A SURVEY OF HISTOCHEMICAL METHODS FOR THE IDENTIFICATION OF POLYSACCHARIDES AND THEIR DERIVATIVES, AND A DESCRIPTION OF A NEW METHOD FOR USE WITH THE SCHIFF REAGENT

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by

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INTRODUCTION

Progress in our knowledge of the histochemistry of mucopolysaccharides, mucoproteins and mucolipids, has not kept pace with the progress in our knowledge of their chemistry. Sufficient data, however, has been accumulated to indicate their widespread distribution and important role in biological processes.

We shall attempt to survey the histochemical methods available, in order to view the background that led to the development of our new oxidation staining technic, and also the possibility of adapting other methods frequently used in the chemical laboratory, for histochemical studies. This should be feasible when the chemical structure is known. Two excellent reviews were written (Meyer 1945, Stacey 1946) on the chemistry of the polysaccharides indicating the constituents obtained as the identified products of hydrolysis of the carbohydrates. The reader of these reviews soon realizes the fact that the histochemical methods thus far developed do not identify in tissue sections the specific carbohydrate derivatives, or even separate the classes as proposed by these authors.

Methods in use for the histochemical identification of glycogen, carbohydrate-proteins, hyaluronic acid, chitin, 1,2 glycols and their variants are described below.

Glycogen:

Glycogen was one of the first carbohydrates to be demonstrated in tissue sections. Bensley (1939) compared three methods for the microscopic demonstration of glycogen, namely Best's carmine, the iodine method, and Bauer's (1933) modification of the Feulgen technic. Saliva digestion controls were used since none of the three methods described stained glycogen exclusively. Bensley concluded they were

simple staining methods and not microchemical tests. These methods are therefore not specific. Mullen (1944) proposed a convenient and rapid procedure for staining glycogen with Best's carmine, and employed celloidin to hold the deparaffinized sections to the slide. Mancini and Barry (1942) found that the iodine method for glycogen could be made permanent by treating with a dilute solution of mercuric sulphate wherewith the glycogen granules retained a deposit of mercuric iodide. Mitchell and Wislocki (1944) utilized Pap's (1929) ammoniacal silver reticulum method for the staining of glycogen. According to the authors this method compared favorably with Best's carmine and Bauer's methods. However, the drawback of their method, as admitted by the authors, was that in addition to glycogen, the method also stained the reticulum fibers of connective tissue. Many other substances, such as phenols, uric acid, glutathione, vitamic C, etc., may interfere by reducing silver. According to Gomori (1946), his modification eliminates phenols and uric acid. He used chromic acid to liberate aldehyde groups in the carbohydrate, which subsequently reduced ammoniacal silver. Arzac (1947) working in Mexico developed a silver method for glycogen and later Arzac and Flores (1949) reviewed the available silver methods and recommended ammoniacal silver after treatment with chromic acid. None of the technics were thought to be specific. In view of the fact that chromic acid is too severe an oxidant, and that many substances in tissues can possibly reduce silver, there are objections to its use as a microchemical reaction.

Carbohydrate-Proteins:

Another aspect of carbohydrate microscopy is the demonstration of carbohydrate-protein (muco-proteins) complexes in tissue sections. The two early stains for mucin were mucicarmine (Mayer 1896, Dresbach 1947) and mucihaematin (Mayer 1896,

Laskey 1950). Either of these stains, when prepared successfully, often give intense staining of mucin, but the methods are not reliable, and the staining solutions deteriorate quickly at room temperature. The thionin (Mallory 1938) stain for mucin also belongs to this category of stains for mucoproteins. They are all based on the affinity of mucin for the dye by a process of adsorption. Leach (1938) found that if a mixture of equal parts of diacetin and water is allowed to stand for several months, and if sudan black B is present in such a solution, it was found to lose gradually its fat staining properties and instead it stained mucin, in both frozen and paraffin sections. Leach proposed to call the stain mucisudan. Hydrolysis of sudan black B, with acetic acid was found by Leach to produce a solution which also stained mucin. Leach (1947) developed the use of Bismarck brown as a stain for water-stable and water-labile mucoproteins. The stain, which is a combination of 2 molecules of m-phenylenediamine with 1 molecule of tetrazo-m-phenylenediamine, was used in strong alcoholic solution in the presence of ferric chloride. The method was by no means selective in its staining.

The use of metachromatic dyes for the staining of the sulfuric acid containing polysaccharides has been most interesting. The essential idea behind this phenomenon is that, under certain conditions, the mucoid compound acquires a color which differs from the color of the original stain. Various theories have been proposed to account for the metachromatic properties of certain dyes like toluidine blue, methylene blue and other dyes of the quinone-imide group. Kelley and Miller (1935) gave an interesting interpretation of the theory of metachromatic staining. According to them the dyes possess a "dilution shift". In very concentrated, or very dilute solutions, the dye exists in the violet or blue form with varying proportions of

each form existing in concentrations between these two extremes. More recently Michaelis and Granick (1945) made quantitative spectrophotometric studies whereby they proved that the shift from blue to purple was due to polymerization of the dye molecules. The simple form of the dye possessed the normal color (pure blue), whereas the polymers are purple to pink, depending on the degree of polymerization. Alcoholic solutions of the dye show only the blue coloration, therefore when a metachromatically stained tissue is treated with alcohol, the purple form of metachromasia invariably disappears.

