CHAPTER II

MATERIALS AND METHODS

1. Preparation of tissue for section

1.1 Materials

Liver specimens were obtained from patients hospitalized at Siriraj Hospital, in whom primary or metastatic carcinoma of the liver was suspected. They were taken from needle or surgical biopsies of the liver.

Normal adult livers from the patients without liver disease were employed for controls.

1.2 Chemicals

Xylene	(Mallinckrodt)
Ethyl alcohol, absolute	(Mallinckrodt)
Acetic acid	(May and Baker)
Paraffin pellet M.P. 56-58^{o}C	(Will scientific)

1.3 Glasswares

Petridishes	(Pyrex)
Coplin jars with cover	(Arthur H. Thomas)
Graduated pipettes	(Pyrex)
Measuring cylinders	(Pyrex)
Beakers	(Pyrex)
Glass slides	(Clay Adams)

1.4 Instruments

Surgical blades	(Swann-Morton)
Forceps	(A Dumont & Fils)
Paraffin oven (58°C)	(Precision)
Rotary microtome	(American optical company)
Microtome knife	(American optical company)
Tissue floatation bath	(Arthur H. Thomas)
Refrigerator	(General Electric)

1.5 Methods

1.5.1 Trimming: Cut an appropriately selected tissue not more than 5 mm thick, with a sharp surgical blade in a cleaned petridish.

1.5.2 Fixation: Dropped the tissue into precooled 1 % acetic acid in 95 % ethyl alcohol for 20 minutes, at 4°C.

1.5.3 Dehydration: Dehydrated in 4 changes of precooled absolute alcohol, 20 minutes each, at 4°C. Agitation and suspension of tissue above the bottom of the container accelerated dehydration.

1.5.4 Clearing: Cleared by passing through three consecutive baths of precooled xylene for 1 hour each, at 4°C.

After placing the specimens in the last bath of xylene removed the coplin jar from the refrigerator and allowed to come to room temperature.

1.5.5 Infiltration: To infiltrate the tissue with melted paraffin wax 3 baths, 1 hour each in paraffin oven at 56°C.

1.5.6 Embedding: Embedded in paraffin wax with a low m.p., strictly below 58°C.

1.5.7 Cutting: Paraffin-embedded sections were cut on a rotary microtome, with a sharp cleaned microtome knife, 4 μ in thickness.

The sections ribbon was floated onto the surface of warm water, 45°C in tissue floatation bath, should be as brief as possible, to avoid leaching of antigenic material from the sections. The clean glass slide was half submerged in the water to bring the sections with it.

In each case two slides were cut from the paraffin-embedded tissues. One slide was for immunofluorescent study. The serial slides were stained with Hematoxylin and Eosin (H&E) for morphological correlation.

1.5.8 Incubating: All slides were incubated at 56°C for 30 minutes.

Principal (54-55):

The preparation of tissues for microscopic examination is a histopathological technique. As soon as a tissue is removed from the body or cut off from its blood supply, it begins to decompose. The tissue should be placed in an adequate bulk of a fixative as quickly as possible, to preserve tissue as life-like manner.

Fixation: Fixation is the process by which the constituents of the cells, and therefore of the tissues, are fixed in a physical, and partly also in a chemical state, so that they will withstand subsequent treatment with various reagents without loss, significant distortion, or decomposition. Most fixatives act by denaturing or precipitating proteins, which

then form a sponge or meshwork, tending to hold the other cell constituents. Ideally, a fixative should penetrate a tissue quickly, be rapid in action, be isotonic, cause a minimum loss and minimum physical and chemical alteration of the cell and its components, and be cheap, stable, and safe to handle.

Dehydration: Tissue contain large amount of water, both intracellular and extracellular. This water must be removed. The process of water removal is called dehydration. Dehydrating agents must be water-miscible fluids, and the best agent is ethyl alcohol.

Clearing or Dealcoholization: Clearing is to render transparency of tissue elements, and removal of alcohol prior to embedding in paraffin wax, among excellent paraffin wax solvents are xylene.

Infiltrating: This process involves the impregnation of the tissues with a medium that will fill all natural cavities, spaces, and interstices of the tissues, to hold the cells and intercellular structures in proper relation to each other, and to eliminate traces of the clearing agent.

Embedding: Embed is to support and enclose specimens which are to be subsequently cut into thin sections. Embedding media must be substances capable of being converted from liquid to solid form. In the liquid state, the embedding media penetrates into the interstices of the tissue and is converted into a solid.

