THE INFLUENCE OF FIXATIVE FACTORS ON THE AVIDIN-BIOTIN-PEROXIDASE

COMPLEX METHOD (ABC) IN THE DETECTION OF IMMUNE COMPEXES

by

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A THESIS

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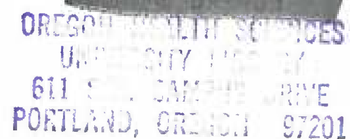

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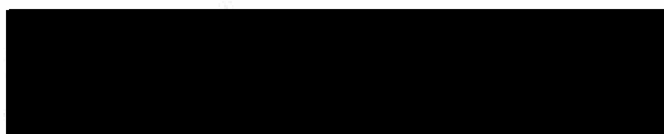

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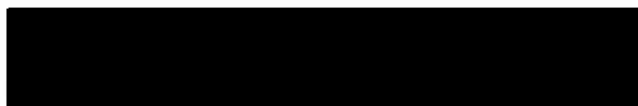

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INTRODUCTION AND LITERATURE REVIEW

Clinical disease is expressed by various constellations of signs and symptoms; all are manifestations of ineffective homeostatic mechanisms. Accompanying these clinical changes, indeed often preceding them, specific variations in the gross and microscopic appearance of cells, tissues and organs can be identified. Even before the anatomic changes can be seen, the reaction between the host's cells and the etiologic agent may have produced biochemical changes, some of which are manifested in body fluids such as plasma and urine. Some diseases are caused by immune complexes. Antibodies and/or sensitized "T" lymphocytes are produced in reaction to antigens, specific substances which immunocompetent cells regard as foreign or "non-self".

This investigation is primarily concerned with antigen-antibody reactions, in which binding sites on the antibody combine with their specific antigenic determinants to form immune complexes. Complement may or may not be involved.

FATE OF IMMUNE COMPLEXES

In animals it has been shown that large immune complexes ($>$19s in their sedimentation characteristics) are removed very rapidly from the circulation by cells of the reticulo-endothelial system. Large complexes are characteristic of pathogen-antibody complexes; these adhere to the Fc receptors on phagocytes, which then ingest and degrade the immune complexes. Very small soluble immune complexes found in antigen excess are phagocytosed least well by the R-E system, probably

because of poor binding with phagocytes. Although immune complexes consisting of large antigen-antibody lattice works are readily removed from the circulation by R-E cells, this removal may be enhanced by the binding of complement to the immune complexes.

Many complexes can initiate the complement cascade by first binding C1q, and most immune complexes can activate the properdin pathway. Immune complex disease results when immune complexes are not properly cleared by the R-E system and are deposited in body tissues, particularly in the walls of the blood vessels and in basement membranes. How are immune complexes deposited beneath the vascular endothelium?

Probably not passively, as deposition does not occur by injection of preformed complexes. Since antihistimines have been shown to prevent or reduce the deposition of immune complexes, it has been suggested that such deposition is an active process requiring increased vascular permeability. Possible release of vasoactive amines could follow: 1) Reaction between the circulating antigen and its specific IgE on mast cells and/or basophils; 2) Activation of platelets due to release of the platelet activating factor from basophils/mast cells. Thus, antigen-antibody complexes become deposited or formed in the blood vessel walls and activate complement, leading to the formation of chemotactic complement fragments, such as C5a. These attract neutrophils which migrate into the area, ingest the complexes and subsequently release lysosomal enzymes, which damage the vessel wall. Microthrombi may also occur due to the combination of stasis of blood flow and platelet aggregation and activation which

follow complement activation. Either microthrombi and/or severe damage to vessel walls tend to cause tissue infarcts to the areas supplied by the vessels.

IDENTIFICATION OF IMMUNE COMPLEXES IN TISSUE (IMMUNOHISTOLOGY)

In order to follow the events described above and/or identify the disease as an immune complex oriented process, it is often necessary to identify immune complexes within the diseased tissues.

Immunoglobulins as well as many antigens can be coupled to fluorescent dyes or enzymes with little or no loss in specificity. The reaction of antibodies or antigens labeled with fluorescent dyes can be rendered visible on histologic slides with a fluorescence microscope. Labeling with specially prepared enzymes makes it possible to use histochemical methods to visualize the reaction. Enzymes employed for this purpose catalyze reaction of substrates that yield colored reaction products. Immunohistologic techniques have been used to identify many substances in tissues. Some examples include: specific antibody, specific antigen, complement, microorganisms, hormones, neoplastic cell markers,[1] subtypes of lymphocytes, blood group antigens, actin myosin, antinuclear factors and more.

IMMUNOFLUORESCENCE

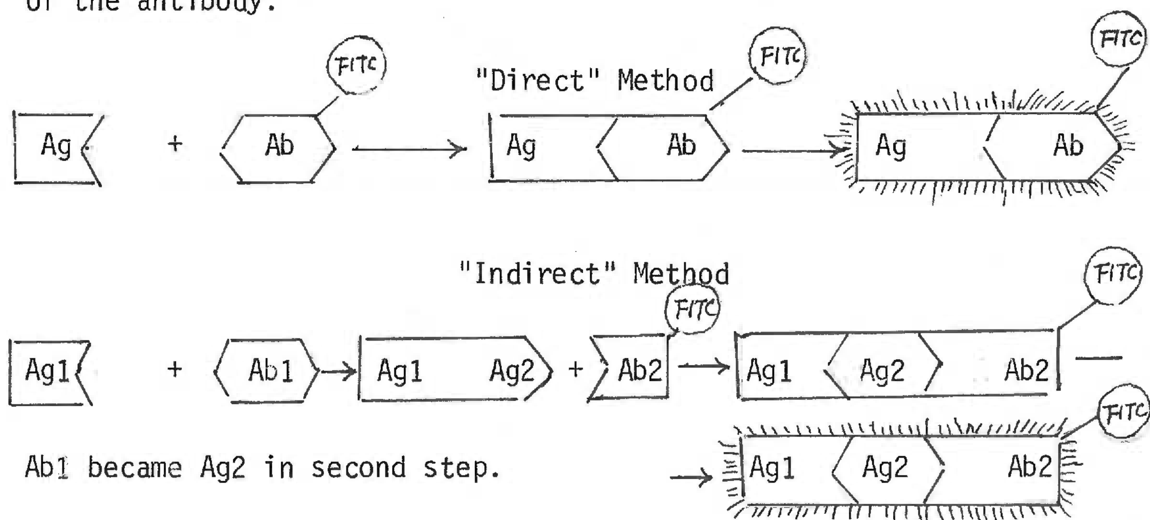
Coons, et al, introduced the era of immunohistology in 1941.[2] Since then, he and a host of other investigators have used a few predominant fluochromes for diagnostic and research immunohistology.

The two most frequently used are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRIC).

Immunofluorescence labeling procedures most often used are the "direct" and "indirect" methods.

(1) "Direct". This utilizes a one step staining procedure with labeled antibodies against the antigen being sought. This antigen can itself be an antibody or complement.

(2) "Indirect". One type of indirect staining utilizes the patient's serum containing the sought-for antibody, as in the diagnosis of pemphigus vulgaris. The serum is brought into contact with test tissue such as monkey esophagus; then the human antibodies combine with the antigen, in this case interepithelial cell desmosomes. Finally, labeled anti-human IgG (usually) demonstrates the presence of the antibody.



PRESENT UTILITY OF IMMUNOFLUORESCENCE

Although immunofluorescence methods are being used very successfully in certain areas of pathology such as in the study of certain renal and skin diseases, they have not achieved widespread

application in diagnostic histopathology. Why?

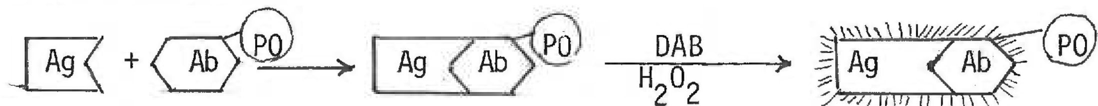
First, immunofluorescence is not generally regarded as suitable for use with formalin-fixed, paraffin-embedded tissues. Second, since sections for immunofluorescence are either unfixed or mildly fixed fresh frozen sections, the morphological detail is poor. Third, the immunofluorescence sections fade rapidly and can not be reviewed at later times. However, Darsett and collegues [3] have used immunofluorescence on Bouin's-fixed, paraffin-embedded sections. The preservation of morphology and preparation permanence have been improved.

IMMUNOENZYME (IMMUNOPEROXIDASE) METHODS - GENERAL

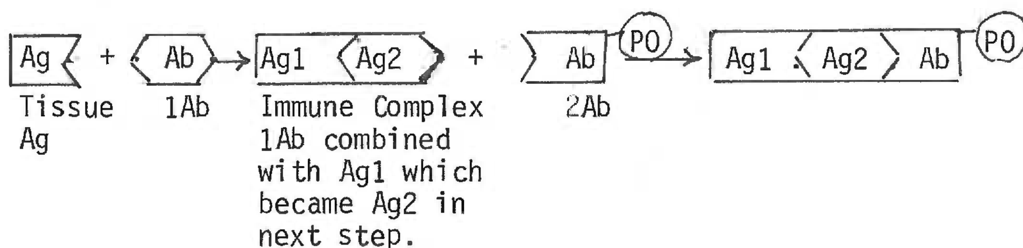
In an effort to overcome the difficulties associated with immunofluorescence, alternative labeling methods, primarily utilizing horseradish peroxidase emerged[4] [5] [6] to solve some of these problems. The horseradish peroxidase label can be localized in tissues by adding a suitable chromogenic substrate to produce a colored reaction product visible by light microscopy. Examples follow:

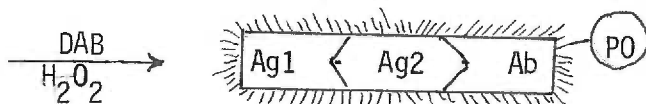
Peroxidase Methods

"Direct" Method:

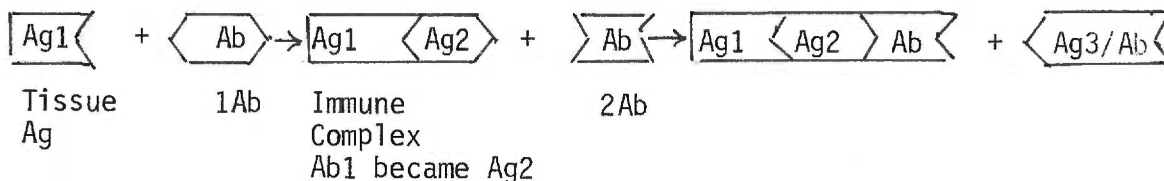


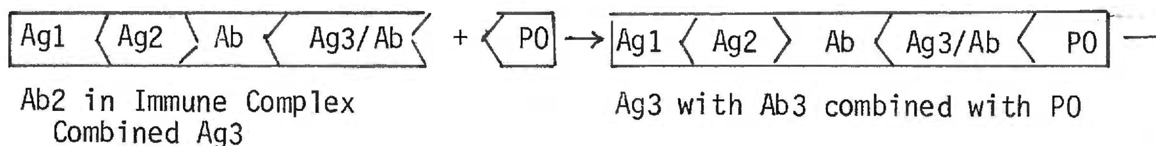
"Indirect" Method:

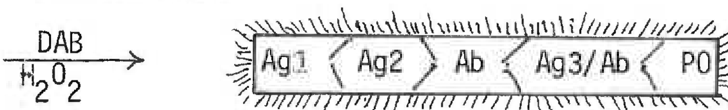




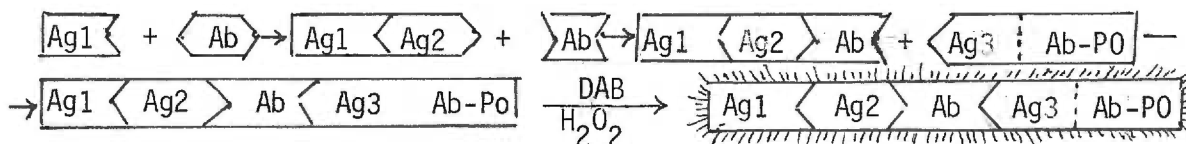
"Unlabeled Bridge" Method:







Enzyme-Anti Enzyme (PAP)



Diagrammatic representation of the four major immunoperoxidase methods.

Ag=antigen; Ab=antibody; PO=peroxidase; PAP complex= peroxidase anti-peroxidase

In any of the immunoperoxidase methods, the sites of peroxidase activity in sections are made visible by the exposure of the sections to a freshly-prepared solution of 3-3' Diaminobenzidine to which H_2O_2 has been added. The peroxidase catalyzes the release of oxygen from H_2O_2 with the subsequent oxidation and polymerization of 3-3' Diaminobenzidine. The resulting precipitate has a mahogany brown color easily visualized in bright field microscopy.

ADVANTAGES OF IMMUNOPEROXIDASE METHODOLOGY

(1) It is appropriate for use by the surgical pathologist, because it is applicable to fixed embedded tissue.

(2) Sections may be examined by light microscopy after counterstaining with hematoxylin.

(3) Morphologic detail of sections is equivalent to H & E sections, therefore traditional morphologic criteria may be used in evaluation of the sections in conjunction with specific immunologic staining.

(4) Retrospective studies are made possible.

(5) Immunoperoxidase methodology can be adapted to electron microscopy. The methods described above (direct and indirect) are directly analogous to similar methods used in immunofluorescence. The indirect methods are more sensitive than the direct methods because the cascade of immunoreactants (Ag + unlabeled primary Ab + labeled secondary Ab) results in greater labeling of tissue constituents than does the direct (Ag + labeled primary Ab).

The reason for this amplified binding of immunoreactants lies in the di or multi-valency of antibody molecules. However, only a single Fab region on the primary antibody, which has been added in excess, combines with the specific antigen, leaving the remaining Fc regions free to combine with four or five labeled secondary antibodies binding to each primary antibody associated with the specific antigen.

IMMUNOPEROXIDASE METHODS - NON-CONJUGATE PROCEDURE

The purpose of these methods is to attain greater sensitivity

than those just described while retaining specificty and minimal background staining. One of the advantages of these methods is the preservation of antibody in its natural state, rather than the denaturation which often occurs when antibody is conjugated.

Of the several older non-conjugate procedures which have been developed in the past decade, only the peroxidase-antiperoxidase method is in general use today, primarily because of its greater sensitivity and the wide availability of many immunoreactants.

THE PEROXIDASE-ANTIPEROXIDASE (PAP) METHOD

In 1970, Sternberger et al [7] introduced this procedure as an alternative to the older direct and indirect immunofluorescent and immunoperoxidase methods. It has been estimated that the PAP technique is 100 to 1000 times more sensitive than the older procedures. Recently, however, it was claimed that the sensitivity of the labeled indirect antibody technique with the use of affinity purified antibodies is almost equal to that of the PAP method [8]. In some systems, the sensitivity of the PAP procudure approaches that of radioimmunoassay with useful working dilutions of the primary antibody approaching 1:100,000 [9] [10].

In order to appreciate the development of the PAP method, its seldom used predecessor, the "unlabeled antibody bridge" method will be described. For this method, primary antibody and antiperoxidase antibody are raised in the same animal, such as the rabbit. A "bridge" antibody, e.g. anti-rabbit IgG, is applied in sequence after the primary antibody (to the antigen in tissue section)

and before the addition of the antiperoxidase to the tissue surface. This secondary antibody "bridges" the primary and secondary antibody by virtue of its specificity for the antigen determinants in the primary and tertiary reagents. Finally, peroxidase is applied to the tissue section and the reaction product is developed.

Use of this particular reagent has been virtually eliminated by availability of the sensitive PAP unlabeled technique, which uses a preformed soluble PAP complex.

The unlabeled PAP procedure differs from the unlabeled antibody bridge method only in that the tertiary reagent consists of a preformed soluble complex of peroxidase and anti-peroxidase, which is not only stable, but contributes to the sensitivity of the method. The stability of the preformed PAP complex precludes the loss of bound peroxidase in the multiple saline washes involved in the procedure. The anti-peroxidase antibody in the PAP complex is bound by the free Fab portion of the bridge antibody and is actually antigenic to the link antibody. The PAP complex is used in a single incubation or to replace the final of two steps of the unlabeled antibody bridge method. The complexed HRP retains its catalytic function and when permitted to react with a substrate such as Diaminobenzidine and H_2O_2 a mahogany brown final reaction is produced. Thus, the tissue antigen is visualized by the application of multiple layers of unlabeled antibody that are linked together by the bridge antibody.

ENZYME MARKERS OTHER THAN HORSERADISH PEROXIDASE (HRP)

Alkaline Phosphatase [11] has been used as a substitute for peroxidase in immunoenzymatic technique, as well as in double-label

methods where two antigens are visualized in situ. Because of possible carcinogenicity of 3,3'Diaminobenzidine Hydrochloride Monohydrate (DAB), other chromogens have been tried as markers: Tetramethylbenzidine (TMB)[12], Aminoethylcarbazole (AEC), P-Phenylenediamine and Pyrocatechol or Hanker Yates reagent (HYR)[13]. The Hanker Yates reagent is the only chromogen generally regarded to be definitely non-carcinogenic. DAB, the most widely used chromogen, in a recent study[14] did not demonstrate carcinogenesis in experimental animals, therefore it is probably not a carcinogen or may not be.