According to the extensive work of the Swedish investigator Sylvén (1941, 1945), the metachromatic staining by toluidine blue of mucoid compounds containing polysaccharide esters of sulfuric acid is specific for these compounds, provided that the toluidine blue method of Lison (1935, 1936) is strictly adhered to. According to Glick (1949), Sylvén (1941) made a thorough study of this method. Sylvén emphasized the fact that "false" metachromatic staining can be obviated by the prompt removal of water by alcohol after staining, whereas the "true" staining is assured by the presence of alcohol. In its presence the red stain is characteristic of, and specific for, the polysaccharide sulfates in tissues. The fixative plays an important role in the preservation of these compounds. Holmgren and Wilander (1937) found that 4% basic lead acetate solution was an excellent fixative for tissues to be stained metachromatically. Sylvén used a mixture of basic lead acetate and formalin in order to reduce the period required for fixation. The time for staining was shortened by ageing of the dye, and when more intense staining was obtained the alcohol concentration in the dye solution was not too high. An alcohol concentration of 30% or higher, in the staining solution

brought out the most cell granules beautifully. Hess and Hollander (1947) claimed to have overcome the loss of metachromatic effect while dehydrating in strong alcohols. They ascribed the success of their technic to the change of pH of the staining and washing fluids from acid to alkaline. Out of a variety of fixatives they found that Zenker's solution with acetic acid gave satisfactory results, without the addition of sodium sulfate. They also found that metachromasia was preserved when the sections were treated with 0.5% potassium permanganate. The procedure, however, was admitted to have some disadvantages.

Hempelman (1940) claimed that the two forms of polysaccharide esters of sulfuric acid (chondroitin sulfuric acid and mucoitin sulfuric acid) could be differentiated from one another in tissue sections by means of toluidine blue stain. With extreme dilutions of 1:1,280,000 of the dye in aqueous solution the chondroitin material stained violet-red color, while the mucoitin complex remained unstained at that dilution. In order to explain this differential staining the author assumed that the attraction of chondroitin sulfuric acid for the purple form of the dye was greater than that of the mucoitin complex. In a dilution of 1:410,000 in alcohol, when the proportion of 95% alcohol to water in the solution was 10 to 45, differential purple staining was also obtained. He stated that if the alcoholic content was less than this both chondroitin and mucoitin sulfuric acids would be stained; if it was greater, neither would stain. After staining with dilute solutions of toluidine blue, methylene blue and Goodpasture's polychrome methylene blue, Hempelman reported red violet metachromatic color in (1) the intercellular matrix of adult and embryonic cartilage, as well as chondromas and chondrosarcomas, (2) the walls of arteries with mucoid degeneration

(aorta, pulmonary, renal and umbilical arteries), (3) heart valves, (4) sclera, (5) tendon, (6) intracellular mucus and secretions of the epithelial mucous glands (respiratory, salivary and gastrointestinal including those of benign intestinal polyps and mucoid carcinoma, biliary and pancreatic ducts), (7) Wharton's jelly, and (8) the mucoid substance of egg yolk. This list, according to the author, included practically all of the tissues from which the above substances have been isolated chemically. According to Glick (1949) the claims of Hempelmann's differential method for staining chondroitin and mucoitin sulfuric acids have not been confirmed, and several attempts to do so have failed.

The effect of vitamin C on mucopolysaccharide production in wound healing was studied by Penney and Balfour (1949). The authors attempted to increase the specificity of the metachromasia with toluidine blue by using hyaluronidase, and nucleases. Not only the polysaccharide esters of sulfuric acid were responsible for the metachromasia, but also hyaluronic acid when present in high concentration (Meyer 1947), and nucleoproteins (Wislocki et al 1947). Penney and Balfour were unable to find metachromatic substances in the wounds of guinea pigs depleted of vitamin C, but within twelve hours after intramuscular injection of vitamin C to depleted animals the metachromasia reappeared in the area.

Acid Polysaccharides:

A method for the demonstration of acid polysaccharides of the hyaluronic acid type was proposed by Hale (1946). Tissues were fixed in a dehydrating fixative (Carnoy's fluid) in order to preserve the acid polysaccharides in situ. The deparaffinized sections were hydrated and then treated with an acid solution of dialysed iron. The iron was combined with acid polysaccharides, but not with neutral

polysaccharides or proteins. The combined iron was then demonstrated by means of the Prussian blue reaction. In order to prove the identification of hyaluronic acid Hale used hyaluronidase for the control. Rinehart and Abul-Haj (1951), working from the basic principle presented by Hale, modified the method without using any special fixation, were able to prepare an iron complex which they claimed was more stable, in a more acid solution, than the colloidal iron used by Hale. According to these authors, when it was carried out in the higher acid concentration, the stain proved to be more selective for acid polysaccharides. Hyaluronidase digestion controls were also used in their experiments.

Neutral Polysaccharides:

The neutral polysaccharide found in chitin has attracted histochemists for it occurs in some plants, fungi, animals, and crustacea as skeletal material, where it functions as a highly resistant protective substance. Chitin requires softening for the preparation of paraffin sections. Murray (1937) used a chloral hydrate-phenol reagent for softening chitin. The sections may then be stained by a variety of methods; Zander, Bethe, Mayer, Hoffman and Orton (Lee, 1928, p. 515).