2. The procedure for immunofluorescent antibody techniques.

2.1 Materials

Paraffin embedded sections, 4 μ in thickness

Black-and-white panchromatic films (Kodak)

2.2 Chemicals

Fluorescein conjugated alpha-fetoprotein antibody (Behringwerke, Germany)

Sodium chloride (NaCl) (Mallinckrodt)

Disodium hydrogen phosphate ($Na_2H\ PO_4$) (Mallinckrodt)

Potassium dihydrogen phosphate (KH_2PO_4) (Mallinckrodt)

Glycerin, pure

2.3 Glasswares

Coplin jars with cover (Arthur H. Thomas)

Micropipette (Pyrex)

Cover glasses (Resistance)

2.4 Instruments

Incubator (35°C) (Arthur H. Thomas)

Analytical balance (Mettler B 5)

Humid chamber

Fluorescence microscope model BH-RFL (Olympus)

2.5 Method of preparation

2.5.1 Preparation of solutions for staining

1. Phosphate buffer saline (PBS) pH 7.2

Sodium chloride	8.5 g
Disodium hydrogen phosphate	8.62 g
Potassium dihydrogen phosphate	2.48 g
Distilled water to make	1000.0 ml

2. The antibody solution.

Dissolved the lyophilized fluorescein conjugated alpha-fetoprotein antibody 1 g in 1 ml of distilled water. For working solution, dilute 1:5 with PBS pH 7.2

2.5.2 The method of direct immunofluorescent antibody techniques

a. Deparaffinization: Removed the paraffin by placing the slide in three consecutive baths of cold xylene, 2 minutes each, and with gentle up and down motion.

Removed the xylene by gentle up and down motion in three consecutive baths of cold 95 % ethyl alcohol lasting 2 minutes each.

Removed the alcohol by gentle agitated of the slide in three separate baths of fresh cold staining buffer, one minute for each bath. The slide was now ready for exposure to the conjugated antibody.

b. Exposed the slide to the fluorescein-conjugated alpha-fetoprotein antiserum.

c. The slide was incubated for 30 minutes in humid chamber at $37^{\circ}C$.

d. Washed for 15 minutes in a phosphate buffered saline pH 7.2, wiped dry except for the area of the section.

e. A drop of buffered-glycerol (1:10) was put over the

section, and a cleaned coverslip dropped carefully over it, sealed with enamel.

f. Immediately examined fluoromicroscopically, and grade according to the intensity of AFP immunofluorescence.

g. Photography.

Principal (56,57):

Antibodies are coupled via a stable bond to fluorescent dye stuffs (fluorochromes) without undergoing any significant loss in their specific immunological properties. Such a labelled antibody is designated as a conjugate, the latter can react with the corresponding antigen almost as well as the antiserum prior to conjugation.

If a preparation containing the antigen, is covered with a layer of the conjugate, binding of the fluorescing antibody molecules to the antigen takes place. Excess conjugate is removed by washing, and those sites of the preparation where antibody molecules are bound fluorescence on excitation with a suitable light source. This fluorescence contrasts with the dark background, which does not contain the antigen. The immunofluorescence method combines immunological specificity with the sensitivity of histochemistry and the precision of microscopy. It permits not only the demonstration of an antigen-antibody reaction, but also the exact localization of the latter.

Fluorochromes:

Various fluorescent dyes are used for the labelling of antibodies. Derivatives of fluorescein-particularly fluorescein isothio-

cyanate (FITC) are the most commonly used fluorochromes. It has been successfully employed as protein labels.

FITC may be synthesized by reacting 4-nitrophthalic acid with resorcinol to produce 4-nitrofluorescein, reducing this to aminofluorescein by catalytic hydrogenation, and finally converting the amine to isocyanate with liquid thiophosgene.

The diagram is shown in Figure 3.