SOME RECENT MODIFICATIONS OF IMMUNOPEROXIDASE PROCEDURES

1. PROTEIN A MODIFICATION[15]. Protein A, a cell wall protein from staphylococcus aureus, binds specifically to the Fc portion of IgG from several mammalian species. Coupling of protein A to substance such as fluorescein [16], peroxidase[17] or alkaline phosphatase[18] does not appear to affect their biological properties. In the two stage method, a primary antiserum that has specificity to the tissue antigen is applied, followed by the protein A conjugated with horseradish peroxidase[19].

In the three-stage technique, unlabeled protein A is used as a bridge reagent between the primary antibody and the PAP immune complexes. Some advantages do occur: a) the methods are relatively rapid; b) the two-stage method exhibits minimal non-specific background staining as protein A peroxidase may be used at a very high dilution, taking advantage of the high affinity of protein A for the Fc portion of IgG; c) the methods are extremely versatile, for protein A can be used with many different primary antibodies

from many different species. It can, for example, link a primary antibody from one species to a PAP reagent from another. This can be important if it is desired to detect antibodies of human origin, because it is not feasible to produce anti-peroxidase IgG in the human.

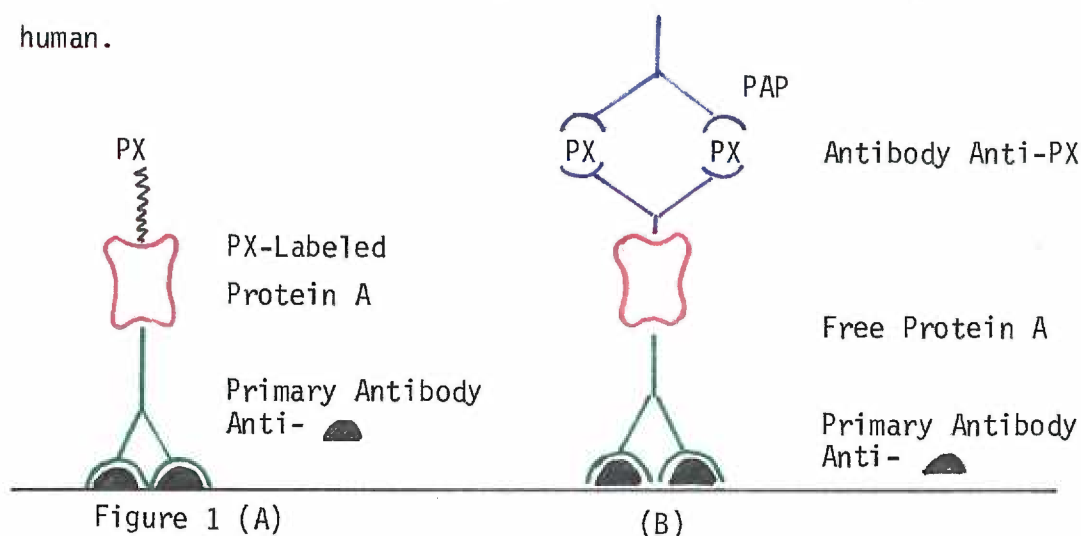


Figure 1 (A) (B)

A shows two stage protein A-peroxidase (PX) method; B, three-stage protein A-peroxidase procedure. Solid semicircle indicates antigen; open H-shaped figure, protein A; and PAP indicates peroxidase-antiperoxidase. (Diagrams from the reference (17)).

THE AVIDIN-BIOTIN-PEROXIDASE COMPLEX METHOD (ABC) [20] [21] [22] [23]

This procedure has several theoretical advantages over other immunoperoxidase methods. 1) It has been found to be eight to forty times more sensitive than the unlabeled PAP method; 2) It reportedly yields immunostained sections having negligible or no background staining; and 3) It is cost effective (about 5% of the cost of the average PAP procedure). The two unique components of the system are:

1) Biotin. A water-soluble low molecular weight vitamin which can be readily covalently coupled through an active ester to the amino groups of an antibody. It is widely distributed in mammalian tissues and is present in high concentration in liver, adipose tissue, mammary gland and kidney [24][25][26].

2) Avidin. An egg white glycoprotein with four binding sites that have high affinity for Biotin.

The formation of complexes between Avidin and Biotin is not inhibited by coupling either to fluorescein isothiocyonate (FITC)[27] or enzymes[20][21]. Three varieties of the ABC method have been described.

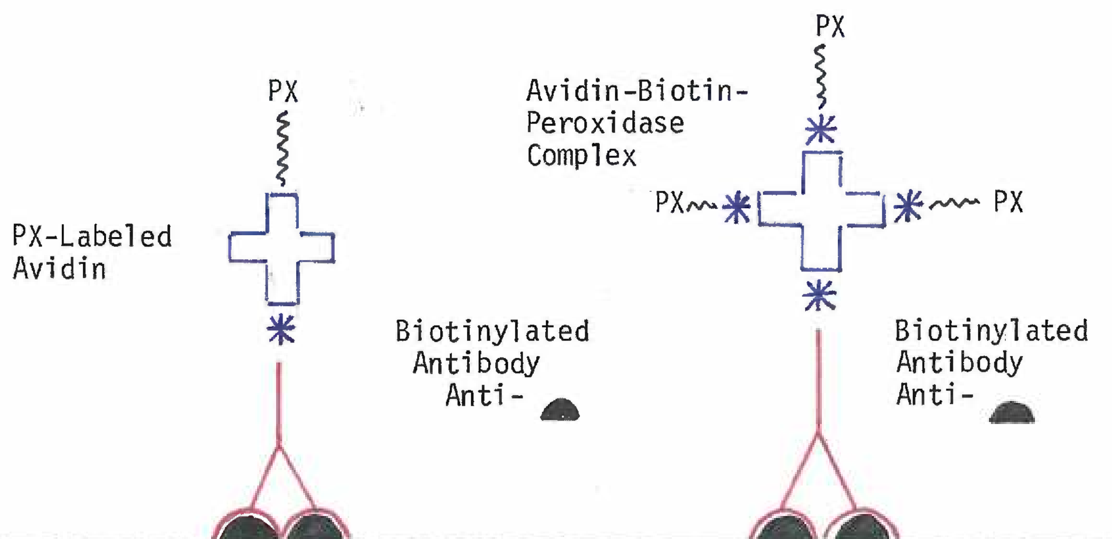


Figure 2.

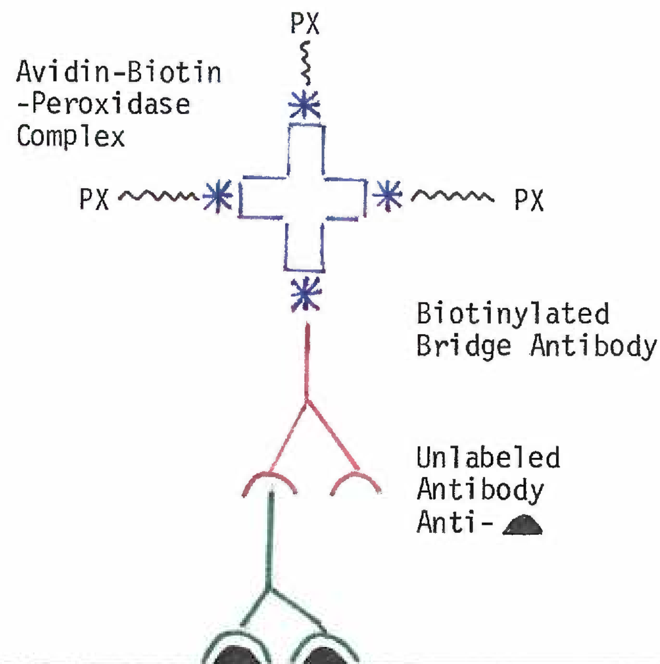


Figure 3.
Biotin-avidin immunoenzymatic techniques. Solid semicircle indicates antigen; PX, peroxidase; asterisk, biotin; and shaded open+, avidin. (Diagrams from the reference (17).

In a direct method, tissue sections are incubated with specific biotinylated primary antibody, followed by an application of an avidin-peroxidase complex. Finally, the HRPO is visualized as in all peroxidase methods with a final step incubating the tissue with DAB and H_2O_2. Another direct method also first incubates tissue sections with specific biotinylated primary antibody followed by unlabeled avidin and biotinylated horseradish peroxidase.

A three-stage technique has been claimed by Hsu and collegues to be more sensitive than the above methods. In this technique, unlabeled specific primary antibody is added to tissue sections, followed by a biotinylated secondary bridge antibody, which in turn is followed by the avidin-biotin-perioxidase complex.

Hsu and colleagues reported optimum staining using an ABC conjugate prepared at a ratio of 4:1 of avidin to biotin-peroxidase. They suggested that the high sensitivity of the method might be due to the formation of a lattice that contained many peroxidase molecules, whereas the PAP complex contains only three molecules of horseradish peroxidase. However, Naritoku and Taylor[28] found that under optimum conditions the sensitivity of the ABC procedure is comparable with the PAP method. A potential problem in using the ABC method is due to the wide distribution of endogenous biotin in mammalian tissues. Since the free avidin or avidin conjugates employed in avidin-biotin systems will bind endogenous biotin, a potential problem arises in the application of these system to tissues high in endogenous biotin. Also non-specific binding of avidin to tissues may occur at neutral pH and physiologic ionic concentration.

If the tissue being studied could present false-positive staining for either or both of these reasons, Wood and Warnke(1981)[29] have suggested a method for suppressing this endogenous avidin-binding activity. Fortunately, this activity has little relevance to the present study, in which immune complexes are detected in the basement membranes of renal glomeruli. Wood and Warnke found that the endogenous avidin-binding acitivity of the kidney was localized to the renal tubular epithelium. Glomeruli and renal interstitial tissue were negative for the endogenous avidin-binding acitivity.

PROBLEMS COMMON TO ALL IMMUNOPEROXIDASE METHODS

A) Endogenous peroxidase and pseudoperoxidase can be a

misleading source of background staining. Endogenous peroxidase are present in high concentration in granulocytes, and pseudoperoxidase in red blood cells. Blocking of these endogenous enzymes is usually accomplished by first exposing the tissue sections to H_2O_2 in methanol or acid alcohol, either of which destroys the enzymes.

B) Background staining may occur due to non-specific absorption of heterologous serum to the tissue. This cause can be eliminated by a combination of prolonged incubation with high dilution of the primary antibody and preincubation of rehydrated sections in non-immune serum.

CONTROLS

To evaluate the results of immunoperoxidase staining a variety of immunologic and non-immunologic controls should be included. These are as follows: [30][31]

1) Replacement of specific primary antiserum with normal serum from the same species. Globulin concentrations of both specific and control sera should be approximately the same.

2) Replacement of specific primary antiserum with a second primary antiserum prepared in the same species but directed to an antigen unrelated to the one under investigation.

3) Absorption of the specific primary antiserum with purified antigen.

4) In the peroxidase-antiperoxidase technic deletion of the second antibody or in ABC technique, omission of one or more reagents.

PROBLEMS OF FIXATION AND PROCESSING IN IMMUNOPEROXIDASE METHODS[32][33]

Many variations on techniques designed to identify antigens in tissue sections have been prepared over the past 20 years. Each of these methods is a compromise between the need for good morphologic fixation of tissues and the preservation of antigenic specificity. Because of the great sensitivity of methods such as PAP and ABC procedures, it may be possible to detect an antigen even when nine out of ten of its antigenic determinants are rendered unavailable to their specific antibodies.

Some variables which can affect antigen identification and quality of the tissue sections include: 1) Specific fixative; 2) Time of tissue in fixative; 3) Temperatures during fixation; 4) Pre- or Post-fixation washing; 5) Post-fixation in other than initial fixative; 6) pH.

Probably the most important consideration in selection of a fixative is the nature and location of the antigen itself. The choice will vary depending on whether the antigen is: 1) An immunogloulin, hormone, unique tissue antigens (myoglobin, actin, hemoglobin, fibronectin, collagen, amyloid, fibrin), microorganisms, tissue enzymes, oncodevelopmental antigens; 2) Membrane bound; 3) Intracellular; or 4) Present as immune complexes on blood vessel walls or basement membranes.

GENERAL COMMENTS ON FIXATION

Whatever tissue preparation method is selected for detection of antigens in tissue, it is always a compromise between crisply detailed

morphology and optimal demonstration of antigens with the best possible "signal-to noise" ratio. No single procedure can at one time, 1) immobilize all types of antigens, 2) preserve their antigenicity, 3) provide optimal access of their corresponding antibody reagents and 4) retain structural integrity of tissues and cells at the microscopial level.

Even though cryostat sections may accomplish the foregoing with varying degrees of acceptability, fixation is usually required to immobilize antigens and to preserve morphology. For immunohistochemical purposes, fixation is necessary to stop enzymatic activity rapidly enough to avoid structural decomposition, to hinder diffusion of peptides and proteins into and out of cells and, if possible, to help prevent deleterious tissue effects during the stages of section preparation. These effects may be due to: 1) changes prior to fixation, 2) the fixative, 3) length of fixation, 4) tissue processing (dehydration, clearing embedding, flotation, drying, dewaxing, incubation and washing), 5) pH.

CONSIDERATION OF FIXATION OF SECTIONS IN WHICH IMMUNE COMPLEXES ARE TO BE DETECTED ON BASEMENT MEMBRANES

Although a modified Saint Marie method[34] of cold ethyl alcohol fixation may be the fixative of choice for these complexes, it was not selected for this investigation, primarily because most tissues which are routinely submitted to a pathology laboratory are immersed in 10% aqueous commercial Formalin. When ethanol is used as a fixative, it immobilizes proteins and carbohydrates by precipitation. Denaturation of proteins is mild and often reversible, thus preser-

ving the antigenicity of antibodies and complement in the immune complexes.

Against these advantages, it has been found that: 1) even large protein antigens may not always be sufficiently immobilized by ethanol fixation to avoid their loss during incubation and washing of tissue sections and 2) dehydration takes place during ethanol fixation, so that shrinkage may distort morphology.

Aldehydes, of which Formalin is the most common fixative, immobilize peptides and proteins by bifunctional cross-linking, which also better preserves structural integrity of tissues and cells. Unfortunately, cross-linking leads to more severe masking than does precipitation, expecially for large protein antigens.

Aldehyde-based fixatives induce both intermolecular and intramolecular bridges. Because of this extensive formation of cross-linkages formaldehyde may, besides masking antigens by denaturation, accentuate the problem by steric hindrance. The number of methylene bridges formed depends on the concentration of the formaldehyde, the temperature, pH and time of exposure. The resulting reduced antigenicity may be partially reversed by extensive washing in water or treatment with sucrose[35].

Commercial formalin usually contains variable amounts of methyl alcohol, which further aggravates the antigen-masking effect of formaldehyde. Paraformaldehyde has been found by some to produce a lesser masking effect than formaldehyde, which itself is better than glutaraldehyde.[36] [32] Unmasking of antigens has been attempted by applying proteolytic enzymes to tissue sections before immunohis-

tological staining. These have included trypsin[37], pepsin, papain and pronase[35]. Another possible advantage of proteolytic digestion is the elimination of xylene-induced antigen impairment, occurring during the "clearing" process. But, the results of proteolytic digestion may vary for different antigens. It may also cause the loss of tissue sections from the glass slides during subsequent procedures. Further, too much proteolytic digestion will destroy tissue integrity.

TIME OF FIXATION

Most reports indicate that fixatives containing aldehyde should be exposed to tissue for less than 24 hours. It has been recommended that when tissues are exposed to formalin for longer periods, that Bouin's fluid, Zenker's, B5 or FAA (Formalin-acetic acid-alcohol) be used as post fixatives to avoid excessive non-specific background staining, which can be caused by Formalin immobilization of antigens or complement in interstitial tissue.

PURPOSE OF THIS STUDY

This investigation is concerned with cellular and tissue changes at the level of the light microscope. The main thrust of this work was to determine which concentrations and fixation times of several fixatives result in the most informative paraffin sections for detecting immune complexes in tissue by an immunoenzyme procedure.

Since most tissues arrive at the laboratory after immersion in buffered neutral 10% Formalin for from 1-9 days, it was decided to compare positive immunostaining in varying concentrations and times of exposure to commercial Formalin, paraformaldehyde and paraformaldehyde with glutaraldehyde.

All these results were compared to the baseline data from standard direct immunofluorescence of quick-frozen, cryostat cut tissue.

It might, therefore, be possible to determine whether routinely submitted tissues, fixed in Formalin for many days are worthy of immunohistologic evaluation for immune complexes. It is possible that the great sensitivity of the ABC procedure might detect sufficient antigens for diagnostic purposes, even though most of the antigenic reactive sites were masked.

MATERIAL AND METHODS

Fifteen MRL/lpr mice (8 female and 7 male) were used in this immunoenzyme study. The mice were sacrificed at ages from three months 24 days to 6 months. Fifteen mice were divided into four groups.