Current methods in use for staining aldehydes after their liberation from the polysaccharides:

Subsequent to the publication of a method by Feulgen and Voit (1924) for the histochemical identification of "plasmalogen" in tissues, carbohydrate compounds have been demonstrated in tissue sections utilizing the Schiff reagent. The two major reactions in these methods are the liberation of aldehyde groups in the polysaccharide chain after the treatment of the sections with an oxidant, and their identification by the purple color they produce with fuchsin sulfurous

acid (PSA). In Bauer's (1933) method chromic acid was used as the oxidant. As Hotchkiss (1948) points out, the aldehyde groups produced by chromic acid are destroyed by further oxidation if incubation is prolonged, notwithstanding the fact that little is known about chromic acid oxidation. Similarly the use of potassium permanganate (Casella 1942, Lillie 1947b) and of sodium persulfate (Bignardi 1940) to produce substances yielding a color with PSA has not yet been worked out from the standpoint of their significance. Lillie (1951) reported a histochemical comparison of the Casella, Bauer, and periodic acid-Schiff (PAS) methods.

The use of periodic acid in histochemistry:

Periodic acid as an oxidizing agent of glycols was introduced by Malaprade (1928, 1934), and its oxidation reactions were reviewed by Jackson (1944). The method was later applied to tissues by McManus (1946), Lillie (1947a, b), and Hotchkiss (1948). Other publications (McManus 1948a, b, McManus et al 1951) dealt with the application of the periodic acid-Schiff technic to the kidney. The same author (1948c) reviewed the histological and histochemical uses of periodic acid and introduced the technic for frozen sections.

Catchpole (1947) using the PAS method demonstrated stainable material believed to be a carbohydrate in the cells of the anterior lobe of the pituitary. Pituitaries were studied in various stages of physiological activity, and preliminary studies showed a glycoprotein material to be associated with the granules of castration cells. In a later report Catchpole(1949) found the PAS positive material in the basophil cells was increased after castration both in female and male rats. It was high in the diestrus part of the oestrus cycle, and decreased progressively through the cycle. On the basis of its solubility properties and physiological correlations, part of

this material was identified as follicle stimulating hormone. He was unable to identify the stainable material that accumulated in the basophils after thyroidectomy. According to Pearse (1949), the method can be used as a histochemical test for the localization of the gonadotrophic hormone, which was found to be present in the basophils and in the "colloid" of both the stalk and the parenchyma of the hypophysis. Similarly Zehnman-Duplessis (1951), applying the PAS technic, came to the conclusion that the basophils in the anterior lobe of the pituitary contained polysaccharides. Laquour (1951) studied the basophilic changes in the hypophysis in diseases of the adrenal cortex. He observed after adrenal insufficiency hypertrophied nongranulated cells which were identified as basophils with the PAS technic. These changes in basophilic activity were interpreted as cytologic evidence of increased function related to an increased output of ACTH as a result of adrenal hypofunction. Conversely, the hyaline basophilic changes in Cushing's disease and after injections of ACTH and cortisone, shown by the PAS positive cells, were associated with high levels of circulating cortical hormone.

Wislocki and Dempsey (1948) using the PAS technic on the placenta found that collagenous reticulum, fibrin and fibrinoid, in addition to glycogen and mucus, were stained. A substance of unknown composition, according to them, was stained in the cytoplasm of glandular epithelium of the endometrium. The acid polysaccharides in the placenta were, however, more readily demonstrated by staining with metachromatic dyes than by the PAS technic. Positive staining material was also found in the normal human and animal placenta and in the cells of chorioepithelioma (Pearse 1949). According to the author this may represent the chorionic gonadotrophin.

A carbohydrate (glycogen) was demonstrated by the use of the PAS method in human blood and bone marrow cells (Wachstein 1949). It was found that the intensity of the staining reaction in the myeloid cells increased with their maturation. Yasuma et al (1951) made a short study on the PAS positive material in blood and bone marrow cells and presented their findings in tabular form.

The use of the PAS technic for the demonstration of polysaccharides in normal and diseased skin was reported by Stoughton and Wells (1950). Normal skin displayed intense coloration at the junction of the dermis and epidermis, in the walls of blood vessels, mast cells, some fibroblasts, senile corium from exposed areas, hair follicles, sebaceous glands and smooth muscle. The mucopolysaccharides of skin were investigated chemically by Meyer and Chaffee (1941). Gersh (1948a) studied the basement membranes in skin, developing kidney, thyroid gland, and lung of normal organs. He found a homogeneous component of the basement membrane, which he thought was continuous with the ground substance of connective tissue. The morphological changes that occurred during histogenesis and inflammation suggested to Gersh that the basement membrane was a highly labile, modifiable structure. A more detailed study was reported later (Gersh and Catchpole 1949).

Gersh (1949a) reported the presence of PAS positive material in the form of granules in the Golgi apparatus of the rabbit and the guinea pig intestine. Arzac and Flores (1952) demonstrated Golgi zones, after fixation in Orth's fluid, by three technics; PAS, chromic acid-silver, and periodic acid colored fuchsin. The three methods gave essentially similar results. He considered discrepancies to be due to the tissue's physiological status and the influence of fixation.

