1. On the PAS-positive, Fat-insoluble Non-carbohydrate Components in Tissues

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An attempt was made to examine more in detail the presence of PAS-positive, non-carbohydrate components in tissues. A formalin-fixed deparaffinized section is deaminated with a 10 per cent aqueous solution of chloramine-T at 37°C for 1 hour and, after washing, oxidized for 60-90 minutes in performic acid-8 vol. formic acid, 31 vol. 30 per cent H₂O₂ and 0.22 vol. conc. H₂SO₄: the section is washed and treated with saturated p-nitrophenylhydrazine in N-HCl at 4°C for 2 hours and then stained by the Lillie-type PAS method. The results are shown in the following table.

<table>
<thead>
<tr>
<th>Material</th>
<th>Ceroid Lipofuscin</th>
<th>Macrophages Kupffer cells</th>
<th>Eosinophil leucocytes, Eosinophil mononuclear cells or myelocytes, and Megakaryocytes</th>
<th>Melanosis pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS</td>
<td>++</td>
<td>+ ~ ++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAS after the above-mentioned pre-treatments</td>
<td>++</td>
<td>-</td>
<td>~ +</td>
<td>-</td>
</tr>
<tr>
<td>Performic acid-Schiff (Lillie)*</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Luxol fast blue MBS stain (Pearse)**</td>
<td>±</td>
<td>+ ~ ++</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>


Notes: The following symbols are used: ++ strong positive; + moderate positive; ~ weak positive; - negative. The symbol ± means that some granules appear to be weak positive and occasionally negative in the same section.

From these results it should be emphasized that the PAS-negative substances following the above-mentioned pre-treatments are regarded as being non-carbohydrate lipid or protein complex possessing hydroxy-amino or hydroxy-alkylamino groups, especially protein-bound phospholipids; on the other hand, the PAS-positive substances following the above-mentioned pre-treatments seem to be carbohydrates containing 1:2-glycol groups or fat-insoluble lipid compounds containing reactive groups of carbohydrates.
2. Unspecific Reactions in Acid Mucopolysaccharide Histochemistry

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One of the prominent examples of unspecific staining reaction in acid mucopolysaccharide histochemistry is inability of ordinary methods to demonstrate acid mucopolysaccharides electrostatically bound to proteins. The typical example is acid mucopolysaccharide present in collagen bundles. The author used urea to break the intermolecular bonds and reveal the masked acidic charges. Fresh tail tendon excised was treated in the mixture of 3 ml of 8 M urea and 1 ml of 1% cetylpyridinium chloride at 58°C for 4-16 hours. After short wash in water, it was fixed in 10% neutral formalin containing 1% cetylpyridinium chloride and was processed to make paraffin sections. By staining with Scott's alcian blue in 0.4 M MgCl₂, the acid mucopolysaccharide was thickly demonstrated between denatured collagen fibers. The control section which was not subjected to urea pretreatment did not show any such acidic material. By the same treatment, acid mucopolysaccharide was demonstrated in collagenous tissues such as in atheromatous plaque, healed periarteritis nodosa, and senile skin.

Another interesting results obtained was urea removed the PAS positive mast cell granules rendering the granules intensely alcianophilic. Cell nucleus was also made stainable with alcian blue after urea treatment. These results strongly suggests both mast cell granules and nucleoproteins are susceptible and removable by urea treatment and these are present as electrostatic complex with polyanions such as heparin and DNA. Considering the fact that the stainability of these polyanions with small molecular dyes such as toluidine blue, methylene blue and neutral red is less influenced by urea treatment than the stainability with larger basic dyes such as alcian blue and colloidal iron, the urea susceptible proteins seem to be loosely combined with the polyanions so as to permit smaller dyes to contact with negative charges of these polyanions. This seems to explain the high specificity of larger basic dyes such as alcian blue and colloidal iron.
3. Erratic Reactions in Enzyme Histochemistry, Particularly Negative Erratic Reaction of Enzymes