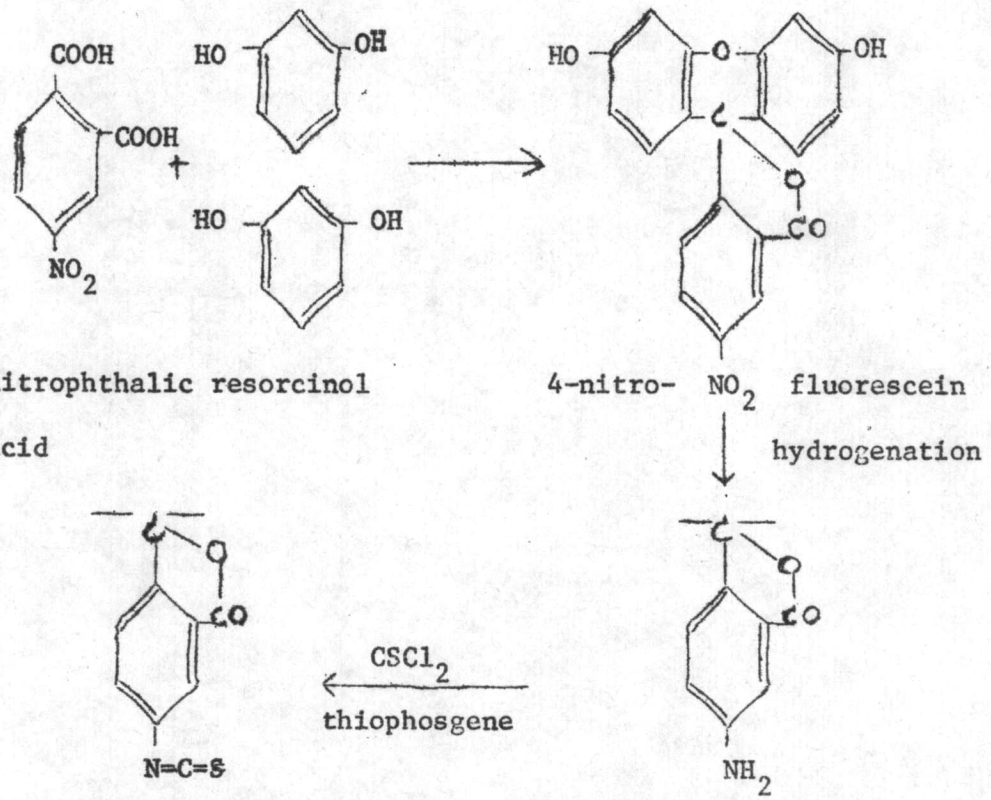

Fig. 3 Synthesis of fluorescein isothiocyanate starting with nitrophthalic acid and resorcinol.

Humid chamber needs to prevent drying of conjugate, is easily prepared by adding wet paper to the covered tray, and incubate in an incubator.

Fluorescent antibody technique

Direct staining:

In the direct test, the conjugate is applied onto the antigen preparation without any other intermediate stage. The antigen is revealed directly by binding to the specific, labelled antibody, as shown in Figure 4.

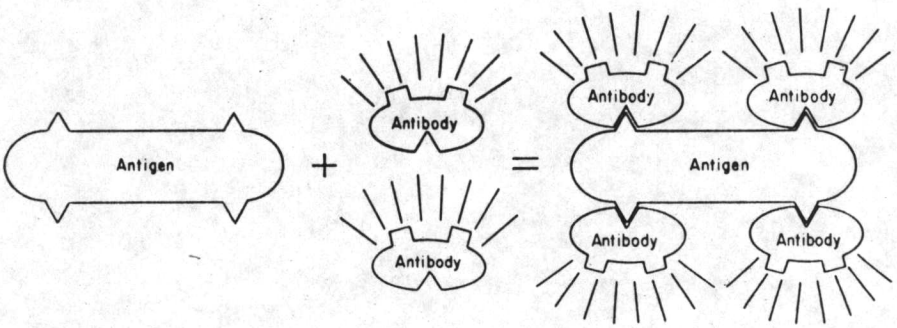

Fig. 4 Schematic representation of direct staining reaction (radiating lines indicate fluorescence)

3. Preparation of histopathological specimens

3.1 Material

Paraffin embedded sections, 4 μ in thickness

3.2 Chemicals

Hematoxylin crystal	(E.Merck Co.)
Potassium aluminium sulfate	(Mallinckrodt)
Mercuric oxide (red)	(Mallinckrodt)
Eosin, yellowish	(E.Merck Co.)
Potassium dichromate	(E.Merck Co.)
Picric acid	(E.Merck Co.)
Permount	(Fisher)
Xylene	(Mallinckrodt)
Ethyl alcohol, absolute	(Mallinckrodt)

3.3 Glasswares

Measuring cylinder	(Pyrex)
Beaker	(Pyrex)

3.4 Instruments

Light microscope	(American optical Co.)
Analytical balance	(Mettler B 5)
Hot plate	(Chromalox)

