstaining was obtained with methyl green. Mann (1902) advised a solution in 25% methyl alcohol to minimize the formation of precipitates; he counterstained with hematoxylin. Conn (1940) modified the method of List slightly.

Only by using strong alcoholic staining solutions were Hardie and Wesbrook (1895) able to retain the very water-labile mucoproteins in the mast cells of the villi

of the intestine. List (1885), Langeley (1901) and others in the past emphasized the great lability of muco-proteins to water even after fixation. So it seemed desirable to use Bismarck brown in strong alcoholic solution as did List, rather than in the aqueous solutions more recently recommended. Addition of hydrochloric acid, aluminum chloride, or ferric chloride further increases specificity. Mann (1902) pointed out that it is essentially the addition of alcohol and aluminum chloride which converts hematoxylin and carmine from nuclear to mucin stains.

An alcoholic nuclear stain must be employed when a water labile mucoprotein is to be demonstrated. A modified Weigert's hematoxylin (Weigert, 1904) is known to give a suitable color contrast. It gives the best results when ferric chloride has been used in the Bismarck brown solution.

Mucoproteins vary in their lability to water. Some are quite resistant and may be stained by Bismarck brown without any special precautions. Others show extreme lability and can only be demonstrated satisfactorily by using a technique which emphasizes the avoidance of water at all stages subsequent to fixation.

The actual technique is as follows:

a. Any fixation may be used:

Fixing solution:

Neutral formalin. . . . . 1 part

Absolute alcohol. . . . . 9 parts

Fix in this solution for 12-24 hours, then put in absolute alcohol for 2-24 hours with at least two changes.

b. Clear by replacing the alcohol with a fluid which is miscible with paraffin.

c. Imbed in paraffin.

d. Paraffin sections to water.

e. Rinse in acid alcohol for two minutes.

f. Stain in Bismarck brown solution for ¼ - 12 hours.

Dissolve 1.5 grams of Bismarck brown in 100 ml. of 70% alcohol containing 0.5 grams of ferric chloride. The solution should be filtered after standing overnight. Filtration from time to time is necessary.

g. Rinse in 70% alcohol.

h. Counterstain nuclei with hematoxylin - preferably Weigert's hematoxylin (Weigert, 1904).

Preparation of Weigert hematoxylin stain:

A. Hematoxylin 1% in absolute alcohol.

B. Distilled water. . . . . 100 ml.
Hydrochloric acid. . . . . 1 ml.
Ferric chloride (30%). . . . 4 ml.

Mix one volume of A with 1 volume of B.

Add two volumes of absolute alcohol.

i. Place in tap water until blue.

j. Dehydrate, clear, and mount.

So far as the care of the mice was concerned, they were kept in a large cage about one foot square and bedded with excelsior. The animals were of the C-57 variety, a strain which has not been known to develop cancer spontaneously. Each mouse was given one teaspoon of prepared food daily and as much water as he wanted. The food consisted of regular dog biscuits which I pulverized by hand and introduced Nile Blue BB into in 0.25% concentrations. The dye was first administered on April 8, 1949, and the last mouse was sacrificed on May 4, 1949. Within a day and a half after the first administration of the dye, the urine had turned blue, and about three to five days later there was a noticeable bluish tinge to the ears, paws, and tail of the animals.

Very little work has been done on the prostate of the mouse, and consequently I found nothing on the descriptive anatomy of it. The nearest thing to it was a manual on the rat by Griffith & Farris. They say, "Within the pelvis, and surrounding the bladder are five pairs of organs, relatively much larger than the corresponding structures in man. There are two paris of prostate glands, one ventral, one dorsal to the ductus deferens; Two large hook-shaped and convoluted seminal vesicles, and within the same capsule, along the concavity of the vesicles, are the coagulating glands. Surrounding the ductus deferens close to its opening into the urethra, is the gland of the ductus deferens." This is essentially what I found in the mouse, except that the two pairs of ventral bodies are in reality one fused body of three lobes. This is much smaller than the two anterior dorsal horns.