GROUP 1 (mouse A & mouse B). Group 1 consists of two mice (one female and one male, both 6 months old). Both kidneys were taken from each mouse and divided into four longitudinal blocks. From mouse A, one specimen was embedded in O.C.T. (Lab-Tek product), embedding medium for frozen tissue specimens, quick-frozen in liquid nitrogen and the block refrigerated at -80° C for future immunofluorescent and immunoperoxidase procedures. The other three tissue blocks were fixed in (a) 10% phosphate buffered Formalin, 0.075M pH 7.0, (b) 2% paraformaldehyde + 1.25% glutaraldehyde and (c) 0.2% paraformaldehyde +0.125% gluaraldehyde solutions respectively for three hours. Mouse B was processed as was mouse A except that fixation proceeded for one day longer.

A) CRYOSTAT SECTIONS. Eight sections were cut from both mouse A and mouse B for immunofluorescent procedures. Liquid nitrogen frozen blocks were cut to a thickness of 6u for positive control of ABC processed sections. They were stained directly with FITC conjugated anti-mouse IgG (obtained from Miles-Yecia LTD) which identified deposits of mouse IgG in the kidney.

The conjugate was diluted to 1/20, 1/40, 1/80. Alternate sections were counterstained with 0.1% Evans Blue to obliterate nonspecific fluorescence. Additional cryostat sections were subjected to the ABC procedure.

Before the ABC procedure was started, the sections had been

quickly dipped into acetone twice.

B) FIXED SPECIMENS. The fixed specimens were dehydrated, xylene-cleared and embedded in 53^{0} melting point paraffin. Five micron serial sections were cut from the paraffin embedded tissues for ABC immunoperoxidase staining.

Reagents for the ABC immunoperoxidase technique were obtained from Vector Corp. and primary antiserum was obtained from Sigma Corp.

The procedure employed followed the Vector Corp. ABC immunoperoxidase staining method except that some sections were pretreated with 0.1% Trypsin for 10-15 minutes or 0.01% Triton X 100 detergent (from Sigma Corp.) for 10 minutes. Trypsin was also obtained from Sigma Corp. (contains 5% calcium acetate). Primary antiserum was diluted to 1/50, 1/100, 1/500, 1/1000, and 1/2000. Two percent aqueous methyl green was used as a counterstain. The negative controls were: a) omitting primary antiserum, or b) primary antiserum prepared from a species different from the secondary antibody; i.e.the anti-mouse IgG was prepared in the goat instead of prepared in the rabbit; or c) normal mouse's kidney. Thus, each set of specimens consisted of: a) hematoxylin and eosin stained sections; b) frozen sections for direct immunofluorescent; c) frozen sections for ABC; d) ABC stained sections from fixed blocks with different dilutions of primary antiserum and e) negative control sections.

GROUP 2 (mouse C,D,E,F). Group 2 consisted of four mice (1 female aged 3 months 24 days, and 3 males, one aged 4 months 2 days and 2 at 4 months 18 days). Both kidneys from each mouse were divided into a total of nine tissue blocks. From the right kidney, one block

was preserved for frozen sections. The other four pieces from mouse C's right kidney were placed either in 10% buffered Formalin, pH7.0, 4% paraformaldehyde, 2% paraformaldehyde or 2% paraformaldehyde with 1.25% glutaraldehyde for 3 hours in each of the fixatives. The different concentrations of paraformaldehyde and the low concentration of mixed paraformaldehyde with glutaraldehyde were chosen following Elias' suggestion[31]. After routine processing, the tissues were embedded in $53^{o}C$ melting point paraffin. The four pieces of tissues from the left kidney were placed in corresponding fixatives except that they were embedded in $57^{o}C$ melting point paraffin. Similarly, mouse D's tissues were fixed for one day, mouse E's for three days and mouse F's for five days. The remaining procedures were identical to Group 1 except that the trypsinization or detergent processing steps were deleted.

GROUP 3 (mouse G, H, I, J). Group 3 consisted of four mice (3 females and 1 male, all aged 4 months 2 days). The same procedure as Group 2 were used, except that all tissues were fixed for 3 hours, 1 day, 3 days and 5 days.

The specimens from mouse G were fixed in 10% buffered Formalin, pH7.0 mouse H's specimens in 4% paraforaldehyde, mouse I's specimens in 2% paraformaldehyde and mouse J's specimens in 2% paraformaldehyde with 1.25% glutaraldehyde.

GROUP 4 (mouse K, L, M, O). Group 4 consisted of five mice (3 females and 2 males; ages were 4 months 18 days X 2, 5 months 12 days X 3, the average weight of kidneys were 0.31g). Both kidneys of each

mouse were divided into a total of ten tissue blocks. After quick freezing in liquid nitrogen, one specimen was refrigerated at -80°C for subsequent immunofluorescent and ABC procedures. The remaining nine blocks were randomly assigned to nine plots. Each plot involved a factor of a specific fixative vs. a specific time. The three fixatives were 10% buffered Formalin, 2% paraformaldehyde and 4% paraformaldehyde; the three different times were 3 hours, 3 days and 9 days. (Diagrams - see Diagram of Study Design).

EVALUATION OF IMMUNOCOMPLEX DEPOSITION

Semiquantitative counting was used to evaluate the immune complex deposits (brownish lumps on or thickened membranous changes) on the basement membrane of capillaries and the mesangium of renal glomeruli.

The total numbers of glomeruli in each section were counted using a Grid Reticle in a manner similar to making a white blood count. The numbers of glomeruli demonstrating immune complexes were also counted.

The intensity of the chromogenic representation of immune complex deposits were divided into four grades: "0"=negative, "+"=mild positive staining, "++"=intermediate, "+++"=strong positive, staining. The percentage of the total glomeruli stained for each grade of intensity was calculated per slide.

In order to standardize the results, two independent observers carried out separate determinations and results were compared. After reaching agreement on the standards of intensity, this observer

carried out the rest of the observations.

STUDY DESIGN

GROUP 1

Mouse A Both Kidneys	A) Frozen block	a) immunofluorescence. b) Immunoperoxidase (ABC).
	B) 3 hours fixation	a) 10% buffered Formalin, pH7.0. b) 2% paraformaldehyde + 1.25% glutaraldehyde. c) 0.2% paraformaldehyde + 0.125% glutaraldehyde.
Mouse B Both Kidneys	A) Frozen block	a) immunofluorescence. b) immunoperoxidase (ABC).
	B) 1 Day fixation	a) 10 % buffered Formalin, pH7.0. b) 2% paraformaldehyde + 1.25% glutaraldehyde, pH7.2. c) 0.2% paraformaldehyde + 0.125% gluteraldehyde, pH7.2.

Pretreated trypsin or non-trypsin. Pretreated detergent or non-detergent.

GROUP 2

Mouse C Both Kidneys	A) Frozen block	a) immunofluorescence. b) immunoperoxidase (ABC).
	B) 3 hours fixation embedded in 53°C melting point paraffin	a) 10% buffered Formalin, pH7.0. b) 2% paraformaldehyde, pH7.2. c) 4% paraformaldehyde.

d) 2% paraformaldehyde + 1.25% glutaraldehyde, pH 7.2

B) 3 hours fixation embedded in 57°C melting point paraffin

a) 10% buffered Formalin, pH 7.0

b) 2% paraformaldehyde, pH 7.2

c) 4% paraformaldehyde, pH 7.2

d) 2% paraformaldehyde + 1.25% glutaraldehyde, pH 7.2

Mouse D - same as Mouse C's, except 1 day fixation.

Mouse E - same as Mouse C's, except 3 days fixation.

Mouse F - same as Mouse C's, except 5 days fixation.

GROUP 3

Mouse G - 10% buffered Formalin, pH 7.0, 3 hours, 1 day, 3 days, 5 days. Frozen block for immunofluorescence and immunoperoxidase (ABC).

Mouse H - 4% paraformaldehyde 3 hours, 1 day, 3 days, 5 days. Frozen block for immunofluorescence and immunoperoxidase (ABC)

Mouse I - 2% paraformaldehyde 3 hours, 1 day, 3 days, 5 days. Frozen block for immunofluorescence and immunoperoxidase. (ABC)

Mouse J - 2% paraformaldehyde + 1.25% glutaraldehyde 3 hours, 1 day 3 days, 5 days. Frozen block immunofluorescence and immunoperoxidase. (ABC)

GROUP 4

Mouse K - A) Frozen block for immunofluorescence and immunoperoxidase. (ABC).

B) Nine blocks placed randomly in nine plots

	3 hours	3 days	9 days
10% buffered Formalin, pH 7.0	K 1	K 7	K 4
2% paraformaldehyde, pH 7.2	K 5	K 2	K 6
4% paraformaldehyde	K 8	K 9	K 3

Mouse L - A) Frozen block immunofluorescence and immunoperoxidase. (ABC).

B) Nine block placed randomly in nine plots.

	3 hours	3 days	9 days
10% buffered Formalin, pH 7.0	L 5	L 1	L 2
2% paraformaldehyde, pH 7.2	L 3	L 6	L 4
4% paraformaldehyde	L 8	L 9	L 7

Mouse M, N, O - same as Mouse K's and Mouse L's.

ABC IMMUNOPEROXIDASE STAINING PROCEDURE

1. Deparaffinize and dehydrate tissue sections through xylene and and graded alcohol series.
2. Rinse for 5 minutes in distilled water.
3. Incubate the sections for 30 minutes in 0.3% H_2O_2 in methanol. Incubation times may be shortened by using higher concentrations of H_2O_2. (In case where the antigenic determinants may be destroyed by treatment with H_2O_2, step 3 and 4 may be carried out after step 10. If endogeneous peroxidase activity does not present a problem, step 3 may be deleted).
4. Wash in phosphate-buffered-saline (PBS), pH 7.3-7.4, for 20 minutes.
5. Incubate sections for 20 minutes with diluted normal serum which was prepared from the species in which the secondary antibody is made. (In case where non-specific staining is not a problem, step 5 and 6 may be deleted).
6. Blot excess serum from sections.
7. Incubate sections for 30 minutes with primary antiserum diluted in buffer.
8. Wash slides for 10 minutes in buffer.
9. Incubate sections for 30 minutes with diluted biotinylated antibody solution.
10. Wash slides for 10 minutes in buffer.
11. Incubate sections for 30-60 minutes with Vectastain ABC reagent.
12. Wash slides for 10 minutes in buffer (Tris-buffer pH 7.6).

13. Incubate section for 5 minutes in peroxidase substrate solution (mix together an equal volume of 0.02% hydrogen peroxide made in distilled water) and 0.01% (1mg/ml) diaminobenzidine tetrahydrochloride (made in 0.1M Tris buffer, pH 7.2).
14. Wash sections for 5 minutes in tap water.
15. Counterstain in 2% methyl green in distilled water (time variable).
16. Wash slides in tap water.
17. Dehydrate, clear in xylene and mount in synthetic medium.

IMMUNOFLUORESCENCE DIRECT STAINING

1. Wash in PBS 30 minutes (use magnetic stirrer).
2. Apply conjugate 30 minutes (in moist chamber).
3. Rinse each slide individually with wash bottle, place slides in staining rack and wash in PBS for 1 hour (2 changes, 30 minutes each).
4. Counterstain slides indicated - Evans Blue - 0.01%, 1 dip-rinse in PBS-wash in PBS 15 minutes.
5. Mount in Aquamount (TM) or glycerol saline.

CONTROL SLIDES

1. Wash in PBS 30 minutes.
2. Apply non-fluorescent antiserum 30 minutes.
3. Rinse each slide individually with bottle, place slides in staining rack and wash in PBS 30 minutes (use magnetic stirrer).
4. Apply conjugate 30 minutes (in a moist chamber) (use least dilution).
5. Rinse each slide individually, then wash for 1 hour in PBS (2 changes, 30 minutes each use magnetic stirrer).
6. Mount in Aquamount (TM) or glycerol saline.

THE PREPARATION OF 2% PARAFORMALDEHYDE

Take 2gm Paraformaldehyde powder add to 50cc distilled water at $60^{o}C$, stir to dissolve and add drops of NaOH until clear, cool, pH 7.2.

THE PREPARATION OF 2% PARAFORMALDEHYDE + 1.25% GLUTARALDEHYDE

Add 8mls 25% glutaraldehyde to 2% paraformaldehyde water, adjust pH to 7.2 with 1N KOH.

MRL/lpr. MICE

The autosomal recessive gene lpr. controls massive early lymphoproliferation consisting of 88% "T" lymphocytes and the early onset of antinuclear autoantibodies, natural thymocytotoxic auto-antibodies and hypergammaglobulinemia. No leukemic blood picture develops and there is no evidence of malignancy, although the syndrome may be prelymphomatous.

Female mean life-span is 120 days, that of the male, 154 days. Kidneys show subacute proliferative glomerulonephritis, with endothelial and mesangial proliferation and basement membrane thickening and proteinura.

RESULTS

H & E SECTIONS, GENERAL REMARKS. The hematoxylin-eosin stained sections from all specimens demonstrated both renal cortex and medulla. The majority of glomeruli showed different degrees of hypercellularity. The endothelial cells, subepithelial cells and probably mesangial cells of the glomeruli were proliferative. The subepithelium of the glomerulus proliferated to form crescents and adhered to Bowman's capsule and filled Bowman's space. There were fibrinous deposits in the glomeruli and in severe cases, obliteration of the glomeruli by scarring was seen. The basement membrane of capillaries revealed a rigid eosinophilic thickening. Some capillary tufts exhibited a wire loop appearance. The lumina of capillaries became narrowed. Occasionally, hyaline thrombi were seen in a capillary lumina. Lymphocytes and plasma cells focally infiltrated the intertubular areas. In adjacent inflamed areas, the tubular lining cells demonstrated varying degrees of degeneration. There were homogenous eosinophilic stained protein casts or red cell casts. The interstitial vessels showed intimal thickening with narrowed lumina. The histopathological changes varied in degree from case to case. The later the mouse was sacrificed, the more severe were the kidney lesions. The female mouse showed more severe disease than did the male at the same age. These typical histopathological findings were confirmed in our animal model. (See Material and Methods - Page 38).

FITC stained frozen sections from all the specimens revealed two patterns of positive staining for immune globulins and/or complement. One of these demonstrated deposits along the basement membrane of

both glomerular and interstitial capillaries, while the other appearance was of irregular granular deposits (so called "lumpy - bumpy" deposits). Within the lumina of some tubules, fluorescent evidence of numerous globulins and/or complement was seen; these probably "leaked" from the damaged glomerular capillaries into the tubules.

Using the ABC method, five different dilutions of primary antiserum (1:50, 1:100, 1:500, 1:1000, 1:2000) were tested. The optimal concentration was 1:500 with regard to intensity and good definition of specific staining, together with minimal non-specific background staining. The higher the concentration of primary antiserum, the stronger the specific staining was. However, the undesirable non-specific background staining was also enhanced. (Fig. 1, 2, 3). Therefore, 1:500 concentration seemed to enhance definition, while at the same time decreasing undesirable background staining.

The optimal dilution of the conjugated antiserum was found to be 1:80, with regard to intensity and good definition of specific staining with minimal undesirable non-specific background staining.

Avidin-Biotin-Peroxidase Complex-stained sections from all specimens revealed brownish irregular clumpy deposits as well as linear, membranous deposits along the basement membrane of glomerular and interstitial capillaries.

GROUP 1 FINDINGS. Group 1 was a preliminary study to estimate the sensitivity and specificity of the ABC method as applied to the animal model employed in this study. Trials were made to: 1) see

whether using proteolytic digestion or detergent would enhance the sensitivity of the method; 2) to make preliminary comparisons between 10% neutral buffered Formalin and the combination of paraformaldehyde and glutaraldehyde; and 3) to determine the optimal dilution of primary antiserum.

0.1% trypsin pretreated slides prior to immunoperoxidase steps produced no immunological staining. To prove that the negative results were not due to technical error, the trial was repeated six times according to the criterion of the ABC procedures supplied by the Vector Corporation and again, the outcome was the same. In addition, proteolytic digestion increased the indidence of sections floating off the slides during processing. With the detergent, Triton X 100, pretreatment of sections also interfered with the immunoenzyme reaction by destroying cell surface staining. Some of the membrane associated immunoglobulins were eluted. The detergent [38] increased the permeability of the membrane systems of the cells and promoted the penetration of conjugate into the cells to facilitate the detection of intracellular antigens. It was our desire, however, to demonstration the cell surface antigens. It is possible that the concentration of the detergent was too high and/or remained in contact with the section too long.

Non-specific background stain, or "noise", can make interpretation difficult. The 1:2000 dilution actually eliminated all background stain but the specific immunoreaction was too weak to be accurately determined. The 1:500 dilution had maximum reaction with minimal background stain. Therefore, this dilution of primary

antiserum was used in the subsequent studies.

This preliminary study also showed that 0.2% paraformaldehyde with 0.125% gluaraldehyde fixation produced the poorest result, because this mixture had less penetrating capacity before it reached the center of the specimen, which underwent autolysis. It was apparent that only the peripheral tissue had been fixed after one day. The resulting poor morphology of tissue rendered interpretation equivocal, so this fixative was abandoned for subsequent studies.