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In enzyme histochemistry, there are two types of non-specific erratic reactions. One is a positive erratic reaction and the other is a negative. In the former, 1) diffusion of enzyme, 2) diffusion of reaction products produced in the histochemical procedure, 3) absorption of enzyme, and 4) absorption or stain of reaction products were already discussed in the literatures. The auto-substrate enzyme reaction in which the intracellular enzyme activity may act the autosubstrate in cells itself was also previously discussed by Takeuchi. In this occasion, some problems in these phenomena are newly discussed in electron-microscopic level, using metal precipitation techniques and synthesizing technique by Takeuchi.

The more important problem in enzyme histochemistry is a negative erratic reaction in the histochemical procedure. Although the enzyme activity in cells must be detected by the histochemical procedure, it does not often succeed under certain conditions. Here, these conditions for negative erratic reaction were newly discussed in light microscope and partly in electron-microscopic levels: 1) detachment of soluble part of enzyme, 2) damage by fixation with various fixative materials, 3) autolytic phenomena concerning the temperature and time, 4) unsuitable selection of a best condition in the procedure, 5) Unsuitable selection of visualization in the procedure, 6) erratic control by chemical substances such as inhibitors or inactivating factors and 7) Dysfunction of hormonal regulation. These factors were analyzed by the author's own experiments using phosphorylase and UDPG-glycogen glucosyltransferase.
4. Nonspecific Reaction in the Metal Salt Method

—With Special Reference to Ornithine Carbamoyltransferase—

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Metal salts have been widely employed in enzyme histochemistry as capture reagents and their usefulness and pitfalls have been discussed by many authors. A new Gomori type method for the demonstration of ornithine carbamoyltransferase (OCT) activity, reported at this meeting, also contained complicated problems. The specific OCT activity was demonstrated in the mitochondria of the hepatocytes only, but not in other cells of the liver or kidney of the rat, mouse and guinea pig. The following factors, however, were indicated to be considered; 1) lead ions nonspecifically combined with cellular components, 2) nonenzymatic hydrolysis of carbamoyl phosphate, particularly concerning lead-catalyzed hydrolysis, 3) enzymatic hydrolysis of carbamoyl phosphate independently of L-ornithine, such as by acid and alkaline phosphatases. Control experiments and quantitative assays could excluded these nonspecific stainings, for instance in lysosomal reaction, and supported the specificity of mitochondrial activity.

5. Nonspecific Reactions in an Enzyme Ultracytochemistry

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Reale and Luciano (J. Histochem. Cytochem. 15, 413, 1967), who pointed out differences in the localization of alkaline phosphatase activity in the renal proximal convoluted tubule among various authors, concluded that the variability of the results is due to the effect of fixation. However, they not only used the two step method (Ca-Co or Ca-Pb method) which is inferior than the one step method, but also didn't perform substrate-free or inhibitor studies.

Attempts were made to elucidate an exact nature of the variability concerning the localization of alkaline phosphatase activity in the renal pro-
ximal convoluted tubule in the rat. The lead citrate method (Histochemie 11, 88, 1967) was applied to fresh or fixed blocks as well as frozen sections (40 μ or less) from fresh or fixed blocks. Fixatives used were glutaraldehyde (1/2, 1 and 2 hrs) and formaldehyde (2 and 6 hrs). Inhibitors such as EDTA (5-10 mM) or NaCN (5-10 mM) were also incorporated in the medium.

In blocks the variability concerning the localization of enzyme activity was observed as reported by Reale and Luciano, although the zonal distribution was not so evident. Whereas, in sections the enzymatic activity was positive only in the brush border in all the proximal convoluted tubules in the field. Sections incubated in the substrate-free medium as well as the media containing inhibitors were entirely free of deposition, however, nonspecific deposits were encountered in cells in blocks treated similarly.