The histology of the prostate is as follows. The form of the glands is very irregular. Large cavities, sometimes assuming the character of cystic enlargements, alternate with narrow, branching tubules. The blind ends of the secreting portions are sometimes narrower than the excretory ducts. In many places, branching papillae and folds with a thin core of connective tissue project far into the lumen. In sections, they may appear as free, epithelium-lined islands in the cavities. There is no distinct basement membrane, and the glandular epithelium rests upon a layer of connective tissue with dense elastic networks and very numerous blood capillaries. In the larger alveolar cavities it may be low cuboidal or even squamous. In most places it is of a simple or pseudo-stratified columnar variety. The cytoplasm of the cells contains numerous secretory granules - as will be soon shown. The abundant interstitial tissue of the prostate consists of dense connective tissue with collagenous fibers, and elastic networks and many smooth muscles arranged in strands of varying thickness. The connective tissue forms a capsule at the periphery of the organ. Together with the smooth muscles, it is arranged in thick, broad septa, widely separating the glands from one another and radiating from the central region to the periphery. Muscles and connective tissue constitute about one-third or more of the organ. There is an abundance of discrete single muscle fibers arranged in small interlacing bundles. I have been led to believe that this is the only region in the body where this feature is found. The histology of the prostate is essentially the same in man and mouse.

Color pictures were taken of the highlights of the various

aspects of the experimentation. A Leica camera was used along with night kodachrome 135A. The exposure time, lighting, illumination, and magnification varied from picture, but these were all recorded. Most of the exposures were pure guess work so far as the time was concerned, because the light had to be cut down in order to make out the essential features of each specimen. There was not enough light present to register on the light meter. A repeat on the photography will correct many errors that were made - such as they were. Most of the results were quite satisfactory. I would like to thank Dr. Paul Wagner at this time for his kind assistance and advice in this part of the experiment.

Results

The results will be described in the same order as they were in the method.

1. Clusters of black crystals of ascorbic acid seemed to be scattered throughout the section, but appeared to be more toward the middle of the tissue than at the periphery. Connective and muscular tissue areas seemed better supplied with ascorbic acid than did the glandular areas, though it was present in most glands to some extent. When this was so, it seemed to be found only in the nuclei of those cells more remote from the ducts. This nuclear granulation seemed to conform to the linin structure which may indicate that ascorbic acid is in some way connected with nuclear structure as well as function in the prostate.

2. Despite some meager results, I am convinced that Nile Blue BB is not precipitated in the same way that methylene blue is. Slide sections showed slight differentiation

so far as cell outlines were concerned, but no other details. These outlines had a very faint bluish tinge. The cellular contents were virtually translucent and showed no particulars.

The picture of this slide was taken under H. P. (43X), and the Spencer light diaphragm was at 15 at distance of about a foot from the sub-stage condenser. The exposure time was three seconds.

I neglected to mention that the first slide on ascorbic acid had the iris diaphragm at 10 and an exposure time of seven seconds.

Getting back to number two slide again, when the results turned out negative for the Nile Blue fixation, I used a staining method suggested by Masson for mitochondria and other cell contents. The cytoplasm was a light clear brown. No detail was visible. The nuclei contained brownish-black granules. These are mitochondria, and are in about the same dispersion and configuration as those I found using the Altmann stain for mitochondria in a later experiment. Fat cells are a very faint brown while their contents are colorless (the fat, itself). The pockets of prostatic cells are much more vividly stained brown than is the surrounding connective tissue and muscular areas which contain brownish-green fibers with a few scattered brown-nucleated cells.

The photograph of this stained material was taken under oil which gave a magnification of about 540X. The iris diaphragm on the microscope was closed and the shutter on the Spencer light was at 15. The exposure was 25 seconds.

3. In this method, the required hand-cut sections were very unsatisfactory inasmuch as their thickness was not uni-

form - and too thick at that. In addition, so much water crept into the reaction because of the inadequacy of the suggested dehydration method that the results were of no significance. Consequently, I was unable to determine whether acetal lipids were present or not by this technique.

4. This experiment dealt with the presence of glycogen and other carbohydrates.

The entire section was dyed a brilliant fuchsia. However, the prostatic glandular areas were stained much more keenly and distinctly than the surrounding connective and muscular tissues. The glandular cytoplasm was darker than other cytoplasmic areas. All nuclei had clear, large bluish-black granules within them. Each nucleus seemed to have about six or seven granules. The connective and muscular tissue nuclei seemed to be slightly paler than the glandular nuclei. The granules seem to correspond to the location of the mitochondria found in parts 2 and 7.

These results indicate that mitochondria which are believed to be associated with secretion are at least partially carbohydrate in nature - possibly glycogen. This ties in with the known fact that carbohydrates are used in energy transformations physiologically, and the activity of the mitochondria may mean that they are the metabolic center for glycogen or other carbohydrates in the normal cell as well as the secreting cell.