GROUPS 2 and 3 RESULTS. Groups 2 and 3 compared the influence of different fixative times from 3 hours to 5 days. In the statistical evaluation the "F" value was not statistically significant. There was no different influence of fixative times in the immunoreaction between these two groups. It also meant that 3 hours minimal fixation already satisfied the requirement of demonstrating the immunoreaction and after 5 days fixation the antigenic determinants of tissue had not been destroyed.

The analysis of variance also showed that the "F" value was significantly different among four fixatives (Tables 1 & 2). Two percent paraformaldehyde was the most ideal fixative in these groups with demonstration of high immunoreactivity with excellent preservation of tissue morphology. The 2% paraformaldehyde mixed with 1.25% glutaraldehyde fixation was unsatisfactory morphologically.

Theoretically, any fixative may compromise demonstration of antigen. Therefore, as controls, frozen sections should be superior to fixed sections. The identification of antigen by the

FITC method on frozen sections served admirably as controls for the ABC method on fixed sections. However, the combination of quick-freezing and the demands of fluorescent staining made tissue morphology of the FITC sections unsuitable for ABC sections.(Fig. 4-7).

GROUP 4 RESULTS. Although it had been shown statistically in Groups 2 and 3 that 2% paraformaldehyde was capable of preserving antigenic determinants, these experiments did not demonstrate possible biological variation between groups of mice. Thus, it was necessary to make a trial of randomized analysis of variance in the Group 4 experiments. The results were similar to those in Groups 2 and 3. Two percent paraformaldehyde had maintained the antigenic determinants as long as 9 days as well as demonstration of immunoreaction (Tab. 3 and Fig. 8).

In the preliminary study, the specimens from Groups 2 and 3 compared the temperature influence of embedding processes between 53°C and 57°C melting point paraffins. The results were not significantly different. This could be taken to mean that the routine paraffin embedding of specimens would not interfere with immunoenzymatic activity.

DISCUSSION

It is well known that fixation and processing will alter antigenicity of immunoglobulins and will mask the majority of antigenic determinants by chemical alteration. This is the reason why the use of frozen sections for immunofluorescence and immunoperoxidase has been empoyed for such a long time. However, not all of the tissue antigenic degerminants are completely destroyed by fixation. Also, effective fixation can help to stop antigens diffusing away from their accustomed sites within tissues while preserving the structural integrity of that tissue.

Acceptable fixation must provide a high degree of availability of antigenic reactive sites in tissues and morphological integrity as well as minimal non-specific background staining.

Several methods of fixation for immunoenzyme procedures are recommended in the literature. Actually, each of those fixative methods is a compromise between the need for good morphological detail and the preservation of enzyme activity. They can be approximately divided into two major categories. One includes aldehydes such as formaldehyde, paraformaldehyde and glutaraldehyde; the other comprises acid-containing fixatives such as Carnoy's, Bouin's B5 fluid and acid alcohol.

In this study, the aldehyde-containing fixatives were chosen because they are almost universally used by clinicians and pathologists. This fact makes it almost mandatory that immunoenzyme methods be applicable to aldehyde-fixed tissues, otherwise the bulk of routinely

obtained human tissues would be unavailable for further immunohistologic evaluation. Tissues so fixed are suitable for light and electron microscopic examination. The preparation of specimens is simple and adequate morphology is obtained. As a rule, glutaraldehyde is not recommended for preserving the antigenicity of immunoglobulins [32]. The potential for tissue antigen reacting with labeled antibody is low with glutaraldehyde and irreversible due to the striking masking of antigenic determinants. The concentration of glutaraldehyde usually considered necessary for ultrastructural preservation [39] renders cells impermeable. If glutaraldehyde is used at all in immunoenzyme methods, only very dilute solution would be used and then, only in combination with paraformaldehyde. The preliminary portion of this study utilized 0.2% paraformaldehyde with 0.125% glutaraldehyde which resulted in poor fixation. Subsequently, 2% paraformaldehyde with 1.25% glutaraldehyde was chosen. Although the fixation of tissue was improved, the immunoreactions were decreased when compared to the other fixatives evaluated.

Ten percent neutral buffered Formalin is a popular fixative. Unexpected negative reactions and the high background staining are occasionally encountered. Formalin can react with tissue protein such as amino, imino, amido, peptide, guanidy, hydroxyl, carboxyl SH and aromatic rings. It can combine with a number of different functional groups forming bridging links (methylene bridges) between adjacent protein chains. Consequently, it promotes polymerization. The specific reaction of Formalin is its addition to a compound containing reactive hydrogen atoms with the

formation of a hydroxymethyl groups and it may then condense with a further hydrogen atom to form methylene. A methylene bridge may be formed between two similar groups, NH_2, or between NH_2 and peptide CONH, or between NH_2 and NH.

Many of the combinations of formalin with tissue proteins are reversible by the simple process of washing. Others are irreversible. It has been suggested that by reducing fixative concentrations and fixation time, adequate preservation of immunoglobulin antigenicity may be attained. Paradoxically, too short a fixation time can lead to diffusion of labile antigens into the surrounding tissue which would cause false negative results or would increase non-specific background staining. Too low a concentration can cause incomplete fixation.

In this study, although 10% neutral buffered formalin was not an ideal fixative, it still preserved an acceptable amount of antigenicity for up to 9 days fixation.

One percent to four percent paraformaldehyde fixative solutions have been recommended as suitable for immunoenzymatic study, either at the light or electron microscopic level.

In the present study, 2% paraformaldehyde was the most ideal fixative compared to the other fixatives studied or to fresh, unfixed frozen sections. It preserved morphological details and maintained a high degree of immunoreactivity.

It has been suggested that the acid-containing fixatives are better

than aldehyde fixatives for preserving immunoglobulin antigenicity. A Formalin-saline combination with acetic acid is considered by some better than simple Formalin. It is possible that the pH of the fixative will influence positively immunoreactions. The pH of each fixative during tissue treatment was determined from 3 hours to 9 days, (Groups 2,3,4) 10% neutral buffered formalin consistently kept its pH within the range of 7.0-7.2 during the testing period, while tissues treated with 2% and 4% paraformaldehyde gradually declined from pH 7.2 to 6.5 which may be advantageous in preserving the antigenicity for relatively longer periods of time.

Four percent paraformaldehyde offered a lower positive immuno-reation rate than 2% paraformaldehyde, because the number of methylene bridges formed depends on the concentration of the formaldehyde fixative. Therefore, the higher the concentration of formaldehyde, the more antigenicity would be masked [40], even though the morphological preservation was excellent.

It is considered that over-fixation would reduce immunoreaction due to masking tissue antigens, particularly in the aldehyde fixative group. This masking can occur after only a few hours in glutaraldehyde [41]. Semiquantitative studies on the influence of various fixatives revealed a decrease of 10% to 20% for every 24 hours, when the fixative time was prolonged [32]. The risk of over-fixation in the use of aldehyde-containing fixatives is higher than acid-containing fixatives. This was not found to be true

in this study. For fixative periods as long as 9 days, the antigenicity of tissue components was preserved, although there was a tendency to decrease the immunoreaction in 10% neutral buffered Formalin from an average 70% positively staining glomeruli after 3 hours of fixation to 50% after 9 days fixation. One can therefore infer that retrospective studies of routinely submitted biopsy specimens, fixed for up to 9 days and embedded in paraffin, may well yield useful immunohistologic findings.

BACKGROUND STAINING. The demonstration of immunoglobulin by immunohistological procedures depends on the development of contrast between the staining of the antigen and its surroundings. Four problems may be encountered [42]: A) Specific background staining. This may result from the presence of the antigen, such as an immunoglobulin, in varying concentrations throughout the material. Diffusion of antigen from intracellular sites prior to fixation or following inadequate fixation contributes to this type of staining. Similarly any degree of inflammation, tissue necrosis or autolysis may produce diffusion of antigens and thus, staining for the specific antigen in interstitial areas, but not restricted to immune complexes. Prompt fixation is therefore critical.

B) Endogenous peroxidase acitivity. Enzyme activity which is naturally present in red blood cells (pseudoperoxidase) granulocytes (myeloperoxidase) and acid haematin can react with 3, 3'diaminobenzidine to produce a brown reaction product that is indistinguishable

from specific immune staining. Methanol treatment combined with very dilute hydrogen peroxide weakens or abolishes endogenous peroxidase activity. Methanol causes the release of heme from hemoproteins. Hydrogen peroxide following methanol treatment causes the denaturation of granulocytic peroxidase. As little as 0.005% hydrogen peroxide in methanol for 30 minutes quenches endogenous peroxidase activity in non-hematopoietic tissue or 3% hydrogen peroxide in methanol with longer bleaching time for hemorrhagic tissue have been used. But the higher concentration methanol-hydrogen peroxide treatment may also denature tissue antigens. Because most of the antigens are protein, methanol can cause precipitation of protein[30] [40].

C) Non-specific background staining. This may occur because of the presence of small amounts of nonspecific antibody within the antiserum. Exploiting the difference in titer between the specific (desired) antibody activity and those of unwanted specificity, diluting the antisera makes it possible to bring out only the desired specificity.

D) Undesirable nonspecific immunoglobulin staining. This problem is most apparent with connective tissue which often displays a very dark brown staining reaction that can obscure any specific staining. The cause of the nonspecific background staining is not completely understood, but it is believed that the most common source is the second bridge antibody. If the bridge antibody does not bind exclusively to the antigenic determinants of the primary antibody but instead binds to other tissue antigens, any free Fab can

can effectively bind the ABC complex, which results in undesirable nonspecific immunoglobulin staining. The problem of nonspecific background staining can be eliminated by using one or more of the following procdures: 1) Apply 1-2% solution of normal serum derived from the species which provided the second bridge antibody. 2) Apply nonspecific blocking agents such as serum albumin which will show the highest level of nonspecific finding to collagen or reticulin. Therefore, most of the sites of nonspecific binding will have been occupied by the initial serum or albumin application to tissue sites and prevent the primary or secondary antibodies from binding non-specifically. Also, the presence of natural antibodies that do not react or interfere with primary antiserum, but can also block tissue sites which the primary antiserum could bind to nonspecifically will be occupied. 3) Use a high dilution of primary antiserum. The antiserum contains natural antibodies which are present in significantly lesser amounts when compared to specific antibody. But, if low dilution primary antisera is used, natural antibodies may effectively compete for the tissue antigen. Therefore, the use of high dilutions of primary antiserum is the most effective means of reducing nonspecific staining. Similarly, if the primary antiserum contains monoclonal antibodies, the non-specific stain will be greatly reduced.[43] In this study, the optimal dilution of primary antiserum was 1:500 which created a markedly positive immoreaction and reduced nonspecific background stain. Serum dilution appropriate for one tissue may not be suitable for another tissue. Selecting the appropriate concentration for a

given tissue gives the greatest useful contrast between specific positive staining and background staining.

GENERAL DISCUSSION OF CONTROLS. Adequate controls are essential in order to avoid false interpretation of immunoenzymatic reactions. The most useful controls are known positive and known negative.

A) Negative controls include: 1) omitting the primary antiserum; 2) using primary antiserum of like specificity but derived from a different species, e.g. primary antiserum derived from rabbit instead of goat-anti-mouse IgG; 3) using antiserum preabsorbed against the specific antigen IgG. This should result in marked reduction of specific staining. The absorbing antigen may contain the same impurities which will absorb out both wanted and unwanted specificty; 4) using non-autoimmune counterparts. B) The positive control is composed of known autoimmune corresponding tissues.

PRESENT STUDY CONTROLS. In the present study, 1 or 2 and 4 procedures were used for the negative control. The cryostat sections were used as baseline positive controls for the experimental ABC technique.

CRYOSTAT CONTROL. The cryostat sections do have a great disadvantage in the formation of ice crystals of unbound water in the cytoplasm of the cells and in the tissue spaces, which create morphologic artifacts. Ice crystals may destroy cell membranes and cause the antigen to diffuse into the interstitium. It is certainly possible to obtain a small piece of tissue in a

condition where no ice crystal artifact is visible by ordinary light or fluorescence mocroscopes. Electron microscopy, however, reveals that ice crystal formation invariably occurs. Although liquid nitrogen has a sufficiently low temperature, its conductivity is low. In spite of an O.C.T. coat, the formation of a layer of vaporized air around the specimen occurs which prevents the transference of heat. There are three zone formations, an out zone in which ice crystals formation is minimal and preservation of structure is excellent, an intermediate zone in which distortion of structure by large crystals is particularly bad and an inner zone of better but relatively indifferent preservation.[44] Even though immunofluorescent techniques have some disadvantages such as frozen specimens requiring specific instrumentation, and sections can not be maintained or stored for future study as easily, it is still a desirable method for identifying antigens in tissues. It apparantly demonstrates the lumpy-bumpy pattern of immunofluorescence better than other methods.

THE ADVANTAGE OF USE OF ABC METHOD. The use of avidin-biotin interaction in immunoenzymatic techniques provides a simple and sensitive method to localize antigens and in the evaluation of different fixatives. The availability of biotin binding sites in the complex is created by the incubation of a relative excess of avidin with biotin labeled peroxidase. The extraordinarily high affinity between avidin and biotin assures a rapidly formed and stable complex between the avidin conjugate and the biotin labeled enzyme.

Biotinylation has minimal effect on biological activities of

proteins and the peroxidase does not contribute to altering the immunoreaction. The sensitivity of the ABC method seems to be less influenced by aldehyde fixative factors than other immunohistologic methods. This highly sensitive method may partially compensate for the effects of the fixative on the visualization of partially masked antigens. Therefore, the ABC method shows great capability for determining antigens in tissues which have been fixed for up to 9 days, routinely processed and embedded in paraffin.

SUMMARY

Five aldehyde-containing fixatives were tested for demonstrating the influence of fixation on the immunoperoxidase reaction in the MRL mouse model. Aldehyde fixatives are the most commonly used fixation techniques in clinical histopathological and electron microscopic examinations. However, it has been suggested in the literature that aldehyde fixatives would mask antigenic determinants. In this study, unmasking of antigens was attained by using a proteolytic enzyme. Trypsin was applied to tissue sections before immunohistochemical staining. It did not appear to increase the sensitivity of the ABC method. In the trial aldehyde fixative group, 2% paraformaldehyde was found to be an ideal fixative for preservation of morphology and antigenicity. Overfixation also was not a significant problem for 2% paraformaldehyde fixation. It could maintain higher levels of immunoreaction for as long as 9 days. Nine days is an adequate period of time for retrospective immunological studies of clinically submitted specimens. During paraformaldehyde fixation, the pH change from neutral to acidity may be responsible for maintaining antigenic integrity.

Although 10% neutral buffered formalin is not a recommended fixative for immunoreactions, it still is an acceptable fixative. Formalin fixed specimens from 3 hours to 3 days demonstrated a high degree of immunoreaction in ABC method. Thereafter, immunoreactivity gradually declined in intensity of immunostaining due to methylene bridge formation. Temperature variation of paraffin processing between $53^{o}C$ and $57^{o}C$ did not significantly influence immunoreactions.

