Histochemical methods have been used since the beginning of the 18th Century as evidenced by the publication of Raspail's "Essai de Chimie Microscopique Appliquée à la Physiologie" in 1830. A somewhat slow development of techniques occurred until immediately prior to World War II when, with the rapid advancements of biochemistry, renewed interest was created. Since 1944, very rapid progress has been achieved in the development of techniques, types of reactions or substances detectable, and the interpretation of results. In 1950, no journals or periodicals were devoted to the science of histochemistry. Today, no less than seven scientific journals are devoted solely to this field. Pearse (1960) lists over 2000 references, of which a large proportion refer to studies reported since 1950.

Histochemistry involves the application of histological-biochemical techniques to demonstrate the presence of enzymes or other chemical substances in plant or animal tissues. The ability of enzymes (or non-enzymic substances) to form insoluble, localized, and readily detectible precipitates at the site of activity (or localization) when reacted with specific substrates or reagents, is utilized. Three primary advantages of histochemical techniques per se are: (1) information can be obtained as to the exact cellular location of biochemically active compounds, (2) the structural and functional cellular integrity can be characterized, and (3) qualitative tests for numerous reactions can be rapidly conducted on small samples of various tissues from single or multiple sources or treatments. The latter is of particular value when used to supplement investigations which are primarily conducted using biochemical methods.

This morning, it is perhaps in order to briefly review some of the principles and techniques involved in histochemistry and indicate the extent of the types of enzymes and substances currently detectable when using these techniques. In so doing, it is hoped that the potential value of histochemistry as a research technique will become apparent. Lastly, a few specific areas, pertinent to the use of these techniques in meats research, will be suggested.

Mechanical Procedures

The relations of the principle methods of microtechnique are shown in slide 1. In routine histology, a sample of fresh tissue would be passed through the following steps as shown on the slide, enroute to the ultimate examination of the tissue under the microscope:

(1) fixation, (2) washing - optional, according to the fixative used, (3) preparation for embedding - dehydration, infiltration, etc., (4) embedding - paraffin or celloidin, (5) sectioning, (6) affixing to slides, (7) staining, and (8) dehydration, clearing, and mounting.
For a few substances, such as glycogen in muscle tissue, a similar procedure would be followed in histochemistry utilizing a histochemical reaction in lieu of the usual staining step in this procedure. However, almost all histochemical methods, particularly those used in detecting enzyme activity, employ a cold microtome or cryostat. The cryostat is basically a microtome contained in an insulated cabinet which has been suitably adapted for operation at temperatures from -12 to -22 degrees Centigrade, and was first developed and used by Linderström-Lang and Mogensen (1938). When using techniques made possible by a cryostat, materials such as enzymes are not subjected to the denaturing effects of the procedures of routine histology.

A prepared tissue sample, either fresh or fixed, is rapidly frozen by immersion in a low temperature liquid, -78 degrees Centigrade (Acetone/CO₂) for most tissues and -155 to -160 degrees Centigrade (isopentane/liquid nitrogen) in the case of muscle tissues. After freezing, the tissues are ready for sectioning with the cryostat either immediately or after a short storage period in the cold. The latter should not exceed 5-10 days if growth of ice crystals and loss of enzymic activity are to be held to a minimum. After sectioning, the sections are affixed to a cover slip; the cover slip and section are then passed through various reagents, including a histochemical incubation media, and finally mounted to a slide for observation with the microscope. In addition to enzyme preservation achieved by the cryostat procedure, the method is also much more rapid than routine paraffin methods.

Types of Reactions

Four types of reactions are used in histochemistry as shown in Slide 2. In each case, a colored or dense precipitate is produced at the site of reaction. The four reactions are:

Slide 2.

<table>
<thead>
<tr>
<th>PRODUCTION OF DETECTABLE PRECIPITATES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. E + S → altered substrate*</td>
</tr>
<tr>
<td>2. E + S → product → complex*</td>
</tr>
<tr>
<td>3. E + S → product → metal salt or complex*</td>
</tr>
<tr>
<td>4. Substance + reagent → substance-reagent complex*</td>
</tr>
</tbody>
</table>

* colored or dense precipitate
E = enzyme; S = substrate

In slide 3, an example of the first type of reaction is given.
In this case, the substrate is a colorless salt which is converted into a colored precipitate at the site of reaction with an enzyme or other specific reducing substance according to the particular salt used. The compound above is not suitable for a histochemical substrate due to the tendency of the colored product to diffuse from the site of reaction. However, derivatives of tetrazolium salts, such as Nitro-ET, provide excellent substrates for a range of specific histochemical reactions.

An example of the second type of reaction is shown in Slide 4.