PAS positive material was found in the colloid of thyroid follicles regardless of their state of activity (Gersh 1947). The observation was based on a study of normal and hypophysectomized rats and of rats treated with potassium iodide, thyrotrophic extract of the anterior lobe of the pituitary, or with sulfaguanidine. The author stated that at least a part of the visualized material represented hyaluronic acid. Further details were published later (Gersh 1949b).

According to Marshall and Wakerlin (1949), purified hog renin gave a positive PAS reaction. They observed a correlation between the renin content and the granules containing glycoprotein in the juxtaglomerular apparatus. Solubility studies on both dog renin and PAS positive material in the kidney showed that the solubilities of both substances corresponded.

For a thorough survey of the distribution of PAS reactive material in the adult rat we refer to the work of Leblond (1950), which includes 56 figures illustrating various tissues and organs. The PAS method has been adapted by Kligman and Mescon (1950) for the demonstration of fungi in animal tissues. The technic is being applied routinely in some hospital laboratories.

Arzac (1948, 1950) studied the possibility of replacing leucofuchsin by colored basic fuchsin for the histochemical demonstration of the aldehydes produced after periodic acid oxidation. He found that the colored basic fuchsin gave identical results when it was used in place of its leuco-form. He concluded that it could be used as a reagent for detecting aldehydes in tissues, with certain practical advantages thus rendering the technic less expensive, simpler, and shorter. The exact mechanism of the reaction, however, is not known.

Dempsey et al (1950) observed that, besides revealing polysaccharides in tissue sections, periodic acid also brought about the formation of strongly acid, basophilic groups, which were not identified. The authors, however, suggested that sulfide and sulfhydryl groups might be oxidized to the corresponding sulfonic acids which could account for the increased basophilia of tissue proteins after periodic acid oxidation.

As indicated from an abstract by Lhotka (1952a) studies were made on periodic acid oxidations of tissue sections under varying conditions of time, temperature and concentration.

There appeared in the literature some objections to using PAS technic as revealing polysaccharide structures (Jeanloz 1950). The author questions the validity of the PAS reaction for detecting the presence of adjacent free hydroxyl groups within the chain of the polysaccharide. He stated that "numerous sugars having such groups, such as cellobiose, methyl α-D-glucopyranoside, methyl n-acetyl-α-D-glucosaminide, give a negative reaction". Some strongly reacting substances such as hyaluronic acid and chitin consume only a very small amount of periodic acid (0.1-0.4 mole for each repeating unit), while under the same conditions, starch, glycogen, and cellulose consumed one mole for each pair of adjacent free hydroxyl groups. Lillie (1950) suggested that the PAS reaction should be interpreted as indicating the presence of 1,2 glycols, their amino, or substituted amino variants, without inference as to the mucopolysaccharide structure. Lillie referred to the paper by Jeanloz, but without commenting on it. Wolman (1950) reported that the PAS technic could be used to demonstrate the presence of double bonds of unsaturated fatty acids, and concluded that the technic was not specific for carbohydrates. The report of Wolman regarding his findings with

periodic acid was strongly criticized by Lillie (1952). Lhotka and Davenport (1950) noted a similarity in the staining picture of Foot's (1938) method for reticular tissue and the PAS technic.

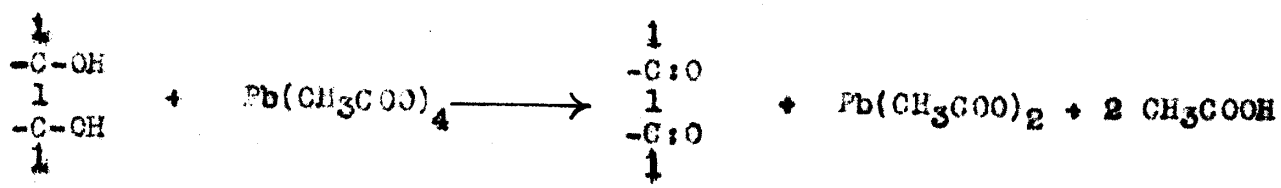
We have been able to develop a technic utilizing lead tetraacetate as a mild oxidant prior to treatment with Schiff's reagent. The work is presented below.

A new method for the histochemical identification of 1,2 glycols:

The fact that lead tetraacetate (LTA), like periodic acid, is commonly used by organic chemists as a specific reagent for 1, 2 glycols, suggested to us the possibility of adopting it for use in a histochemical technic. Criegee (1931) initiated the use of LTA for the study of 1,2 glycols and reviewed the problem (1948). By comparing the oxidative cleavage of 1,2 glycols by various oxidants (manganic acetate, cobaltic acetate, thallic acetate, ceric sulfate and LTA) Criegee et al (1933) concluded that LTA was the superior oxidant for this purpose. The work of Criegee (1934) indicates that LTA and periodic acid react similarly with 1,2 glycols, or variants in which the hydroxyl group is replaced by a primary or secondary amino group, and that the rate of oxidation by each reagent is more rapid for cis- than for trans-glycols. In the same publication Criegee notes that neither oxidising agent is consistently superior, nevertheless important differences exist in individual compounds. LTA oxidizes α-hydroxy acids whereas periodic acid readily oxidizes α-hydroxy aldehydes and α-hydroxy ketones. The two reagents differ also with respect to the kind of solvent in which they function best. Oxidation with LTA can be carried out in inert solvents (Criegee et al 1933), as well as in the presence of water (Baer et al 1939), however water is the most suitable solvent for periodic acid oxidation.