The photograph was taken under H. P. (43X).. The iris diaphragm was shut, the Spencer shutter was at 10, and the exposure was five seconds.

5. The tissue remained colorless in this method which

made use of the Gomori technique in the detection of acid phosphatase. The characteristic brown coloration of the lead sulfide which would have been present had acid phosphatase been in evidence was lacking. This is in apparent contradiction to the human prostate in which acid phosphatase was demonstrated quite clearly in the epithelial lining of the prostatic ducts (alkaline phosphatase was proved to be not present).

No photograph was taken of this.

6. Nucleoli and plastids were demonstrated in this method, although the results were not too satisfactory because the sectioning was too thick. The tissue became a moderate tan with uniform staining throughout. Cell outlines were distinct. Apparently the only nuclear inclusions which took up the stain were numerous nucleoli. Plastids were scattered throughout the cytoplasm. These tannophylic bodies were dark brown. No other inclusions were noticed.

The photograph was taken under H. P. (43X). The iris diaphragm was shut, the Spencer shutter was at 10, and the exposure was twelve seconds.

7. This particular method made use of hydroxybenzene compounds as cytoplasmic fixatives. I used resorcinol. In this fixation, the resorcinol-formalin solution polymerized and formed a rigid gelatinous mass. Slight warming only increased the rigidity. The alcohol used in dehydration seemed to dissolve most of this mass. However, particles of polymerized resorcinol were in evidence under the microscope. The author who suggested this technique (Huseby) made no mention of this polymerizing reaction. However, it did not seem to effect the results too much, because the sections did demonstrate mitochond-

ria with the Masson stain in the same fashion as in part 2. The description is the same.

The photograph was taken under H. P. (43X). The Spencer shutter was at 10 and the exposure time was five seconds.

Using sections fixed the same way, I made use of the Altmann aniline acid fuchsin stain - specific for mitochondria - with methyl green as a counterstain. Here, the cytoplasmic granules were stained very diffusely a light violet. This differentiation is not clear-cut and was of a rather hazy nature. The nuclei were moderately, yet quite clearly and distinctly, stained. The mitochondria and secretion antecedants are well defined as purplish-black granules. Nucleoli were not in evidence. The tissue seemed to be rather uniformly stained except the connective tissue fibers which were a darker purple than the regions they separated. A few mast cells were observed which had rather clear cytoplasmic portions and black nuclei.

The photograph was taken under H. P. (43X). The Spencer shutter was at 10 and the exposure time was five seconds.

8. This technique demonstrated water-stable mucoproteins by the use of Bismarck brown. The cells lining the prostatic ducts seemed to contain a greater concentration of mucoproteins than those cells more remote. These mucoproteins were of a light tannish-brown color. They seemed to be not much in evidence in the cytoplasm, but form small granular clusters in the nucleoplasm. Nucleoli are rather densely stained in many cells. Connective and muscular tissue does not appear to take up this stain especially.

The water-stable mucoproteins which were demonstrated seemed to be in granular form in the nuclei in about the same disposition as chromatin granules generally are found. They may possibly have some influence on the mitotic process either as a source of stored energy or more probably as a constituent of chromatin.

Two photographs were taken under oil immersion (540X). The shutter on the Spencer light was at 10, but the exposure times differed - one was twenty seconds, and the other was forty seconds. The second shot was over-exposed more than the first. Apparently the best exposure time is about ten seconds under the conditions we used. Whereas all other photographs were taken with a concave mirror, these two pictures were taken with a flat mirror.

Some exposures were made also of freshly excised prostatic tissue and the associated seminal vesicle in the hope of noticing some granular differentiation on the surface. The only thing that could be detected was that the prostate, which was colored a pale powder blue as contrasted to the white seminal vesicle, was stained too diffusely to notice any distinct granulation.

Pictures were taken with the Spencer shutter at 30. The magnification was about 5X, and the exposure time was $\frac{1}{2}$ second.

Conclusion

The nature of the blue granules present in living prostatic tissue after the administration of Nile Blue BB is still a mystery. I was unable to get a look at them in their living state and so have something to compare known cellular constituents with. However, I have established and located mitochondria, nucleoli, and

plastids. Ascorbic acid was shown to be present in the prostate in some quantity, as well as glycogen and other carbohydrates, and also water stable mucoproteins. Furthermore, it has been proven that Nile Blue BB is not precipitated in the same manner as is methylene blue. The work on acid phosphatase and acetal lipids was inconclusive.

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