S. S. College, Jehanabad

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Topic: Histochemical localication of proteins with -SS-groups

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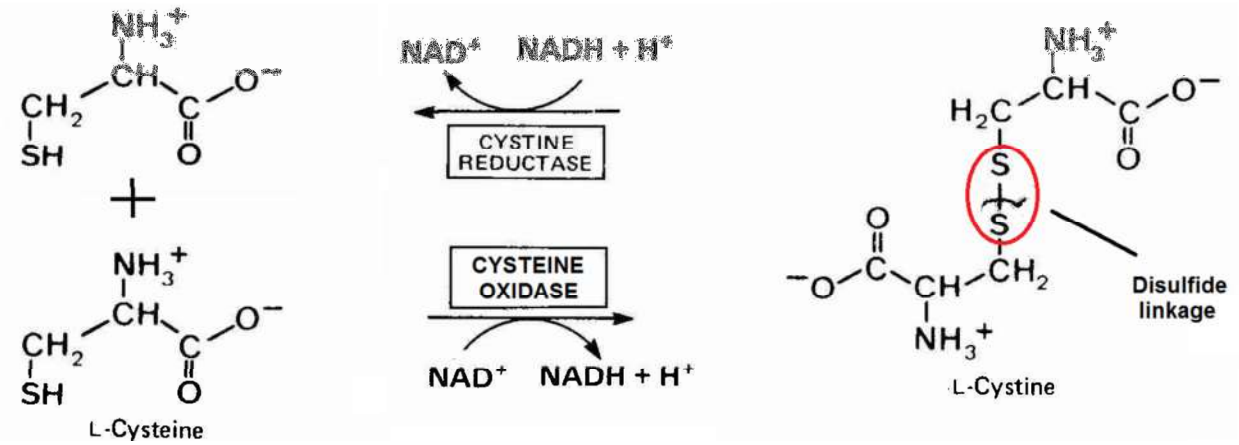
HISTOCHEMICAL LOCALICATION OF PROTEINS WITH -SS-GROUPS

It was **G. Gomori** in the Department of Medicine, University of Chicago in 1955, who found during the course of investigation on the islets of Langerhans by the tetrazolium and the dihydroxydinaphthyldisulfide (DDD) methods that the results obtained by then frequently used both these techniques were not strictly identical. This discrepancy prompted a comparative study of various methods for the demonstration of protein-bound sulfhydryl and disulfide groups. Both sulfhydryl and disulfide groups occur in various proteins which are imparted due to the presence of cysteine or cystine, and methionine amino acid. The sulfhydryl groups can be demonstrated directly by sulfhydryl reagents while the disulfide groups are first reduced to sulfhydryl. Two cysteine molecules are linked together by disulfide linkage make up the amino acid cysteine, which is sometimes listed separately in common amino acid listings. Cysteine is made in the body from serine and methionine and only present in the I-stereoisomer[1] in mammalian proteins.



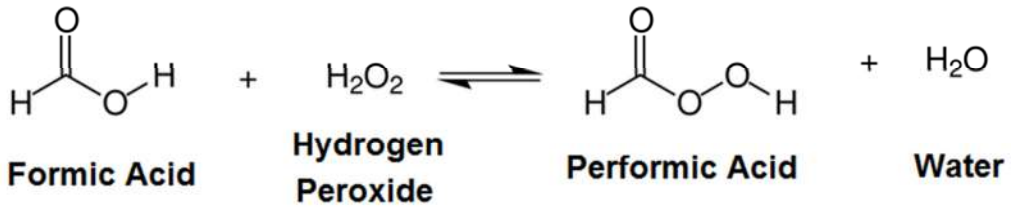
Sulfydryl compounds are quite sensitive to even mild oxidative agents such as exposure to air, and are converted to disulfides. The only fixative which is claimed to preserve sulfydryls is acid alcohol (80% alcohol containing 1% trichloro-acetic acid). This mixture is a poor fixative which has a powerful cytolytic effect (e.g. red cells are largely destroyed; myoglobin is leached out of muscle) and a solvent action on many proteins such as insulin. Whatever sulfydryl is preserved by it is probably only a fraction of that originally present. In addition, an unpredictable percentage of the sulfydryl content will be oxidized in the course of cutting, floating, and drying the sections. For this reason, a positive reaction after fixation by acid alcohol is significant, but a negative reaction is meaningless. On the other hand, mercury salts are very powerful precipitants of proteins and also form insoluble mercaptides with sulfydryl compounds. On account of these features, they are considered excellent histological fixatives, especially in combination with formaldehyde. However, Lugol's solution, used for the removal of mercury precipitates, will oxidize sulfydryls to disulfides. There is every reason to believe that sulfydryl groups produced by reduction in sections of mercury fixed tissues will closely represent the total of sulfydryls plus disulfides originally present in the tissues, provided that reduction is reasonably quantitative, as it appears to be if the right reductant is employed (Mirsky and Anson, 1934-5).