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APPENDIX

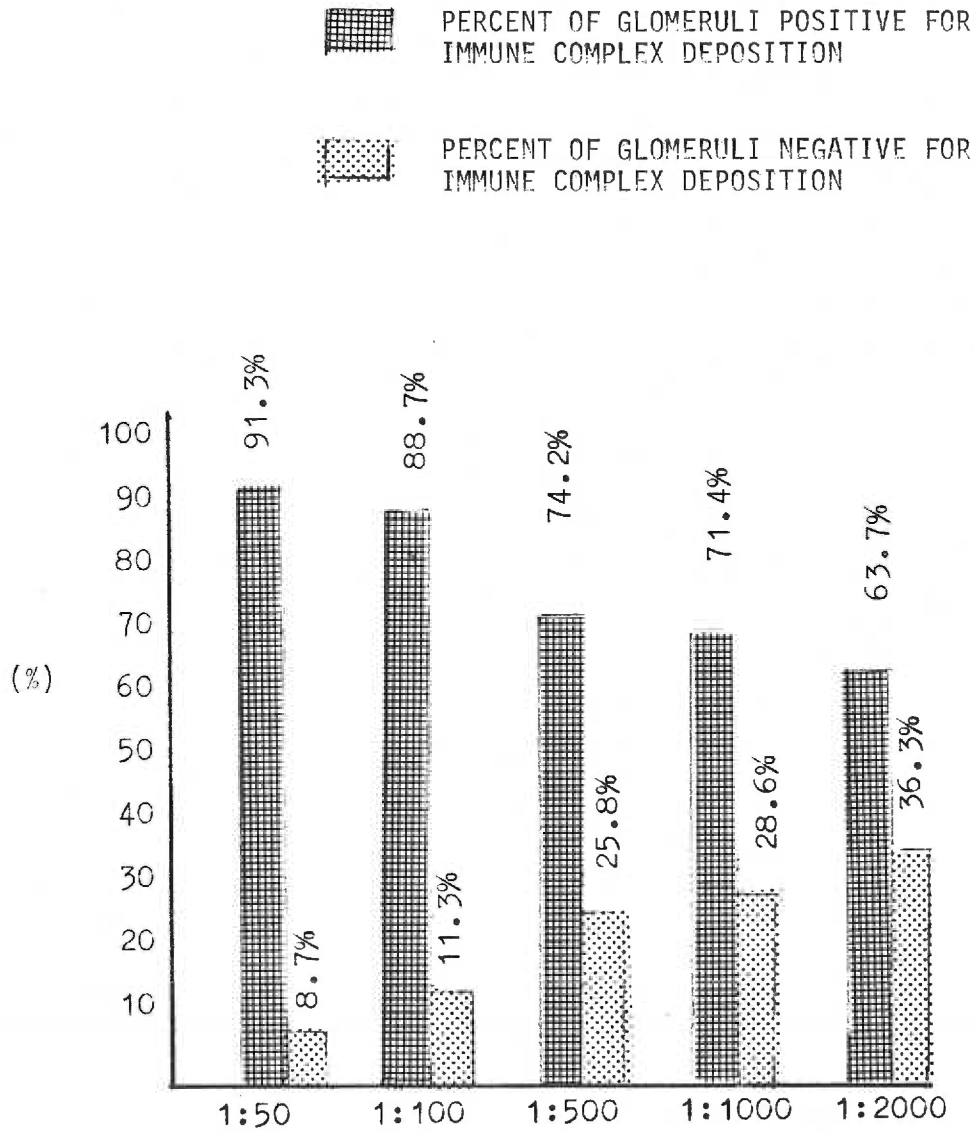


Figure 1. THE INFLUENCE OF DIFFERENT DILUTIONS OF PRIMARY ANTISERUM MOUSE "A" MRL/lpr 6 MONTHS FEMALE 3 HOURS FIXATION 10% NEUTRAL BUFFERED FORMALIN

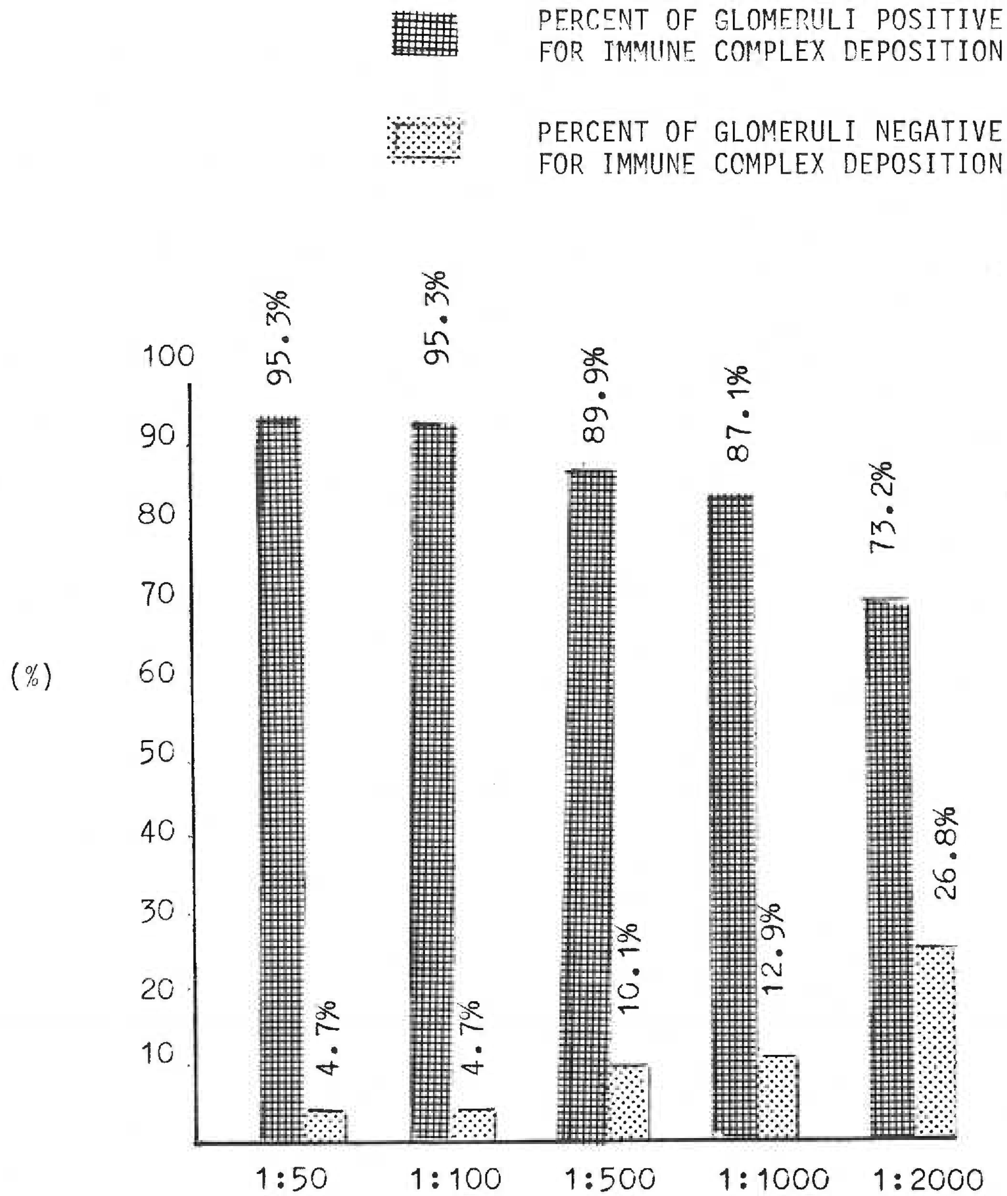


Figure 2. THE INFLUENCE OF DIFFERENT DILUTIONS OF PRIMARY ANTISERUM MOUSE "D" MRL/lpr 4 MONTHS FEMALE 1 DAY FIXATION 2% PARAFORMALDEHYDE

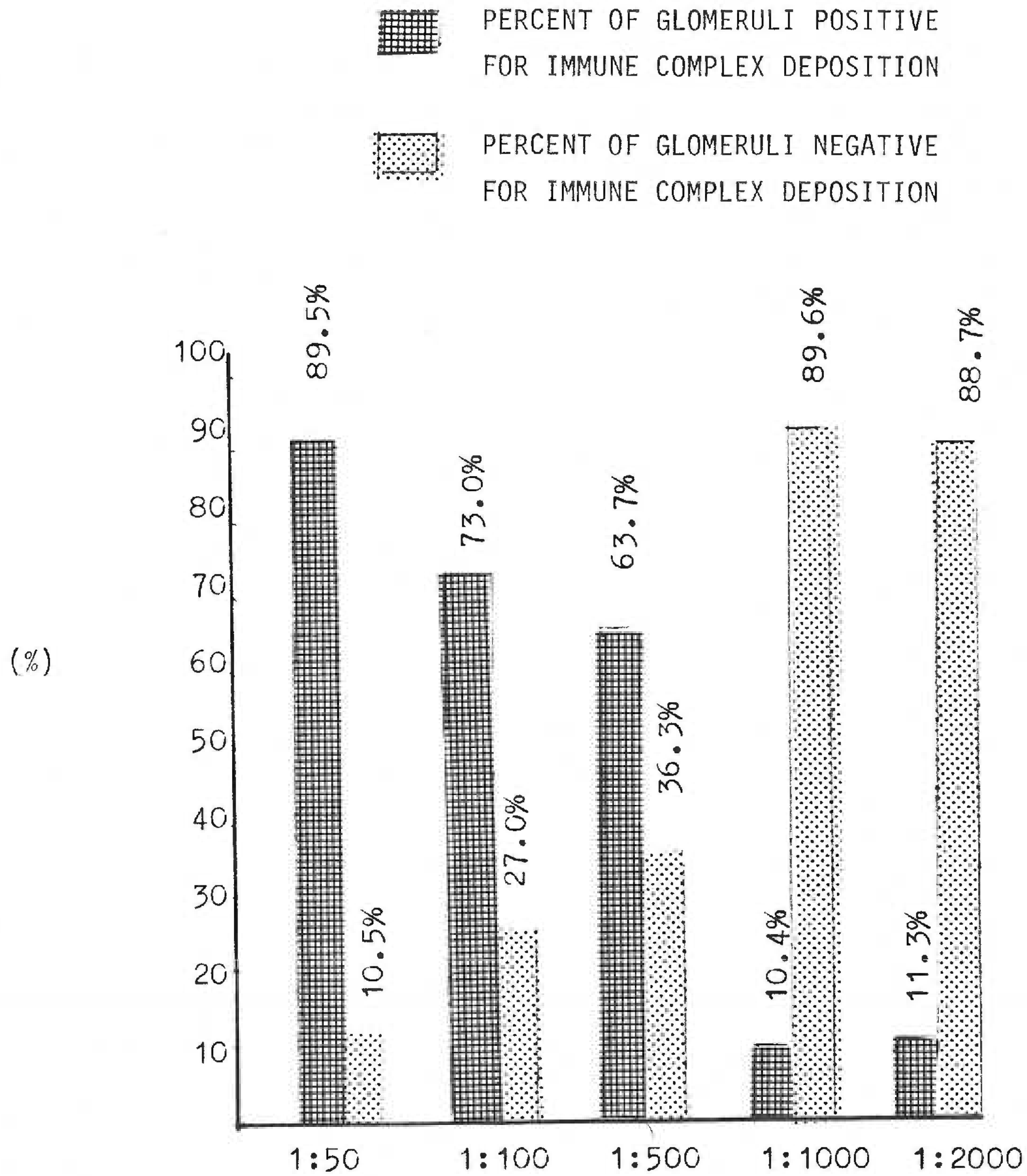


Figure 3. THE INFLUENCE OF DIFFERENT DILUTIONS OF PRIMARY ANTISERUM MOUSE "F" MRL/lpr 4 MONTHS 2 DAYS MALE 5 DAYS FIXATION 4% PARAFORMALDEHYDE

Table 1

TEST FOR FIXATIVE : RAT KIDNEY DATA
ANOVA USING WEIGHTED TOTALS 0, 0, 1, 2, 3

****************** CELL MEANS ************

DEP 1
BY RAT RAT NUMBER
FIX FIXATIVE F1= 10% BUFFERED FORMALIN; F2= 2% PARAFORMALDEHYDE
F3= 4% PARAFORMALDEHYDE; F4= 2% PARAFORMALDEHYDE + 1.25% GLUTARALDEHYDE. F5= CONTROL.

TOTAL POPULATION
133.15
(20)

RAT	1	2	3	4	
	63.20	217.40	157.40	94.60	
	(5)	(5)	(5)	(5)	
FIX	1	2	3	4	5
	187.50	244.50	148.00	55.00	30.75
	(4)	(4)	(4)	(4)	(4)

************** ANALYSIS OF VARIANCE **********************

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	198991.950	7	28427.421	6.403	0.003
RAT	70326.150	3	23442.050	5.280	0.015
FIX	128665.800	4	32166.450	7.246	0.003
EXPLAINED	198991.950	7	28427.421	6.403	0.003
RESIDUAL	53272.600	12	4439.383		
TOTAL	252264.550	19	13277.082		

20 CASES WERE PROCESSED 0 CASES (0.0. PCT) WERE MISSING.

********** MULTIPLE CLASSIFICATION ANALYSIS *********************

GRAND MEAN = 133.15

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
RAT 1	5	-69.95		-69.95			
2	5	84.25		84.25			
3	5	24.25		24.25			
4	5	-38.55		-38.55			

Table 1

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
			0.53		0.53		
FIX 1 10%	4	54.35		54.35			
2 2%	4	111.35		111.35			
3 4%	4	14.85		14.85			
4 2%+1.25%	4	-78.15		-78.15			
5 CONTROL	4	-102.40		-102.40			
			0.71		0.71		
MULTIPLE R SQUARED					0.789		
MULTIPLE R					0.888		

**************** CELL MEANS *************************

DEP 2

BY RAT RAT NUMBER

FIX FIXATIVE

TOTAL POPULATION

60.59

(20)

RAT	1	2	3	4	
	30.22	95.66	71.69	44.80	
	(5)	(5)	(5)	(5)	
FIX	1	2	3	4	5
	84.31	101.30	68.05	30.63	18.66
	(4)	(4)	(4)	(4)	(4)

************* ANALYSIS OF VARIANCE ********************

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	32348.485	7	4621.212	8.439	0.001
RAT	12623.019	3	4207.673	7.683	0.004
FIX	19725.466	4	4931.366	9.005	0.001
EXPLAINED	32348.485	7	4621.212	8.439	0.001
RESIDUAL	6571.580	12	547.632		
TOTAL	38920.064	19	2048.424		

20 CASES WERE PROCESSED 0 CASES (0.0. PCT) WERE MISSING.

Table 1

********* MULTIPLE CLASSIFICATION ANALYSIS **********

GRAND MEAN = 60.59

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
RAT 1	5	-30.37		-30.37			
2	5	35.07		35.07			
3	5	11.09		11.09			
4	5	-15.79		-15.79			
			0.57		0.57		
FIX 1 10%	4	23.72		23.72			
2 2%	4	40.71		40.71			
3 4%	4	7.46		7.46			
4 2%+1.25%	4	-29.96		-29.96			
5 CONTROL	4	-41.93		-41.93			
			0.71		0.71		
MULTIPLE R SQUARED					0.831		
MULTIPLE R					0.912		

Table 1

************* ANALYSIS OF VARIANCE ***************

SOURCE	DF	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB
BETWEEN GROUPS	4	128665.8000	32166.4500	3.904	0.0229
WITHIN GROUPS	15	123598.7500	8239.9167		
TOTAL	19	252264.5500			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINMUM	MAXIUM	95PCT CONF INT FOR MEA
10%	4	187.5000	142.8764	71.4382	83.0000	388.0000	-39.8449 TO 414.8449
2%	4	244.5000	98.1105	49.0552	99.0000	308.0000	88.3866 TO 400.6134
4%	4	148.0000	93.9752	46.9876	63.0000	254.0000	-1.5333 TO 297.5333
2%+1.25 %	4	55.0000	46.2673	23.1337	7.0000	106.0000	-18.6206 TO 128.6206
CONTROL	4	30.7500	13.7204	6.8602	12.0000	41.0000	8.9181 TO 52.5819
TOTAL	20	133.1500	115.2262	25.7654	7.0000	388.0000	79.2225 TO 187.0775
FIXED EFFECTS MODEL			90.7740	20.2977			89.8865 TO 176.4135
RANDOM EFFECTS MODEL				40.1039			21.8055 TO 244.4945

RANDOM EFFECTS MODEL-ESTIMATE OF BETWEEN COMPONENT VARIANCE 5981.6333

**************** MULTIPLE RANG TEST ********************

TUKEY-HSD PROCEDURE

RANGES FOR THE 0.050 LEVEL

4.37 4.37 4.37 4.37

THE RANGES ABOVE ARE TABLE RANGES THE VALUE ACTUALLY COMPARED WITH MEAN(J) -MEAN(I) IS 64.1869* RANGE *SQRT(I/N(I) + I/N(J) =177.402

HOMOGENEOUS SUBSETS (SUBSETS OF GROUPS WHOSE HIGHEST AND LOWEST MEANS DO NOT DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE)

SUBSET 1

GROUP	CONTROL	2%+1.25%	4%	10%
MEAN	30.7500	55.0000	148.0000	187.5000

SUBSET 2

GROUP	2%+1.25%	4%	10%	2%
MEAN	55.0000	148.0000	187.5000	244.5000

Table 1

************ MULTIPLE RANGE TEST *************

SCHEFFE PROCEDURE

SUBSET 1

GROUP	CONTROL	2%+1.25%	4%	10%	2%
MEAN	30.7500	55.0000	148.0000	187.5000	244.5000

************* ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB
BETWEEN GROUP	4	19725.4659	4931.3665	3.854	0.0239
WITHIN GROUP	15	19194.5984	1279.6399		
TOTAL	19	38920.0643			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUN	MAXIMUM	95PCT CONF INT FOR MEAN
10%	4	84.137	54.9441	27.4720	39.2881	161.3431	-3.1133 TO 171.7407
2%	4	101.3034	37.8744	18.9372	45.1786	127.9289	41.0376 TO 161.5691
4%	4	68.0522	39.0871	19.5435	35.0459	112.0414	5.8568 TO 130.2476
2%+1.25 %	4	30.6312	19.2846	9.3423	13.4279	52.6599	-0.0545 TO 61.3169
CONTROL	4	18.6644	6.7215	3.3607	10.4198	24.3355	7.9691 TO 29.3596
TOTAL	20	60.5930	45.2595	10.1203	10.4198	161.3431	39.4109 TO 81.7751
FIXED EFFECTS MODEL			35.7721	7.9989			43.5438 TO 77.6422
RANDOM EFFECTS MODEL				15.7025			16.9966 TO 104.1894

RANDOM EFFECTS MODEL-ESTIMATE OF BETWEEN COMPONENT VARIANCE 912.9316

************* MULTIPLE RANGE TEST *****************

TUKEY-HSD PROCEDURE

SUBSET 1

GROUP	CONTROL	2%+1.25%	4%	10%
MEAN	18.6644	30.6312	68.0522	84.3137

SUBSET 2

GROUP	2%+1.25%	4%	10%	2%
MEAN	30.6312	68.0522	84.3137	101.3034

*********** MULTIPLE RANGE TEST ******************

SCHEFFE PROCEDURE

SUBSET 1

GROUP	CONTROL	2%+1.25%	4%	10%	2%
MEAN	18.6644	30.6312	68.0522	84.3137	101.3034

Table 2

TEST FOR TIME: RAT KIDNEY DATA.
ANOVA USING WEIGHTED TOTALS 0, 0, 1, 2, 3

************** CELL MEANS *****************

DEP 1
BY RAT RAT NUMBER
TIME T1= 3 HOURS; T2= 1 DAY; T3= 3 DAYS; T4= 5 DATS; T5= CONTROL.