The reaction shown here is used to detect alkaline phosphatase activity and is dependent on the simultaneous coupling of a colorless salt with the product of the enzyme-substrate reaction at the site of reaction to
form a suitable precipitate. The remaining two types of reactions result in similarly detectable precipitates which are localized at the site of reaction.

**Reaction Mixtures**

To detect enzyme activity, histochemically, reaction mixtures which include substrate, activators and buffers must be used just as in a biochemical assay. An example of such a mixture is shown in Slide 5.

**Slide 5.**

**LEAD METHOD FOR ATPASE**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ml 125 mg.% ATP(di-Na)</td>
</tr>
<tr>
<td>20 ml 0.2 M Tris-HCl buffer (pH 7.2)</td>
</tr>
<tr>
<td>3 ml 2 % Pb(NO₃)₂</td>
</tr>
<tr>
<td>5 ml 0.1 M MgSO₄</td>
</tr>
<tr>
<td>2 ml distilled H₂O</td>
</tr>
</tbody>
</table>

This is a reaction mixture for ATPase (adenosine triphosphatase) after Wachstein and Miesel (Pearse, 1960). Here, magnesium ions serve as activators for the sarcoplasmic ATPase enzyme, the disodium ATP being the substrate. The phosphate released upon hydrolysis reacts with lead ion to form a lead salt at the site of activity. This is subsequently reacted with ammonium sulfide to give a brown metallic precipitate, lead sulfide. In routine histochemical studies, the incubation of some sections in a reaction mixture void of substrate and/or containing specific inhibitors serves as a control in detecting non-specific, or false, reactions.

**Confirmation of Results**

Where possible, it is also preferable to use at least two different procedures for confirming the activity of a given enzyme or the presence of a particular non-enzymic substrate. In the next slide, a method is shown which provides a "double reaction" at the site of activity. This is a modification of the reaction shown in Slide 4. The phosphate released upon the hydrolysis of naphthyl phosphate by alkaline phosphatase is reacted with calcium ions to yield calcium phosphate at the site of the initial reaction. This is converted to a dense precipitate, cobaltous sulfide, by treatment with cobalt and subsequently ammonium sulfide. An insoluble red-blue pigment is also precipitated at the site of activity. In a true reaction, the locations of the two precipitated products will be identical.
Analogous Materials

Certain compounds give similar reactions with some histochemical tests. Consequently, a series of differentiating reactions must be used. An example of such a series, for carbohydrate containing materials, is given in the next slide (Slide 7, Identification of Carbohydrate Containing Materials; after Pearse, 1960, p. 236). The series of substances include (among others) glycogen, starch, glycoproteins, and glycolipids. By selective use of the series of tests listed, it is apparent that any of the substances can be identified. Likewise, another series, using other suitable reactions, must be utilized to differentiate between certain esterases and lipases (Gomori, 1952).

Quantification

Histochemical methods are primarily qualitative procedures and biochemical methods must be used for quantification in most instances. However, a large proportion of the current methodology studies in histochemistry is concerned with the development of quantitative procedures. The most successful methods utilize spectrophotometric procedures (Nachlas and Seligmann, 1950; Doyle, 1950; Doyle et al., 1951). In these procedures, a precipitated colored reaction product is extracted from tissue sections and the concentration of the extracted material is determined spectrophotometrically using a standard curve of known amounts of the product extracted. This procedure can only be used when the reaction product is suitable for spectrophotometric analysis.
Specialized Procedures

Specialized procedures are used in applying fluorescence microscopy and autoradiographical techniques in histochemistry. The application of such procedures in histochemistry has potential usefulness and is consequently an area of current interest. The employment of histochemical techniques in conjunction with electron microscopy is a further area in which suitable methods are being developed. Pearse (1960) includes a general introduction to these areas of study and refers to several pertinent reviews where detailed information can be found.

Metabolic Enzymes

In addition to the use of histochemical methods to clarify the relations of enzymes and other substances to structure, these methods can also be used to observe changes in structure by observing changes in the functional and structural integrity of the cell. Two groups of enzymes which are relevant to this problem can be extensively followed using histochemical methods.

The first group includes certain enzymes of the metabolic cycles, i.e. TCA (tricarboxylic acid), glycolytic, fatty acid oxidation, and hexose monophosphate (pentose shunt) cycles. These cycles, in abbreviated form, are shown in slide 8. In the TCA cycle, these include isocitrate, malate, and succinate dehydrogenases. A side reaction, catalyzed by glutamic dehydrogenase, yielding products utilized in the TCA cycle, can also be detected.

In the hexose monophosphate pathway the activity of two enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate, can be observed histochemically. Three enzymes, whose primary concern is with glycolysis, can be demonstrated. These include alpha-glycerophosphate, alcohol, and lactic acid dehydrogenases. In the fatty acid oxidation cycle, only one enzyme, beta-hydroxybutyrate dehydrogenase, can be demonstrated.