From the standpoint of histochemistry, it would appear that when LTA and periodic acid are used separately they might be of value in a comparative study of compounds yielding to each of these oxidants.

The oxidative cleavage of 1,2 glycols with the formation of aldehydes, which are subsequently stained violet with PSA, may be represented as follows:



MATERIALS AND METHODS

Preparation of Reagents:

1. **Lead tetraacetate.** LTA was prepared according to an adaptation of the methods of Dimroth and Schweitzer (1923) and McClenahan and Hochet (1938). Into a dry 500 cc. Erlenmeyer flask, provided with a mechanism for continuous stirring, was placed 70 cc. of glacial acetic acid and 5 cc. of acetic anhydride. The glass rod stirrer was inserted into the flask through a suitable bored cork covered with a filter paper to prevent excessive moisture from leaking into the flask. The flask was heated to 60°C (55-60) on a water or glycerin bath. Twelve grams of red lead Pb_3O_4, (Malinkrodt) were added to the flask in one gm. portions, care being taken not to add the subsequent portions before the contents of the flask became colourless. After the reaction was complete, the solution was filtered while still hot on a Buchner funnel using suction. This step should be done as quickly as possible otherwise crystals of LTA will deposit on the Buchner funnel. The clear filtrate was allowed to cool at room temperature. On cooling the LTA crystallizes out as fine white needles. Rapid cooling in ice-cold

water should be avoided as it will also cause crystallization of the glacial acetic acid. The supernatant fluid was decanted and a quantity of fresh glacial acetic acid which was just sufficient to make a saturated solution at room temperature was added.

Due to the hydrolysis of LTA in the presence of moisture, and the difficulty of keeping the crystals in a dry condition under vacuum for long periods, a saturated solution in glacial acetic acid at room temperature was found to be practical.

2. LTA standard solutions. The saturated LTA solution was assayed by the iodometric method of Hochet and McClenahan (1939). To 4 cc. of the LTA solution was added 8 cc. of a solution containing 25 gm. of sodium acetate and 2 gm. of potassium iodide per 100 cc. The liberated iodine was then titrated with 0.1 N thiosulfate using starch as an indicator. Dilute standard solutions, whenever needed, were prepared by appropriate dilution of the stock solution with the desired solvent[1]. The normalities of these solutions were rechecked by titration.

3. Schiff reagent and sulfurous acid rinse. These were prepared according to the description by Ritter and Oleson (1950).

4. Ethylene diamine tetraacetic acid (EDTA)[2] solution. A 2% solution of EDTA was prepared with the aid of gentle heat and the requisite amount of 1N NaOH to bring the solution to pH8. This solution keeps indefinitely, but should be renewed when the concentration of EDTA is greatly diminished through repeated use.

5. Enzyme bath. One gram of Taka diastase was dissolved in 100 cc. of a buffered (pH 6.9) solution containing 8 gm. NaCl, 3.3 g. $Na_2HPO_4 \cdot 12 H_2O$ and 0.8 $Na_2H_2PO_4 \cdot H_2O$ per liter.

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1. Glacial acetic acid, water, methanol, ethanol, toluene and benzene.

2. EDTA (Sequestrene) was kindly supplied by the Alrose Chem. Co., Providence, Rhode Island.

Staining Procedures:

After fixation in a 10% solution of formalin in 90% alcohol, the tissues were embedded in paraffin, sections were cut at 5μ, and deparaffinised in xylol. Two methods were developed in which different solvents were used.

Method A. This method is applicable to procedures which involve the use of LTA in glacial acetic acid, or in a mixture of acetic acid and water. (1) Bring sections down to water, and place 5 minutes in the oxidation bath of LTA (prepared at the time of application when water is used as the diluent). (2) Place 5 minutes in EDTA solution. (3) Wash with distilled water, 2 changes. (4) Immerse in Schiff reagent for 20 minutes. (5) Wash with sulfurous acid rinse, 3 changes of 2 minutes each. (6) Wash with several changes of water, dehydrate, clear and mount.

Method B. This method is applicable to procedures which involve the use of LTA in glacial acetic acid diluted with organic solvents such as benzene or toluene. (1) Wash the deparaffinized sections in the same organic solvent used in the oxidation bath, and oxidize for 5 minutes. (2) Wash with absolute alcohol, bring down to water, and proceed as in method A, starting with step 2.

EXPERIMENTAL

It was found desirable to choose a solvent for LTA that would serve such manifold purposes as to enhance the stability of LTA, to replace or reduce effectively the concentration of acetic acid, and to provide a medium in which LTA functions best and acts quickly without any detrimental effects to the tissue. With this as an objective the following experiments were conducted.

Stability of LTA solutions in aqueous and organic media. The criterion of stability of these solutions was based upon iodometric titrations. A stock solution of LTA in glacial acetic acid, kept in a tightly stoppered container, was found to be stable over a period of 4-5 weeks. A solution of LTA in glacial acetic acid, when diluted with an equal volume of water was stable for at least 5 minutes. When stock LTA solution was diluted (1:5) with either absolute methanol or ethanol, the decomposition of LTA was negligible within 5 minutes. After 40 minutes, the ethanol mixture showed complete loss of LTA, whereas with the methanol mixture the decomposition was 14%. The stability of LTA, when toluene and benzene were used as the diluents, is shown in table 1.