TOTAL POPULATION

112.90
(20)

RAT	1	2	3	4	
	124.00	160.80	123.60	43.20	
	(5)	(5)	(5)	(5)	
TIME	1	2	3	4	5
	81.25	140.75	137.25	138.50	66.75
	(4)	(4)	(4)	(4)	(4)

*************** ANALYSIS OF VARIANCE ***********************

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	57572.800	7	8224.686	3.056	0.043
RAT	36951.000	3	12317.000	4.577	0.023
TIME	20621.800	4	5155.450	1.916	0.172
EXPLAINED	57572.800	7	8224.686	3.056	0.043
RESIDUAL	32295.000	12	2691.250		
TOTAL	89867.800	19	4729.884		

20 CASES WERE PROCESSED 0 CASES (0.0 PCT) WERE MISSING.

********** MULTIPLE CLASSIFICATION ANALYSIS *****************

GRAND MEAN = 112.90

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
RAT 1	5	11.10		11.10			
2	5	47.90		47.90			
3	5	10.70		10.70			
4	5	-69.70		-69.70			
			0.64		0.64		

Table 2

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	ETA	ADJUSTED FOR INDEPENDENTS DEV'N	BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	BETA
TIME 1 3 HOURS	4	-31.65		-31.65			
2 1 DAY	4	27.85		27.85			
3 3 DAYS	4	24.35		24.35			
4 5 DAYS	4	25.60		25.60			
5 CONTROL	4	-46.15		-46.15			
			0.48		0.48		
MULTIPLE R SQUARED					0.641		
MULTIPLE R					0.800		

*************** CELL MEANS ********************

DEP 2

BY RAT RAT NUMBER

TIME

TOTAL POPULATION

53.44

(20)

RAT	1	2	3	4	
	60.88	69.08	59.66	24.16	
	(5)	(5)	(5)	(5)	
TIME	1	2	3	4	5
	39.56	68.39	64.83	61.72	32.71
	(4)	(4)	(4)	(4)	(4)

*********** ANALYSIS OF VARIANCE ****************

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	10156.600	7	1450.943	2.860	0.053
RAT	5980.239	3	1993.413	3.929	0.036
TIME	4176.361	4	1044.090	2.058	0.150
EXPLAINED	10156.600	7	1450.943	2.860	0.053
RESIDUAL	6088.625	12	507.385		
TOTAL	16245.225	19	855.012		

20 CASES WERE PROCESSED 0 CASES (0.0. PCT) WERE MISSING.

Table 2

********* MULTIPLE CLASSIFICATION ANALYSIS **********

GRAND MEAN = 53.44

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
RAT 1	5	7.44		7.44			
2	5	15.63		15.63			
3	5	6.21		6.21			
4	5	-29.29		-29.29			
			0.61		0.61		
TIME 1 3 HOURS	4	-13.88		-13.88			
2 1 DAY	4	14.94		14.94			
3 3 DAYS	4	11.39		11.39			
4 5 DAYS	4	8.28		8.28			
5 CONTROL	4	-20.73		-20.73			
			0.51		0.51		
MULTIPLE R SQUARED					0.625		
MULTIPLE R					0.791		