It is concluded that the variability of the results is due primarily to the use of blocks per se which caused difference in the rate of penetration of constituents of an incubation medium. It is not unlikely that sections (supposedly 40 μ) used by Reale and Luciano were in actuality thicker than 40 μ, creating the blocklike nature. Additionally, the two step method used by them might have aggravated the non-enzymatic deposition of metals in the tissue.

6. Electron Microscopic Cytochemistry of Adenosine Triphosphatase

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Adenosine triphosphatase is one of the most important enzymes which are widely distributed in the plasma membranes, mitochondria, and other biological membrane systems. The nature of this enzyme is not uniform among a variety of membrane systems, as being activated by Mg²⁺, or Mg²⁺ + Na⁺ + K⁺, or Ca²⁺. Furthermore, many other enzymes, which liberate inorganic phosphate from ATP, or ADP, or AMP, exist in some membrane systems. An electron cytochemical reaction using ATP as substrate, however, appears in general only on the plasma membranes of a variety of cell types. In order to clarify the specificity and nonspecificity of this reaction, cytochemical electron microscopic observation and biochemical assay of ATPase activity have been made on the membrane fractions isolated from rat liver cells or ascites hepatoma cells, microvilli membrane of intestinal epithelial cells, and beef heart mitochondria. In unfixed specimens, Mg²⁺-ATPase activity is
high in the plasma membranes and mitochondria, and a part of Mg$^{2+}$ -ATPase in the plasma membrane is activated further by Na$^+$ and or K$^+$. The plasma membrane also contains high 5'-nucleotidase activity. However, most of these activities, except Mg$^{2+}$ -ATPase activity of the plasma membrane, are almost completely inactivated by glutaraldehyde fixation. Non-enzymatic reaction does not occur and the reaction has substrate-specificity in the present condition using histidine-buffer medium (Oda, T.: J. Electron Microscopy, 14, 343, 1965). Data indicate that Mg$^{2+}$ -ATPase in the plasma membrane only can be demonstrated specifically using ATP as substrate with the electron microscope after glutaraldehyde fixation.

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7. Non-specific Staining in Fluorescent Antibody Technique

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The fluorescent antibody method, introduced by Coons et al (1941), employs immune serum globulin labeled with fluorescent dye to locate the corresponding antigen. Its widespread application is clear from the many thousands of publications in this field which have appeared during the last decade. It has been used for the visualization and identification of bacterial, viral, protozoal, helminthic, fungal and tissue antigens, in modified form for the localization of antibody in tissues, and also for the detection and characterization of serum antibody, or various hormones and enzymes. The immunofluorescence technique combines the sensitivity and specificity of immunology with the precision of microscopy; it is complementary to traditional methods and has yielded an information some of which could hardly have been discovered in any other way.

Fluorescent staining of microscopical preparations which is not due to specific reaction between a particular antigen and its corresponding labeled antibody is spoken of as non-specific. It may be caused by unreacted material, or by conjugated serum proteins, or by unwanted conjugated antibodies.

At the same pH, the net charge of the conjugate may be different from that of the native protein; the titration behaviour of the amino acid residue which reacts with the fluorochrome will obviously be changed and any ionizable groups in the fluorochrome molecule may contribute to the net charge.
Differences in the degree of conjugation of one type of protein will give a wider range of net charge than is found with the native material. The net charge of the conjugated protein is an important factor in non-specific protein interaction in the fluorescent antibody method.

Nowaday, by the advantagement of the purification method by DEAE-Column chromatography, the purified conjugates could be used satisfactory when the mol ratio of dye and protein in the conjugates are between 1.0-3.0.

8. On the Non-specific Staining in the Fluorescent Antibody Technique

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Since the fluorescent antibody technique is based on the high specificity of the antigen-antibody reaction, there is no scope for discussion on the specificity of the method itself from the theoretical point of view. But in practice, the non-specific attachment of the conjugated \( \gamma \)-globulin on the tissue occasionally bothers the observation of the precise localization of antigens.