Effect of Time of Oxidation on Staining. Tissue sections[3] were placed in either a 0.018 or 0.022N LTA solution, prepared by diluting stock LTA solution with either benzene or toluene, for varying lengths of time (1, 5 and 30 minutes). The results are presented in table 2.

Effect of Concentration of LTA on Staining. Portions of LTA stock solution were diluted according to the following scheme: One series consisted of dilutions ranging from 0.09-0.005 N made with glacial acetic acid. Immediately before use equal volumes of water were added to each and the tissues were stained according to method A. The results are presented in table 3a. A second series of dilutions ranging from 0.035-0.015 N were made with benzene, and a third with toluene. Series 2 and 3 were stained according to method B. The results are presented in table 3b.

Oxidation in glacial acetic acid consisted in placing tissue sections for 5 minutes in an oxidation bath of a saturated solution of LTA. This

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3. For purposes of comparison serial sections of the kidney and intestine of the rat were used throughout these experiments.

Table 1. Decomposition of LTA in a mixture of acetic acid and benzene or toluene

LTA stock soln. plus	Decomposition of LTA in %			
	5 mins.	18 hrs.	4 days	8 days
Benzene	0	0	10	30.3
Toluene	0	3.2	37.5	55.6

Table 2. Effect of time of exposure of tissues to LTA

Diluent	Norm. of LTA	Tissue	Schiff reaction		
			1'	5'	30'
Benzene	0.018	Kidney	±	1+ to 2+	4+ to 5+
		Intestine	±	1+ to 2+	4+ to 5+
	0.022	Kidney	1+	3+	5+
		Intestine	1+	3+	5+
Toluene	0.018	Kidney	±	2+	4+ to 5+
		Intestine	±	2+ to 3+	4+ to 5+
	0.022	Kidney	+	2+ to 3+	4+ to 5+
		Intestine	+	2+ to 3+	4+ to 5+

Staining intensities, in this table and in tables 2a and 2b, are designated as ± to 5+. ± = extremely faint, 1+ = faint, 2+ = fair, 3+ = good, 4+ = slightly overstained, 5+ = overstained.

Table 3a. Effect of concentration of LTA in 50% acetic acid on the Schiff reaction.

LTA norm. in 50% acetic acid	Schiff reaction	
	Kidney	Intestine
0.045	5+	5+
0.023	3+	4+
0.017	3+	3+
0.011	2+	3+
0.003	±	±

Table 3b. Effect of concentration of LTA in toluene and benzene on the Schiff reaction.

Norm. of LTA in benzene or toluene	Schiff reaction			
	Benzene		Toluene	
	Kidney	Intestine	Kidney	Intestine
0.035	4+	4+	4+	4+
0.025	3+ to 4+	3+	3+	3+
0.022	3+	3+	2+ to 3+	2+ to 3+
0.018	1+ to 2+	1+ to 2+	2+	2+ to 3+
0.015	1+	1+	1+ to 2+	1+ to 2+

method has certain disadvantages. Glacial acetic acid is known to cause swelling effects on tissues (McClung and Allen, 1950), and to retard the oxidation rates (Criegee et al 1933), thus requiring a longer time of exposure of the tissue. Apparently the high concentration of LTA compensated for the short period of exposure. However, with prolonged oxidation periods and high concentrations of LTA (beyond an optimal range), the probability of rendering other compounds in the tissues susceptible to the action of LTA, cannot be overlooked.

With regard to oxidation in aqueous media, the oxidation baths consisted of (a) a 1:5 mixture of stock LTA solution (0.125 N) and water. The tissues were placed in the bath for only 2 minutes, which was adequate time for oxidation and served as a precaution against excessive deposition of PbO_2 which is a product of LTA hydrolysis. (b) A 1:1 mixture of standard glacial acetic acid solution of LTA and water, in which the tissues were placed for 5 minutes. A solution of LTA in 50% acetic acid of 0.017 N (0.011-0.023N) was found to be optimal. Fawaz and Seraidarian (1947) found that in 50% acetic acid the reaction was complete in 5 minutes, before the onset of hydrolysis of LTA. Both methods gave satisfactory results, however, the use of LTA in aqueous media is considered a handicap from the standpoint of routine histological technics, since the reagent has to be discarded after use and prepared newly each time needed.

Oxidation in mixtures of acetic acid and toluene or benzene gave optimal results with 0.023 N LTA (0.020-0.026 N), at an exposure time of 5 minutes. Benzene may be recommended for routine purposes since it renders the reagent serviceable for 3-4 days. Inert solvents, as a rule increase the reaction rate (Criegee et al 1933), and thus contribute to the shortening of the time of exposure.

During the initial experiments it was observed that the rinsing of excess LTA reagent resulted in the hydrolysis of the compound with subsequent deposition of PbO_2 on the slides. Furthermore, LTA was found to colorize FSA. According to the Technical Bulletin of the Alrose Chemical Co. Sequestrene (EDTA) is a powerful complexing agent that forms nonionic soluble complexes with a large number of metallic ions. We demonstrated in vitro experiments that EDTA inactivates LTA, as determined iodometrically, and that in its presence no PbO_2 is formed. It has been noted by Sreebny and Nikiforuk (1951) that EDTA solution preserves the structural detail and staining properties of soft tissues.