[1] **Stereoisomers** are isomers that have the same composition (that is, the same parts) but that differ in the orientation of those parts in space, and this phenomenon is called stereoisomerism or spatial isomerism.

Histochemical localization of disulfide group containing proteins is achieved by adopting two methods viz. Performic acid – Schiff's method and Performic acid – Alcian blue (PFAB) methods.

Performic acid – Schiff's (PFAS) method

Performic acid (PFA) is an organic compound with the formula CH_2O_3. It is an unstable colourless liquid which can be produced by mixing formic acid with hydrogen peroxide by the following equilibrium reaction;



It is a very strong oxidizing agent. Owing to its oxidizing and disinfecting property, it is used in the chemical, medical and food industries also.

Performic acid cleaves the disulfide bonds formed between two molecules of cysteine amino acids (disulfide linkage of cystine amino acid) into sulfhydryl group of cysteic acid molecules. This also has the effect of converting methionine residues to methionine sulfone. This sulfone group, which is a highly-ionized acid radicle, may be linked to certain basic dyes. Now, if the dye is dissolved in strong acid (pH 0 2), the dissociation of all other "tissue" acid-radicles is suppressed, except sulfate and the sulfone or sulfonate radicle of oxidized cystine. In practice sulfate can be excluded by treating an unoxidized section with the dye alone. It follows that staining seen only in the oxidized section is due to cystine.

In this method, tissue section is preferentially fixed in Formol – calcium solution. However, somewhere other formaldehyde base fixative solution is also taken. After fixation, further tissue processing is carried out as usual.

Equipment

- Slides
- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- Formic acid (CH_2O_2), 90%

- Hydrogen peroxide (H2O2), 20%
- Concentrated Sulfuric acid (H2SO4)
- Basic fuchsin
- Potassium metabisulfite (K2S2O5)
- Hydrogen chloride (HCl)
- 100% ethyl alcohol
- Distilled water
- Activated charcoal
- Xylene
- DPX

Solution

1. Performic acid solution

- Prepared by mixing 40 ml of 90% formic acid with equal volume of 20% hydrogen peroxide.

2. Schiff's reagent soultion

- Prepared by dissolving 5 g of basic fuchsin in 900 ml of boiling water which is then cooled to 50°C and thereafter 100 ml 1N HCl is added and again cooled to 25°C.
- Finally, 10 g of potassium metabisulfite is dissolved in the solution , shaken for 3 minutes, incubated for 24 hours in the dark and then 5 gm of activated charcoal is added before storing to refrigerator.

2. 1 N HCL

- Prepared by adding 3.067 (~3.1) ml HCl (36.46 mg of HCl is equivalent to 3.067 ml of 37.5% HCl) in 100 ml of distilled water.

3. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

4. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

5. Alcohol 80 %

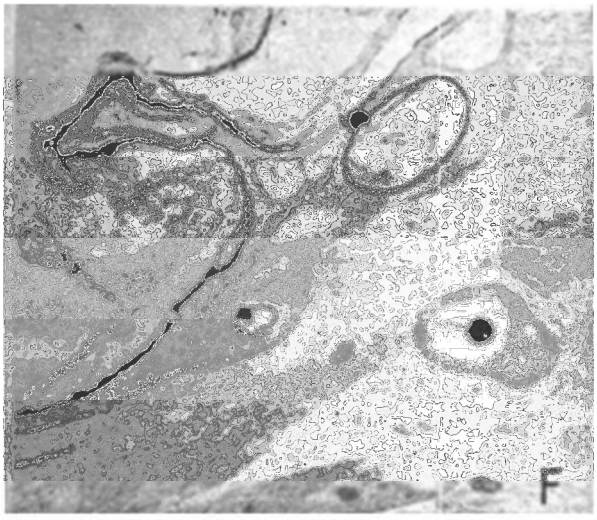
- Reagent alcohol ~80 ml
- Deionized water ~20 ml