The non-specific staining, in the narrow sense, is a sort of the staining phenomena by the conjugated \( \gamma \)-globulin solution on the site where is not lodged by the target antigen. The condition of the staining should be confined within the range of the optimum circumstance for the antigen-antibody reaction. Therefore, autofluorescence of the tissue components and the fluorescence on the tissue section caused by the attachment of the free fluorescent dye on the tissue, are all excluded from the original category.

Within the limits of the definition mentioned above, the possible nonspecific staining is encountered under the following conditions:

1. Impurity of the antigen used for the sensitization of animals.
2. Possibility of the non-specific antigen-antibody reaction.
3. Heterogeneity of the composition of the \( \gamma \)-globulin.
4. Error in the course of the conjugation procedure.
5. Inadequate purification of the conjugated antibody.
6. Carelessness in the preparation of the tissue specimens, specifically no consideration on the choice of the appropriate fixatives.
7. No control on the condition of the staining procedure. Duration of the staining and the pH of the rinsing medium are worth due consideration.
To eliminate the non-specific staining caused by the erroneous handling of antigen, anti-serum and tissue, repeated control examinations on the side of the anti-serum and the model examinations using agar blocks in place of the tissue are requested, as well as the reconsideration of the counterplan against seven items described above.

9. How to Avoid Erroneous Interpretation in the Electron Microscopic Autoradiography

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In the present symposium, I discussed appearance of various kinds of grains in an electron microscopic autoradiograph (EM-ARG).  
1. Specific reaction products or silver grains reduced by beta rays emitted from a radiation source in the section distribute predominantly directly above the source, but some may, though very rarely, scatter a few microns afar. This is because beta rays are emitted from the atom in all the directions with various velocities. Beta rays with highest velocity emanated from H³-atom may reach as far as 7 microns in the tissue section, although in the nuclear emulsion their trajectory rarely exceeds 1 micron. Therefore, probabilistic consideration is indispensable in interpreting the significance of silver grains in an EM-ARG.  
2. Non-significant silver grains or background grains appear not infrequently. Sometimes they are located on certain ultrastructures to which experimenter pays particular attention to prove the incorporation of the radio-isotope. It is necessary not to select favorable scenes only, under electron microscope, to meet the experimenter's desire. Furthermore, any attempt to remove gelatin from an EM-ARG after the nuclear emulsion is applied to it results in dislocation of significant grains increasing non-significant grains.  
3. Contaminations or non silver grains occur frequently as a result of staining. Sometimes they are present in the tissue section itself. I examined plain, i.e. not autoradiographed, electron micrographs published in a series of textbooks, atlases, and journals and found that small round or ovoid dense particles frequently appear on nucleus, mitochondria, endoplasmic reticulum, cell surface or on any fine structures of the tissue. Sometimes, their close spatial relationship to the ultrastructure, for example attachment to the cell surface, indicates that they are contaminations contained in the resin section itself.
In my EM-ARG, silver grains are developed to assume a shape of comma or of a pair of small dots. Thus, it is easy to distinguish silver grains from non-silver contaminations. It is, therefore, recommended not to suppress development of EM-ARGs beyond this point.

4. Autoradiography on the light microscopic level is much easier and more reproducible. I make it routine to prepare thicker sections of the resin embedded material for light microscopic autoradiography (LM-ARG) when thinner sections from the same block are processed for EM-ARG. Comparison of both LM- and EM-ARG assures significance of silver grains in the EM-ARG.

In Summary, in order to avoid erroneous interpretation of EM-ARG, I suggested,

1. To develop silver grains at least until they are distinguishable from particulate contaminations,

2. To avoid any degelatinization procedure to the emulsion applied on the grid, and

3. To compare EM-ARG with LM-ARG prepared from the same specimen, to exclude incompatible pattern of grain distribution in the EM-ARG.