Effect of an enzyme on LTA reactants. Sections of the liver, heart, kidney, small intestine and spleen of the cat were incubated for one hour at 37°C in Taka diastase solution, prior to treatment with LTA and FSA. Parallel sections, not subjected to the action of the enzyme, were run for comparison. In the enzyme treated sections, staining was prevented in the liver, heart and the cytoplasm of the distal convoluted tubules of the kidney. The amount of stained material in the intestine was not reduced, while that of the spleen was moderately decreased. The loss of staining in the liver and heart muscle can be attributed to the hydrolysis of glycogen. For an exhaustive study of this kind, it is necessary to apply many enzymes that act on specific compounds under certain conditions. A possible control for LTA oxidation is to block the glycol grouping in such a way as to cause it to resist the action of LTA. Boric acid has been extensively used as a blocking agent for adjacent free hydroxyl groups. All these are interesting problems for future study.

DISCUSSION

1, 2 glycols are oxidizable by LTA to form aldehydes or ketones. The reaction may also be applied to α-hydroxy acids, α-amino acids, and α-hydroxyamines. As a guide to histo-chemical interpretation, the following considerations are taken into account.

1. The susceptibility of the particular compound to the action of LTA with the production of aldehydes. Glycols with partially or entirely masked hydroxyl groups are more or less stable to oxidation by LTA.

2. The products of the oxidative cleavage by LTA of certain compounds are either of low molecular weight and hence soluble and diffusible, or of such high molecular weight as to resist dissolution.

3. The chemical or physical nature of the compound; whether it is originally present in solution in the cell of the fresh tissue in which case the possibility of diffusion will come into play when ordinary technics are employed; or whether it is chemically bound to protoplasmic entities thus rendering it fixed, insoluble and non-diffusible.

4. The aldehydes produced must be of such an amount as to meet the sensitivity requirements of the Schiff reagent.

These factors increase the specificity of the method. The monosaccharides and individual α-amino acids are washed out during the process of histological manipulation. The carbohydrate component of RNA and DNA, and the α-hydroxyamines of proteins are chemically substituted so that the free glycol grouping is masked, and do not therefore give the reaction. In the case of the α-amino acids of proteins, their reactive groups are also masked except probably at the end of their linkages, and the aldehydes produced at the end of the chain do not give a detectable color with the Schiff reagent.

The double bond of unsaturated fatty compounds is stable to LTA oxidation. The common practise has been to introduce two OH groups to the double bond by treatment with an oxidizing agent like hydrogen peroxide, and the glycol thus formed is subsequently split by LTA. Scanlan and Swern (1940) hydroxylated oleic acid and oleyl alcohol with hydrogen peroxide. The hydroxylated products, 9, 10-dihydroxystearic acid, ethyl 9,10-dihydroxystearate, and 9,10-dihydroxyoctadecanol were oxidized by LTA to yield aldehydes identified as pelargonic, azelaic half aldehyde and 9-hydroxypelargonic aldehyde. All this goes to show that the double bond of unsaturated fats has to be treated with a hydroxylating agent to yield glycols that can be oxidized by LTA. In this respect it might be of value to take a cirrhotic liver which is rich in ethylenic groupings, hydroxylate it, oxidize it by LTA and stain it with the Schiff reagent.

The chief materials in animal tissues that are expected to react positively are substances such as glycogen, mucins, mucoids, sulfomucins, cerebrosides and chitin. The sulfuric acid esters of polysaccharides will also show the stain because they contain two groups for oxidation in their molecule. (for formula see Lison 1936, p. 236).

The applicability of the method described has been extended to the demonstration of 1, 2 glycols in algae, bacterial capsules, fungi and protozoa (figs. 1, 3, 5, and 7). Further work on this line is in progress. Figures 2, 4, 6, 8 and 9 represent mammalian tissue preparations stained by the technic described in the text.

In conclusion we can state that the adaptation to tissues of oxidation by LTA has been successful in different media. The selection of a medium is governed by such factors as the stability of LTA in the medium, which determines its fitness for routine work, the rate

of the reaction, the solubility of the substance to be demonstrated and the preservation of the finer structural details of the tissue. For the mammalian intestine and kidney we recommend benzene as the medium with a concentration of 0.023 N LTA and a reaction time of 5 minutes.

After this work was completed a paper by Lhotka (1952b) dealing with the histochemical use of lead tetraacetate came our attention, however it only appeared in abstract form.