Procedure

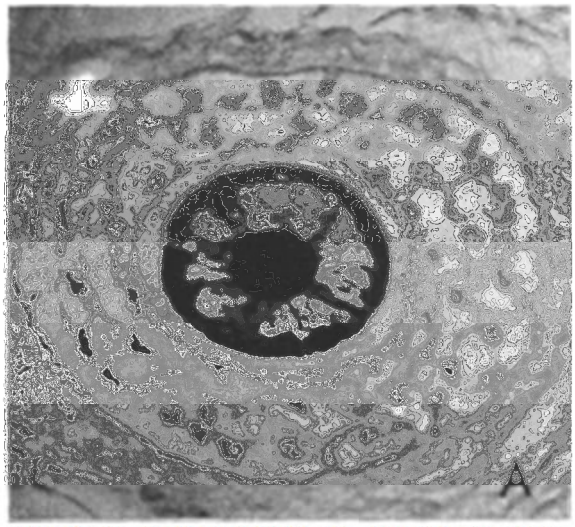
1. Deparaffinize tissue specimen.
2. Hydrate in distilled water.
3. Rinse with running tap water.
4. Treat with performic acid solution with 0.5 ml concentrated sulfuric acid for 30 minute.
5. Wash in running tap water for 15 minute.
6. Place the specimen slide in Schiff's reagent solution for 40 minute.
7. Wash in running tap water for 1 minute.
8. Dehydrate tissue specimen in graded alcohol.
9. Clear with xylene
10. Mount in DPX.

Results

The protein which contain disulfide linkages stain bright magenta to bright magenta red and unsaturated lipids stain red in colour.



Keratin in hair and stratum corneum stained bright magenta- red. PFAS method I. x 75.



The hair shaft and Huxley's layer both stain bright magenta-red. PFAS method I. X 360.

A. G. Everson Pearse, Quarterly J. Microscop. Science 1951; 92(4): 393-402.

Performic acid – Alcian blue (PFAB) method

In this method, generally tissue specimen is fixed in 10% neutral formalin and thereafter dehydration, and infiltration and embedding in paraffin are followed. Paraffin block of tissue is cut with thin section of 4 or 5 µM with the help of a microtome and proceeded for the staining procedure.

Equipment

- Slides

- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- 10% formalin
- Formic acid (CH_2O_2), 90%
- Hydrogen peroxide (H_2O_2), 20%
- Concentrated Sulfuric acid (H_2SO_4)
- Alcian blue
- 100% ethyl alcohol
- Distilled water
- Xylene
- DPX

Solution

1. Performic acid solution

- Prepared by mixing 40 ml of 90% formic acid with equal volume of 20% hydrogen peroxide.

2. Alcian blue soultion

- Prepared by dissolving 1 g of alcian blue in 100 ml of 10% sulfuric acid.

2. 10% Sulfuric acid

- Prepared by adding 10 ml of LR grade concentrated sulfuric acid in 50 ml distilled water pre-filled container through the edge of a glass rod with stirring and then bringing the volume up to 100 ml by adding 40 ml more distilled water.

3. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

4. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

5. Alcohol 80 %

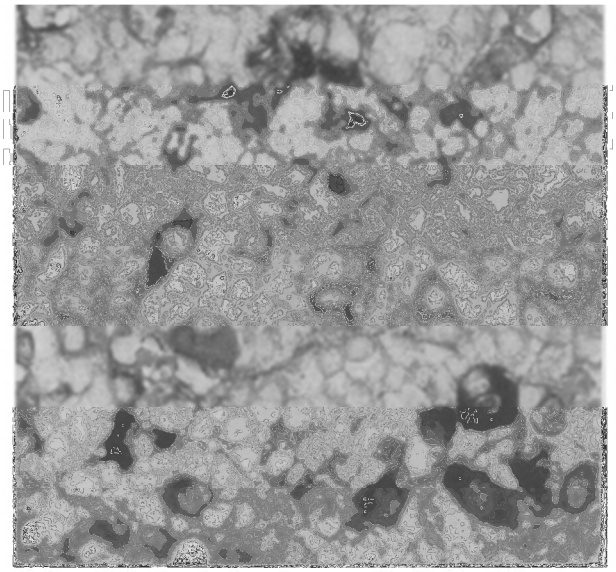
- Reagent alcohol ~80 ml
- Deionized water ~20 ml