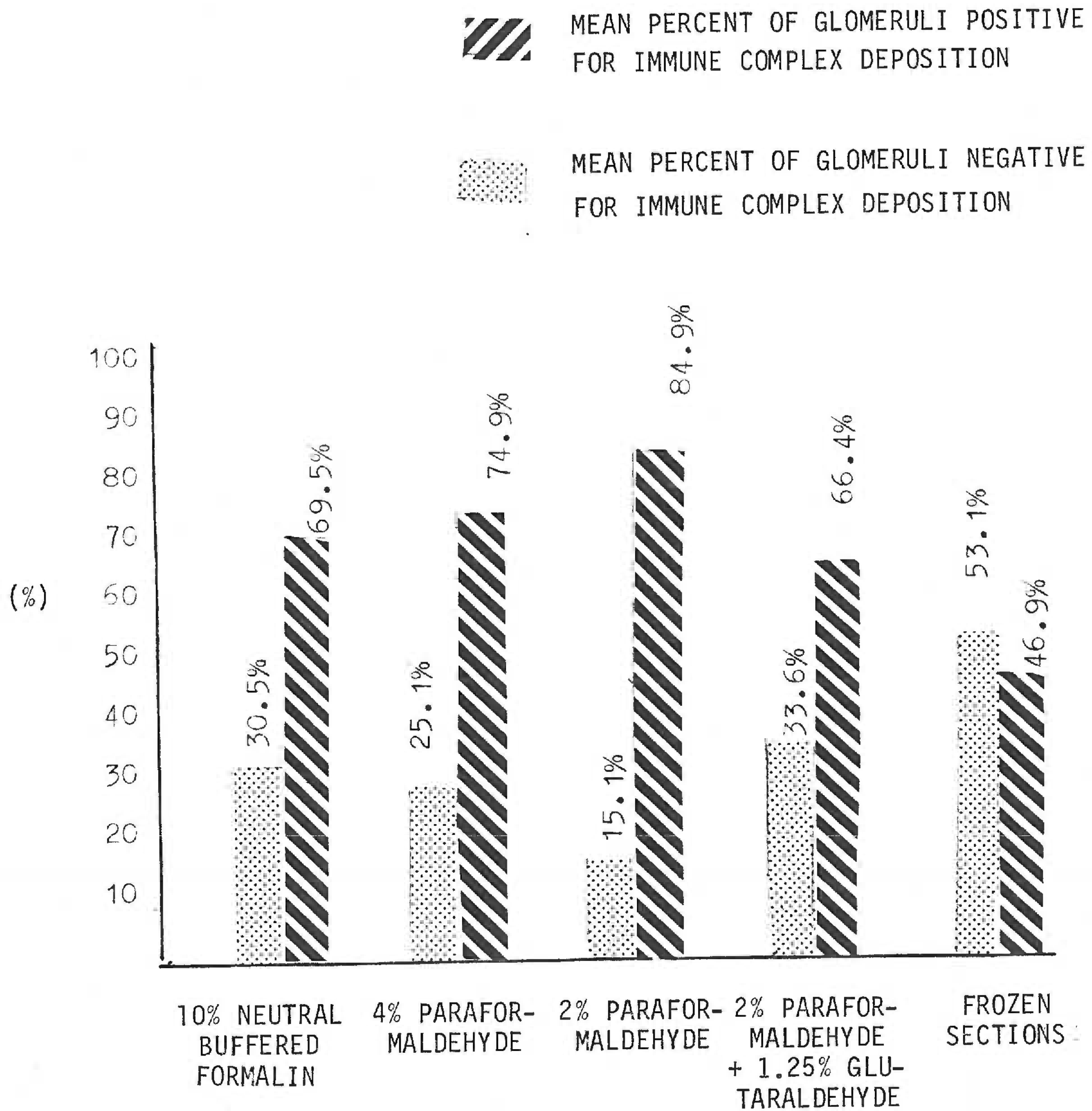


Figure 4. INFLUENCE OF FIXATION ON IMMUNE COMPLEX DEPOSITION (GROUPS 2 AND 3) 3 HOURS FIXATION

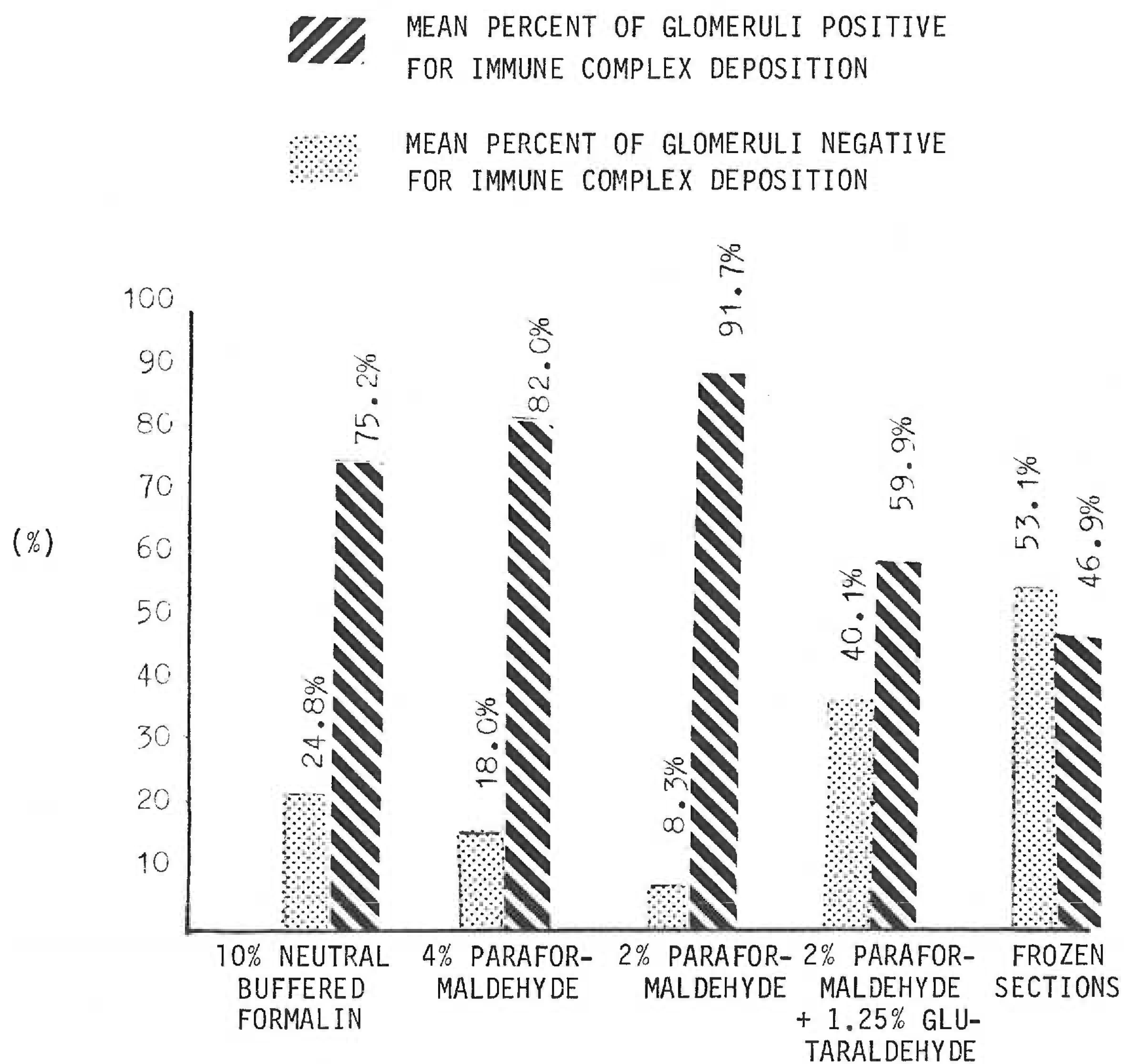


Figure 5. INFLUENCE OF FIXATION ON IMMUNE COMPLEX DEPOSITION (GROUPS 2 AND 3) 1 DAY FIXATION

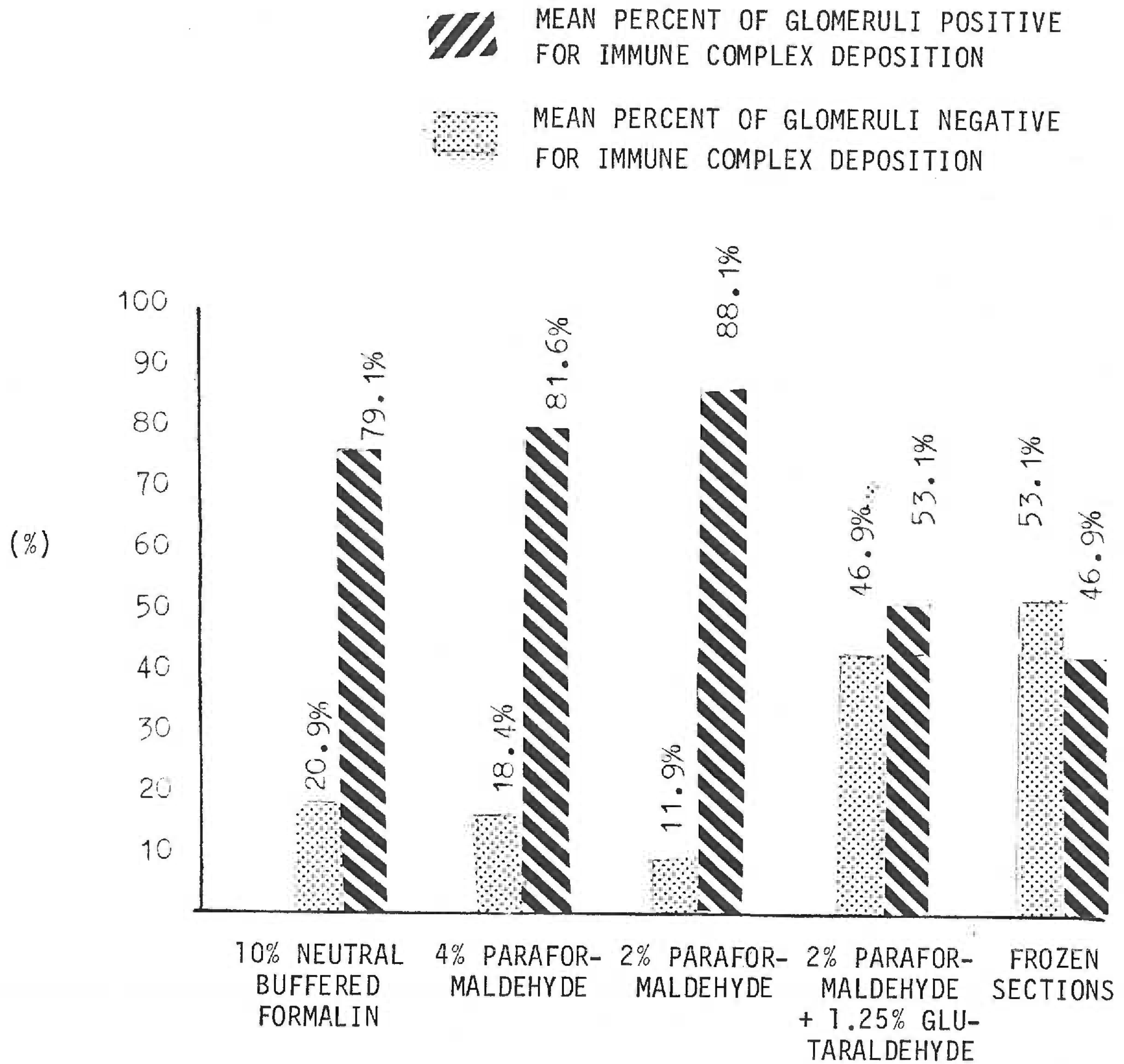


Figure 6. INFLUENCE OF FIXATION ON IMMUNE COMPLEX DEPOSITION (GROUPS 2 AND 3) 3 DAYS FIXATION

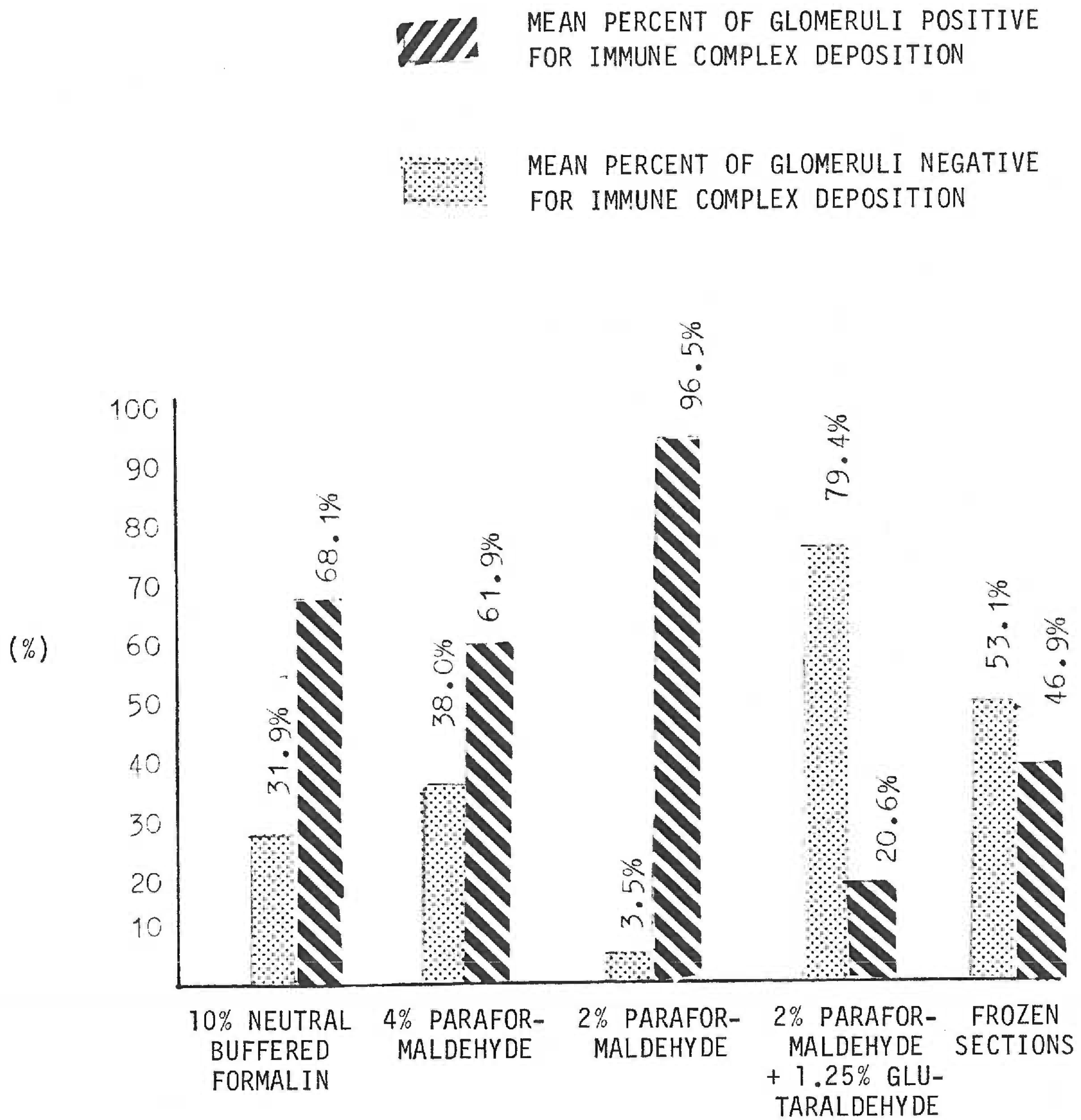


Figure 7. INFLUENCE OF FIXATION ON IMMUNE COMPLEX DEPOSITION (GROUPS 2 AND 3) 5 DAYS FIXATION

Table 3

ANOVA OF RAT KIDNEY DATA

ANOVA USING WEIGHTED TOTALS 0, 0, 1, 2, 3

* * * * * * * CELL MEANS * * * * * * * * * *

DEP 1

BY REP RAT NUMBER

F FIXATIVE F1= 10% BUFFERED FORMALIN; F2= 2% PARAFORMALDEHYDE; F3= 4% PARAFORMALDEHYDE.

T TIME T1= 3 HOURS; T2= 3 DAYS; T3= 9 DAYS.

TOTAL POPULATION

0.87
(45)

REP	1	2	3	4	5
	1.22	1.09	0.79	0.61	0.66
	(9)	(9)	(9)	(9)	(9)

F	1	2	3
	0.69	1.05	0.89
	(15)	(15)	(15)

T	1	2	3
	0.95	0.86	0.82
	(15)	(15)	(15)

REP \ F	1	2	3
1	0.97	1.45	1.23
	(3)	(3)	(3)
2	0.82	1.29	1.17
	(3)	(3)	(3)
3	0.64	0.90	0.84
	(3)	(3)	(3)
4	0.66	0.70	0.46
	(3)	(3)	(3)
5	0.34	0.91	0.73
	(3)	(3)	(3)

Table 3

F \ T	1	2	3
1	0.66	0.78	0.61
	(5)	(5)	(5)
2	1.23	0.90	1.03
	(5)	(5)	(5)
3	0.97	0.88	0.81
	(5)	(5)	(5)

******** ANALYSIS OF VARIANCE ***********

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	3.743	8	0.468	4.683	0.004
REP	2.587	4	0.647	6.473	0.003
F	1.002	2	0.501	5.016	0.020
T	0.154	2	0.077	0.770	0.480
2-WAY INTERACTIONS	2.157	20	0.108	1.080	0.444
REP F	0.428	8	0.053	0.535	0.813
REP T	1.461	8	0.183	1.828	0.145
F T	0.268	4	0.067	0.671	0.621
EXPLAINED	5.900	28	0.211	2.109	0.060
RESIDUAL	1.598	16	0.100		
TOTAL	7.498	44	0.170		

45 CASES WERE PROCESSED 0 CASES (0.0 PCT) WERE MISSING

******** MULTIPLE CLASSIFICATION ANALYSIS **********

GRAND MEAN = 0.87

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	ETA	ADJUSTED FOR INDEPENDENTS DEV'N	BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	BETA
REP							
1	9	0.34		0.34			
2	9	0.22		0.22			
3	9	-0.08		-0.08			
4	9	-0.27		-0.27			
5	9	-0.21	0.59	-0.21	0.59		
F							
1	15	-0.19		-0.19			
2	15	0.18		0.18			
3	15	0.01		0.01			

Table 3

VARIABLE + CATEGORY	N	UNADJUSTED DEV'N	ETA	ADJUSTED FOR INDEPENDENTS DEV'N	BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	BETA
			0.37		0.37		
T_1	15	0.08		0.08			
2	15	-0.02		-0.02			
3	15	-0.06		-0,06			
			0.14		0.14		
MULTIPLE R SQUARED					0.499		
MULTIPLE R					0.706		

ANOVA OF RAT KIDNEY DATA
ANOVA USING RIDIT-WEIGHTED TOTALS
************* CELL MEANS ***********

DEP 2
BY REP RAT NUMBER
F FIXATIVE
T TIME

TOTAL POPULATION
0.58
(45)

REP	1	2	3	4	5
	0.68	0.66	0.58	0.49	0.51
	(9)	(9)	(9)	(9)	(9)

F	1	2	3
	0.52	0.65	0.59
	(15)	(15)	(15)

T	1	2	3
	0.61	0.60	0.54
	(15)	(15)	(15)

REP	F	1	2	3
	1	0.62	0.74	0.68
		(3)	(3)	(3)
	2	0.59	0.71	0.69
		(3)	(3)	(3)
	3	0.50	0.62	0.61
		(3)	(3)	(3)

4	0.51	0.54	0.41
	(3)	(3)	(3)
5	0.37	0.62	0.55
	(3)	(3)	(3)

REP \ T	1	2	3
1	0.73	0.59	0.72
	(3)	(3)	(3)
2	0.66	0.64	0.69
	(3)	(3)	(3)
3	0.53	0.58	0.62
	(3)	(3)	(3)
4	0.58	0.59	0.30
	(3)	(3)	(3)
5	0.56	0.59	0.39
	(3)	(3)	(3)

F \ T	1	2	3
1	0.50	0.57	0.48
	(5)	(5)	(5)
2	0.71	0.61	0.62
	(5)	(5)	(5)
3	0.63	0.61	0.53
	(5)	(5)	(5)

************ ANALYSIS OF VARIANCE ****************

SOURCE OF VARIATION	SUM OF SQUARES	DF	MEAN SQUARE	F	SIGNIF OF F
MAIN EFFECTS	0.436	8	0.055	5.787	0.001
REP	0.263	4	0.066	6.975	0.002
F	0.132	2	0.066	6.997	0.007
T	0.041	2	0.021	2.200	0.143
2-WAY INTERACTIONS	0.359	20	0.018	1.904	0.098
REP F	0.079	8	0.010	1.045	0.444
REP T	0.241	8	0.030	3.198	0.023
F T	0.039	4	0.010	1.032	0.421
EXPLAINED	0.795	28	0.028	3.013	0.012
RESIDUAL	0.151	16	0.009		
TOTAL	0.946	44	0.022		

45 CASES WERE PROCESSED 0 CASES (0.0 PCT) WERE MISSING

Table 3

***** MULTIPLE CLASSIFICATION ANALYSIS **********

GRAND MEAN = 0.58

VARIABLE + CATEGORY		N	UNADJUSTED DEV'N	UNADJUSTED ETA	ADJUSTED FOR INDEPENDENTS DEV'N	ADJUSTED FOR INDEPENDENTS BETA	ADJUSTED FOR INDEPENDENTS + COVARIATES DEV'N	ADJUSTED FOR INDEPENDENTS + COVARIATES BETA
REP	1	9	0.09		0.09			
	2	9	0.08		0.08			
	3	9	-0.01		-0.01			
	4	9	-0.09		-0.09			
	5	9	-0.07		-0.07			
				0.53		0.53		
F	1	15	-0.07		-0.07			
	2	15	0.06		0.06			
	3	15	0.00		0.00			
				0.37		0.37		
T	1	15	0.03		0.03			
	2	15	0.01		0.01			
	3	15	-0.04		-0.04			
				0.21		0.21		
MULTIPLE R SQUARED						0.461		
MULTIPLE R						0.679		

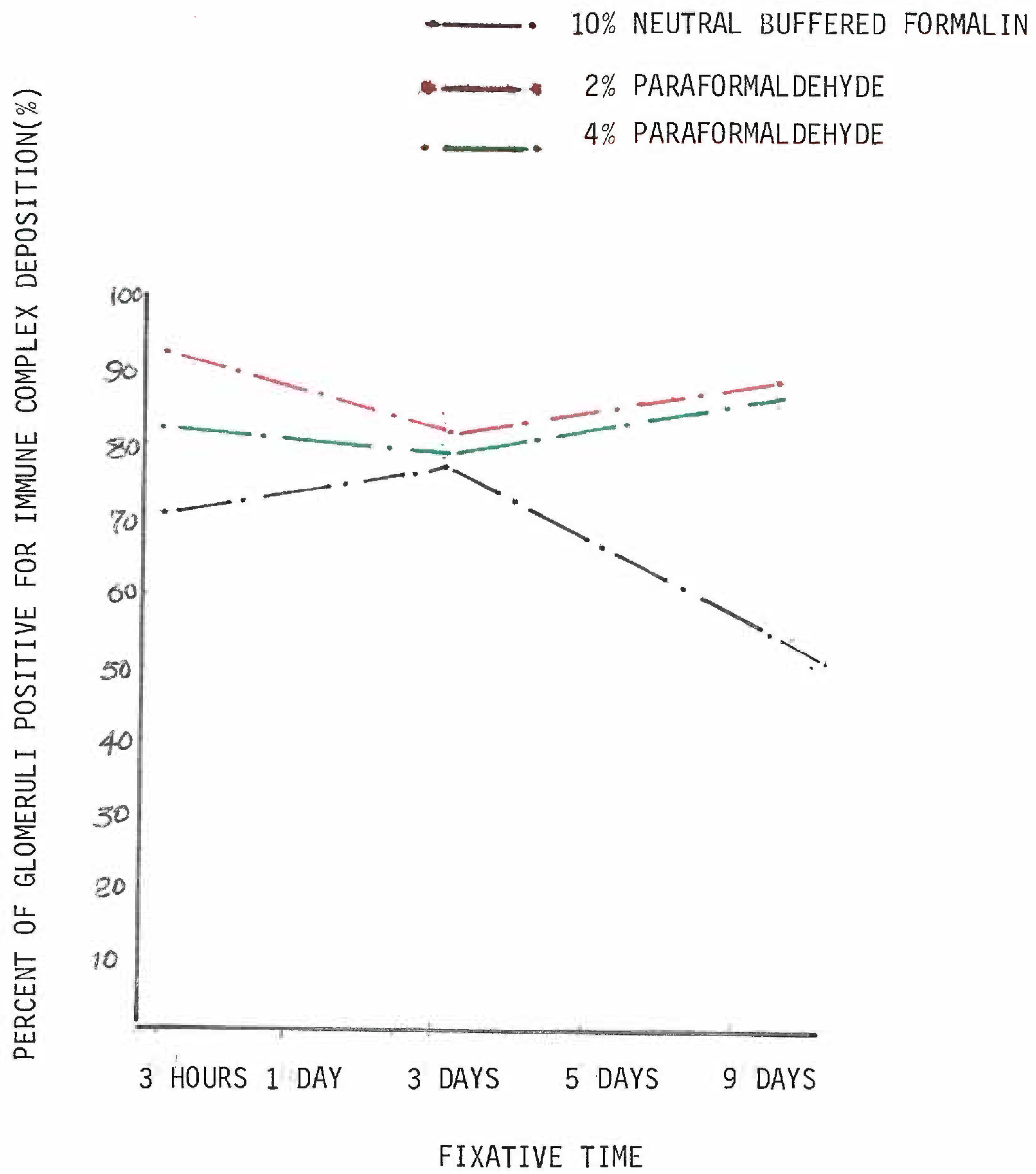
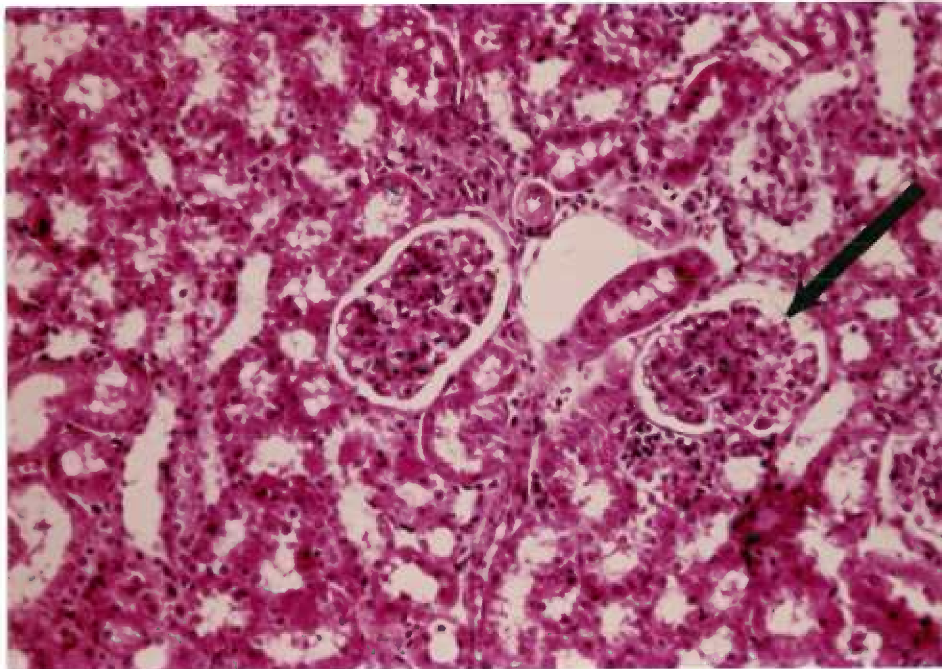
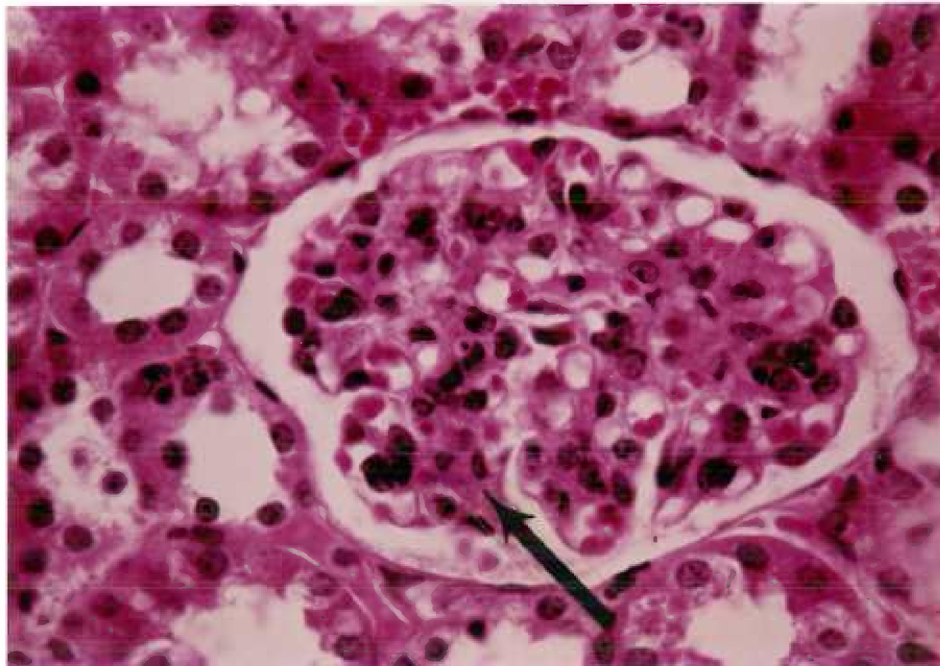