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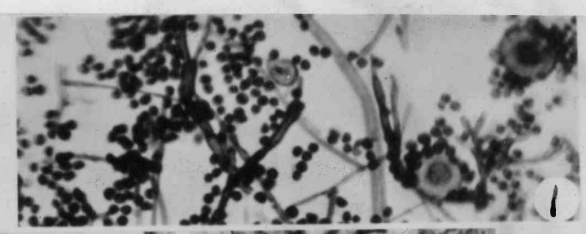
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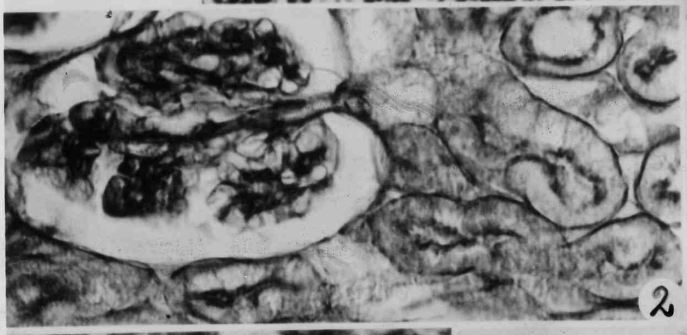
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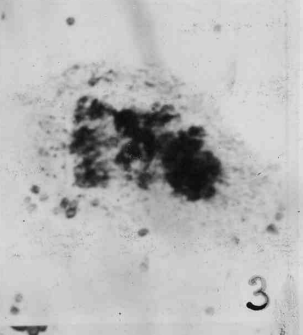
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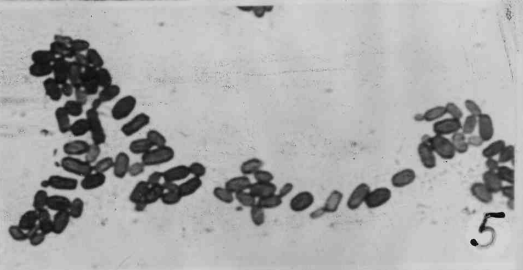
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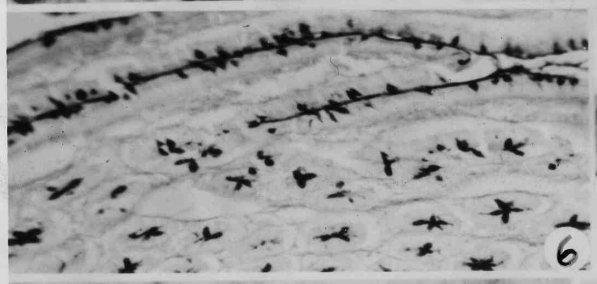
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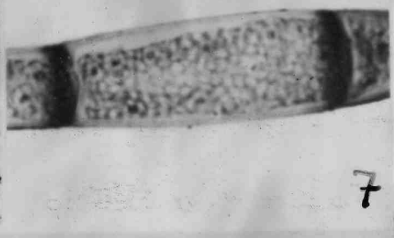
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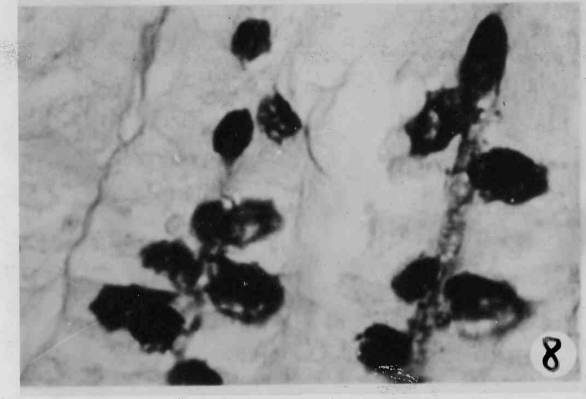
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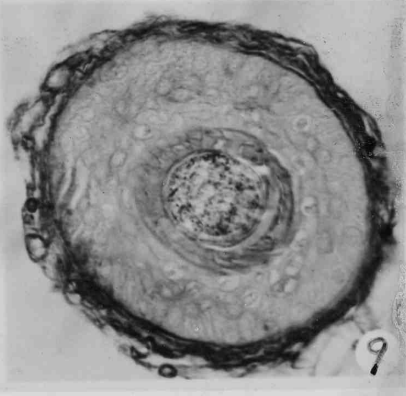
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Explanation of Figures

Following oxidation by lead tetraacetate (LTA) all preparations were stained by the Schiff reagent.

Fig. 1. Fungus (Penicillium). Oxidation by LTA in 50% acetic acid (0.018 N). 500x.

Fig. 2. Kidney of rat. Oxidation by LTA in benzene (0.023 N). 400x.

Fig. 3. Protozoon (Paramecium). Oxidation by LTA in glacial acetic (0.125 N). 400x.

Fig. 4. Liver of rat. Oxidation by LTA in benzene (0.023 N). 600x.

Fig. 5. Yeast (Torulopsis utilis). Oxidation by LTA in 50% acetic acid (0.022 N). 625x.

Fig. 6. Intestine of rat. Oxidation by LTA in 20% acetic acid (0.021 N). 125x.

Fig. 7. Alga. Oxidation by LTA in toluene (0.022 N). 625x.

Fig. 8. Intestine of rat. Oxidation by LTA in 20% acetic acid (0.021 N). 625x.

Fig. 9. Hair follicle of man. Oxidation by LTA in benzene (0.020 N). 300x.

ABSTRACT OF THE ORIGINAL MATERIAL PRESENTED IN THIS PAPER

The oxidative cleavage of 1,2 glycols, or their variants by lead tetraacetate (LTA), with the production of aldehydes which stain with fuchsin sulfurous acid (FSA), forms the basis for the histochemical identification of such linkages.

Sections from tissues fixed in a 10% solution of formalin in 90% alcohol were treated for 5 minutes with LTA in different solvents, the excess reagent being removed with 2% solution of ethylene diamine tetraacetate (EDTA) at pH 8. The sections were then stained with FSA for 20 minutes.

The chemical stability of LTA in different solvents, the effect of its concentration and the time of exposure on the stainability of tissues, were studied. Satisfactory results were obtained with mammalian tissues, algae, bacteria, fungi and protozoa. Some of the preparations obtained by using the technics described are illustrated in an accompanying plate of photomicrographs.