Procedure

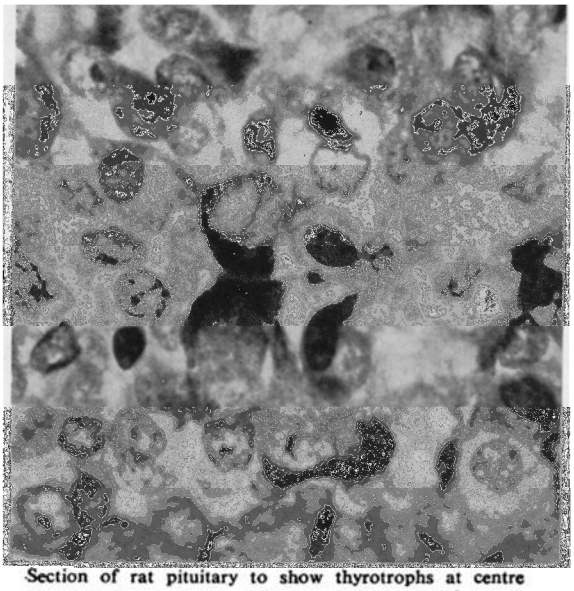
1. Deparaffinize tissue specimen.
2. Hydrate in distilled water.
3. Rinse with running tap water.
4. Treat with performic acid solution with 0.5 ml concentrated sulfuric acid for 30 minute.
5. Wash in running tap water for 15 minute.
6. Rinse in 70% alcohol and blot with filter paper.
7. Again rinse in absolute alcohol.
8. Wash in running tap water for 1 minute.
9. Immerse in alcian blue solution for 1 hr at room temperature.
10. Wash in running tap water for 5 minute.
11. Dehydrate tissue specimen in graded alcohol.
12. Clear with xylene
13. Mount in DPX or Canada balsam.

Results

Protein rich in cysteine amino acid residues is observed as pale blue in colour to dark steely blue.



Section of pituitary to show strong reaction in basophils after pretreatment with buffered performic acid. P.A.S. × 540.



Section of rat pituitary to show thyrotrophs at centre stained with alcian blue. Buffered P.F.A.B.-P.A.S. × 920.

Swettenham K. J. clin. Path. 1960; 13: 256.

Sulfhydryl group in a protein is detected by Ellman's test which utilized nitrobenzoic acid thiobenzoic acid dyes. This test is specially designed for the histochemical analysis of sulfhydryl groups enriched proteins in a specimen.

Ellman's Test (Sulfhydryl Reaction)

This method is used for the demonstration of the presence of a free sulfhydryl group (cysteine) in the unknown or protein sample.

Principle

Free sulfhydryl group reacts with DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid), which gives the product of mixed disulfide and TNB (2-nitro-5-thiobenzoic acid). TNB produced in the reaction gives a yellow color to the solution.

Requirement

Test sample, Ellman's reagent {DTNB+Tris buffer (1M, pH 8) + distilled water), pipette, and cuvette.

Procedure

1. Add 50 µl DTNB solution and 100 µl of tris buffer followed by 840 µl of distilled water in a cuvette (Ellman's reagent). Mix the solution well by the use of a pipette.
2. At last, add 10 µl of the test sample in the same cuvette making the final volume up to 1000 µl. Note: You can also directly use the commercially available Ellman's reagent for this method.
3. Mix the prepared solution well by the use of a pipette and incubate the mixture at room temperature for 5 minutes.
4. Take the absorbance of the solution at 412 nm.
5. Prepare different dilutions of the test sample and repeat the same procedure.
6. For a standard calibration curve, cysteine acetate can be used and the same steps can be repeated.

Reference

1. Gomori G. Histochemical Methods for protein-bound Suphhydryl and disuphide groups. 1956; 97(1): 1–9.
2. Mirsky A. E. & Anson M. L. Sulfhydryl and disulfide groups of ptoteins III. Sulfhydryl groups of native proteins-hemoglobin and the proteins of the crystalline lens. J. Gen. Physiol. 1935, 439–450.
3. Jesse P. Greenstein Sulfhydryl groups in proteins: Egg albumin solution of urea, guanidine, and their derivatives. J. Biologic. Chem. 1938; 125: 501–513.
4. Everson Pearse A. G. The histochemical demonstration of keratin by methods involving selective oxidation. Quarterly J. Microscop. Science 1951; 92(4): 393-402.

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