3.5 Methods

3.5.1 Preparation of solutions for staining

1. Harris alum hematoxylin

Hematoxylin, crystal	5.0 g
Ethyl alcohol, absolute	50.0 ml

Potassium aluminium sulfate	100.0 g
Distilled water to make	100.0 ml
Mercuric oxide (red)	2.0 g

a. Dissolved the hematoxylin in the alcohol, the alum in water by the aid of heat. Removed from heat.

b. Added hematoxylin solution to potassium aluminium sulfate solution, brought to a boil as rapid as possible. Removed from heat.

c. Placed the flask in cold water and added mercuric oxide quickly. This substance is oxidizing agent.

d. Kept in cold water until the mixture developed dark purple colour.

e. When cold, added 2 to 4 ml of glacial acetic acid per 100 ml of solution, to increase selectivity of the stain for the nucleus.

f. Filtered before use.

2. Picro-eosin solution

Eosin yellowish, water soluble	10.0 g
Potassium dichromate	5.0 g
Picric acid, saturated aqueous	100.0 ml
Ethyl alcohol, absolute	100.0 ml
Distilled water	800.0 ml

a. Dissolved the eosin y and potassium dichromate in water, in separated portion.

b. Mixed the two solutions.

c. Added picric acid, saturated aqueous to the

mixtures.

d. Added ethyl alcohol and distilled water to the volume.

e. Filtered before use.

3. Differentiated solution (acid alcohol solution)

Dissolved hydrochloric acid concentrated 10 ml in ethyl alcohol 70 %, 1000 ml.

4. Ammonia water

Dissolved strong ammonia water 3 ml in 1000 ml tap water.

3.5.2 Method of Hematoxylin and Eosin stain (H&E)

a. Deparaffinization: The slide was placed in xylene, 3 baths, 2 minutes each, to dissolve the paraffin.

b. Hydration:

1. The slide was taken out of xylene, it should appear quite clear and was transferred to absolute alcohol, 3 baths, 2 minutes each, when it would become opaque.

2. The slide was removed from the absolute alcohol, drained, and placed in 95 % ethyl alcohol, 2 minutes.

3. Rinsed in distilled water.

c. Staining:

1. The slide was transferred to hematoxylin solution for 3 minutes.

2. Rinsed well in running tap water, until the section was blue. The change of colour had caused this stage to be known universally as "blueing sections."

3. The slide was dipped into acid alcohol for a few seconds, then returned to water.

4. Blue section in ammonia-water mixture.

5. Washed in running tap water for 10 minutes to remove excess of ammonia.

6. Counterstained with eosin solution, 30 seconds.

7. The slide was transferred to water.

d. Dehydration: Dehydrated in 95 % ethyl alcohol, 2 changes and absolute ethyl alcohol 3 changes.

e. Clearing: Cleared in xylene 3 changes, 2 minutes each.

f. Mounting: Put one or two drops of permount, depending on the size of the tissue. Placed the coverslip on the slide, to clear of the section.

g. Results: Nuclei was blue, and cytoplasm was pink to red.

Principal (54,55)

H & E is the most commonly used in the histopathological laboratory.

Hematoxylin: one of the most valuable natural basic dyes. It is a constituent of the heart wood of the log wood tree (Hematoxylon campechianum) which grows in Campeche, Mexico.

By itself, hematoxylin has little affinity for tissues and the

actual colour formed in solution is caused primarily by the oxidation product, hematein, in a process known as ripening or oxidation by the addition of an oxidizing agent, mercuric oxide. In the ripening process hematoxylin (C16 H14 06) loses two hydrogen atoms to become hematein (C16 H12 06).

The hematein mentioned by itself has little affinity for tissues and must be used with a mordant, potassium aluminium sulfate to produce good nuclear staining.

The hematoxylin staining is a regressive staining method, in which sections are first overstained, and the stain then selectively removed in acid alcohol. The advantage of this method is that the degree of staining is controlled, and a perfectly clear cytoplasm and background can be obtained.

Eosin, is the most widely employed cytoplasmic counterstain in hematoxylin techniques, and the most frequently used is eosin y. Eosin itself derives its name from its dawn-like colour, and the "y" stands for yellowish which is the predominant colour of its solution. Eosin is most commonly used as a backgound or contrast stain because it gives a pleasing and useful contrast to nuclear stains such as hematoxylin.