By observing the activities of these metabolic enzymes, it is possible to determine to a considerable extent the general cellular metabolic condition and if irregularities occur, where the breakdown in normal metabolism is present.

Respiratory Enzymes

The second group of enzymes demonstrable by histochemical techniques which give information concerning the structural and functional cellular condition include the respiratory enzymes as shown in slide 9. This is a schematic adaption from Hartree (1957) showing those portions of the electron transport system of interest to the histochemist. These reactions are important in being specific indicators of the state of the mitochondria of the cell. TPNH (reduced triphosphopyridine nucleotide) and DPNH (reduced diphosphopyridine nucleotide) related diaphorase and cytochrome a3 oxidase activities can be determined directly by histochemical methods. Through use of selective inhibitors (amytal, B.A.L., antimycin A, etc.), it is possible to histochemically characterize the other portions of the transport system.
Examples of Histochemical Reactions in Muscle Tissues

In the next few slides, examples of histochemical reactions are shown as detected in striated muscle tissue (lamb, beef, poultry) including (1) leucine amino peptidase, a catheptic enzyme, (2) acid phosphatase, (3) succinic dehydrogenase, (4) sarcoplasmic ATPase, and (5) the periodic acid-Schiff reaction for carbohydrate containing materials. Lastly, as an example of the application of histochemical technique in conjunction with electron microscopy, is a photomicrograph of striated muscle tissue which illustrates the histochemical demonstration of the hydrolysis of thiolacetic acid in rat muscle in the M band (Barnett and Palade, 1959).

Histochemical Methods in Meats Research

In considering some possible areas of meats research in which histochemical techniques are of potential value, an area which immediately is of interest is that of changes occurring in the muscle during rigor. Particularly in reference to beef, several questions can be tendered. Are all of the enzymes of the metabolic cycles active throughout rigor?

Andrews, et al. (1951) reported that succinic dehydrogenase, ATPase, and the overall glycolytic system were active through 2-4 weeks after slaughter in intact beef muscle stored at 2°C. Aldolase, a glycolytic enzyme, was found to decrease markedly during the same period. What is the activity of the other metabolic enzymes during rigor, especially those of the pentose shunt and of the fatty acid oxidation cycle? For how long a period after slaughter is cellular respiration operative, as indicated by the activity of the electron transport system? At what point does the cell membrane actually break down? Do different animals vary in the initial levels and/or rate of decrease in enzyme activity during rigor of the carcass? These are all questions of basic concern, for (in beef) such possible changes or differences may have considerable effect on practical problems such as color and tenderness.

In pork, studies by Bendall and Wismer-Pederson (1962) have shown that watery pork can be induced with specified treatments and that an abnormal accumulation of denatured sarcoplasmic protein on the myofilaments can be detected on the histological level. Are the enzyme activities here comparable to that of the normal or non-watery pork under the same conditions? Are the sarcoplasmic and myosin ATPase activities normal prior to the accumulation of the sarcoplasmic proteins? Is it possible that a breakdown in metabolic or respiratory enzyme activity is involved in some manner? These are all pertinent questions to a histochemical approach to a problem of current interest in meats research.

Likewise, histochemical studies would possibly be helpful in elucidating some of the chemical aspects of muscle tissue in relation to possible structural changes as produced by the use of stilbesterol implantation or feeding. Histochemically detectable changes would be of interest when using other feeding regimes as well, especially if biopsy sampling was utilized during the feeding period.

Lastly, histochemical methods are of value when observing changes in various tissues of experimental animals on specified diets. In meats
research, two areas of interest would be the effects of feeding smoked products and the feeding of animal fats in the diet.

Conclusion

Histochemical methods can be used to indirectly study changes in muscle structure. Of greater importance, such methods enable the research worker to relate chemical changes to muscle structure. This has been a very precursory treatment of a rapidly developing field of study. The techniques involved would appear to provide a potentially useful approach to certain problems of interest in meats research.

REFERENCES


MR. BRISKIY: Thank you very much. The person preparing our next report is the distinguished international scientist, Dr. Fujimaki. He is an associate professor at the University of Tokyo and since he hasn't been introduced this morning I would like to have Dr. Fujimaki stand. We certainly are pleased that he is here.

As you know he is here to work with Dr. Deatherage. The fact that Dr. Deatherage has a reputation to encourage scientists from all over the world to work with him certainly is a credit in itself. Dr. Deatherage, who, as you know is Chairman of the agricultural Biochemistry Department, Ohio State, will give the report which Dr. Fujimaki has prepared.

I might add that the reason for this is, Dr. Fujimaki has only been here a few weeks and doesn't speak the English language too well. We'll hear from him directly.