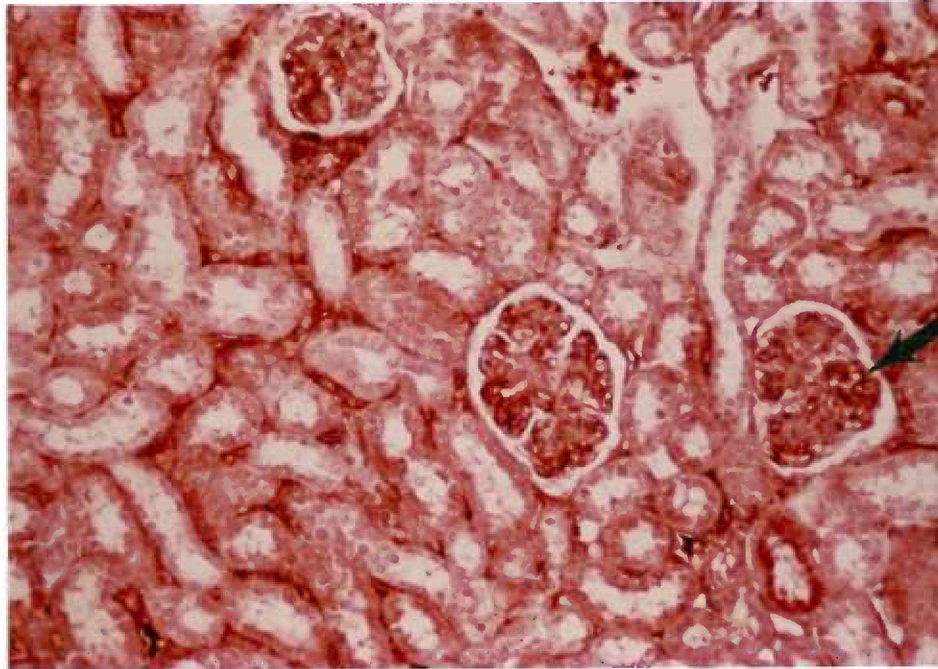
Figure 8



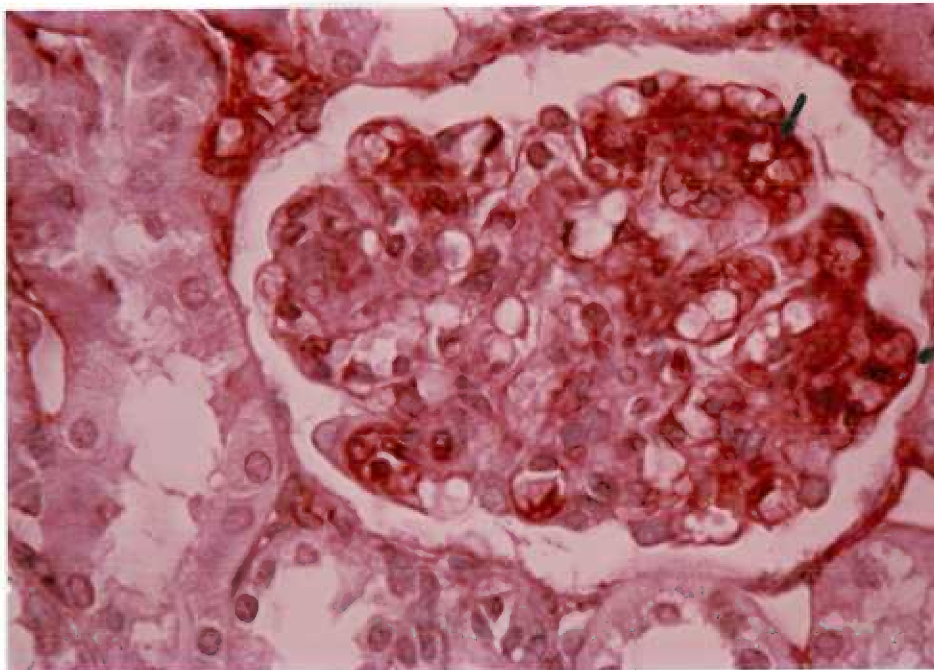
P1 MOUSE "I" GLOMERULI FEMALE SACRIFICED AT 4 2/31 MONTHS, H&E STAIN, THE PICTURE SHOWS SUBACUTE PROLIFERATIVE GLOMERULONEPHRITIS 25 X 10X



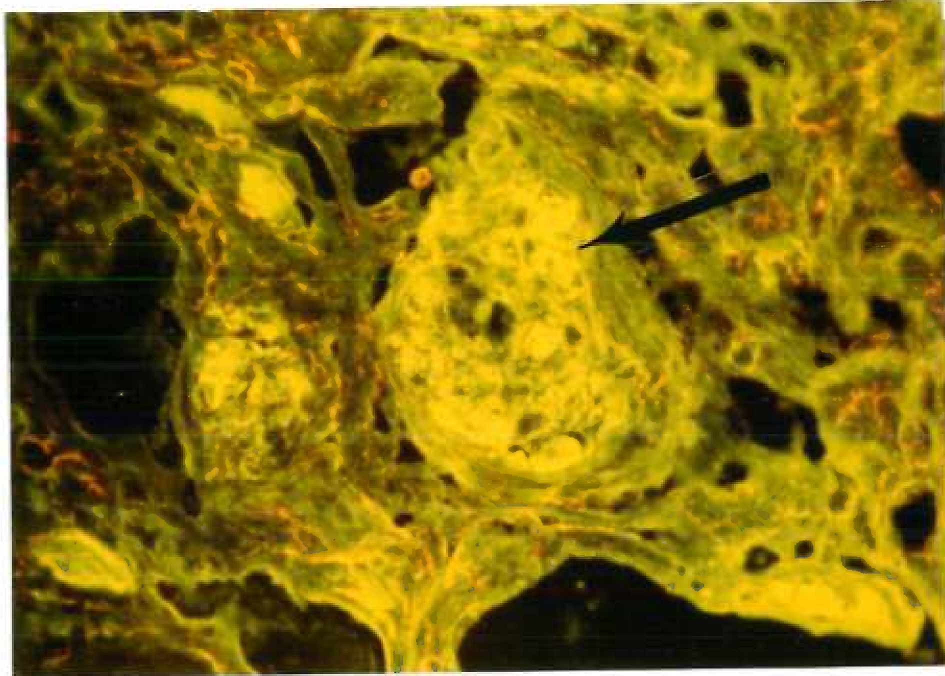
P2 MOUSE "I" GLOMERULI, H&E STAIN, THE PICTURE SHOWS SUBACUTE PROLIFERATIVE GLOMERULONEPHRITIS WITH ENDOTHELIAL AND MESANGIAL PROLIFERATION AND BASEMENT MEMBRANE THICKENING. 25 X 40X



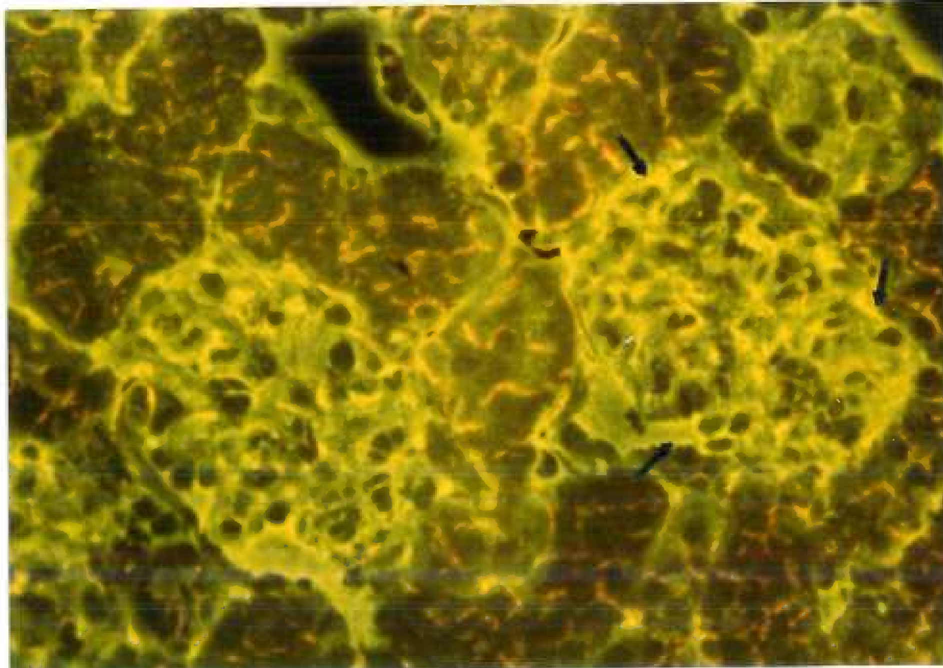
P3 MOUSE "I" GLOMERULI, 2% PARAFORMALDEHYDE FIXATION, 5 DAYS
ABC IMMUNOSTAIN, ANTI-MOUSE IgG PRIMARY ANTISERUM, 1:500 DILUTION,
THE PICTURE SHOWS BROWN MEMBRANOUS OR GRANULAR DEPOSITS ON THICKEN
ED BASEMENT MEMBRANE. 25 X 10X



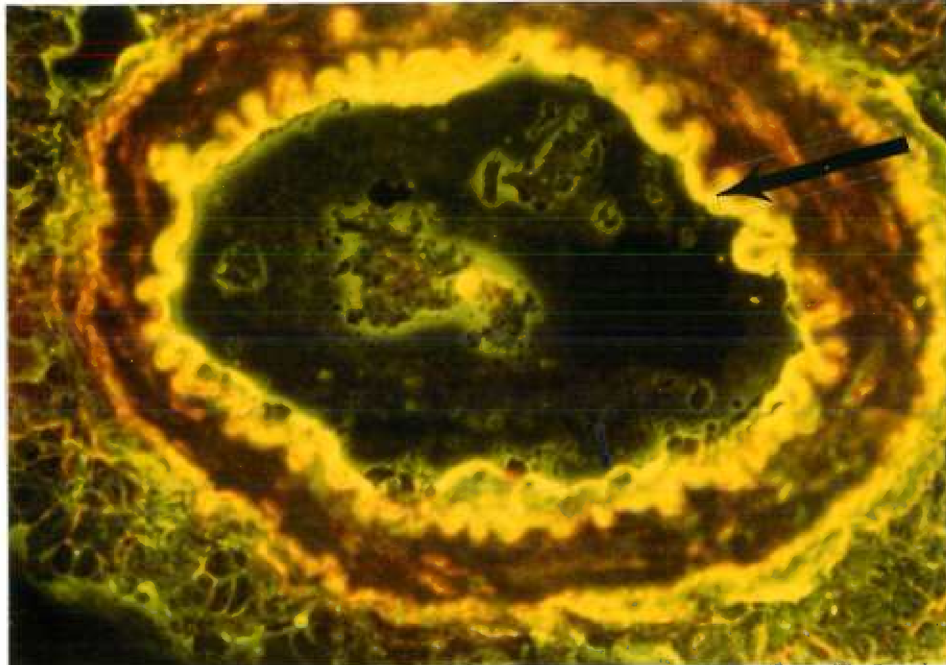
P4 MOUSE "I" GLOMERULES, 2% PARAFORMALDEHYDE FIXATION, 5 DAYS ABC
IMMUNOSTAIN, ANTI-MOUSE IgG PRIMARY ANTI-SERUM 1:500 DILUTION,
THE PICTURE SHOWS BROWN MEMBRANOUS OR GRANULAR DEPOSITS ON THICKENED
BASEMENT MEMBRANE. 25 X 40X



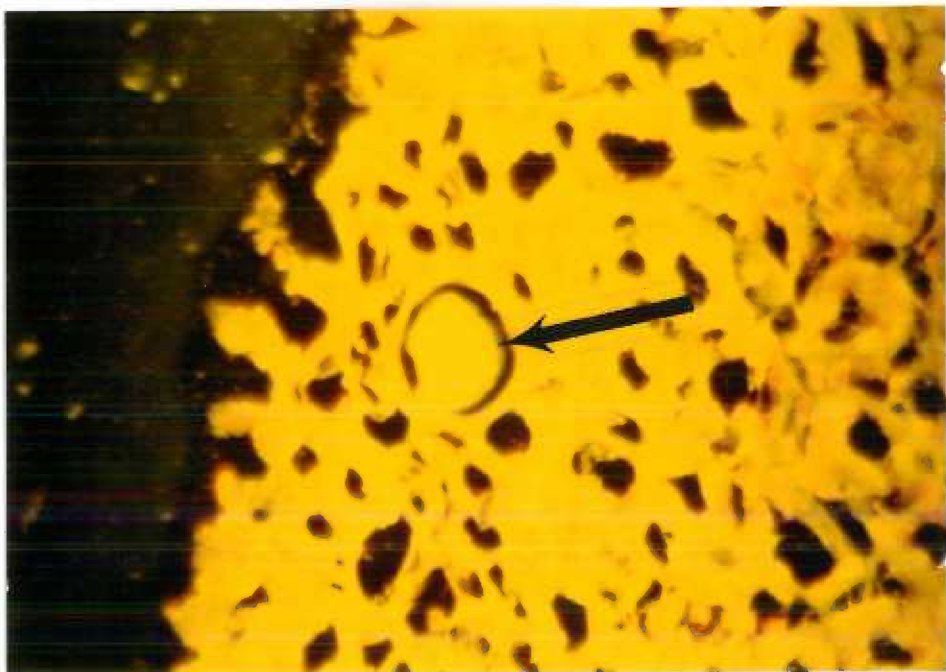
P5 MOUSE "I" GLOMERULI, FROZEN SECTION WITH DIRECT FITC CONJUGATED ANTI-MOUSE IgG, 1:80 DILUTION, THE PICTURE SHOWS HIGH IMMUNO-FLUORESCENCE ON THICKENING BASEMENT MEMBRANE OF GLOMERULI. 25X40X.



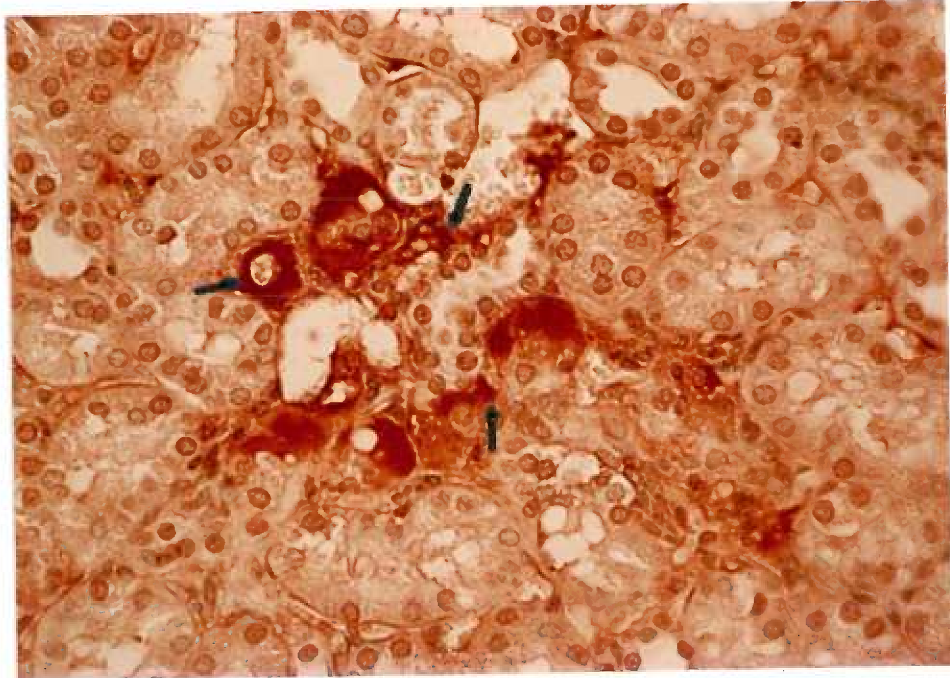
P6 MOUSE "I" GLOMERULI, FROZEN SECTION WITH DIRECT FITC CONJUGATED ANTI-MOUSE IgG, 1:80 DILUTION, THE PICTURE SHOWS HIGH IMMUNOFLUOR-ESCENCE ON THICKENING BASEMENT MEMBRANE OF GLOMERULI. 25 X 40X.



P7 MOUSE "F" KIDNEY SACRIFICED AT 4 18/31 MONTHS, MALE, FROZEN SECTION WITH DIRECT FITC CONJUGATED ANTI-MOUSE IgG 1:80 DILUTION THE PICTURE SHOWS HIGH IMMUNOFLUORESCENCE ON THICKENED BASEMENT MEMBRANE OF A SMALL ARTERY. 25 X 40X



P8MOUSE "F" KIDNEY, FROZEN SECTION WITH FITC CONJUGATED ANTI-MOUSE IgG, 1:80 DILUTION, THE PICTURE SHOWS A CAST IN A DILATED TUBULE 25 X 40X



P9 MOUSE "G" KIDNEY, FEMALE, SACRIFICED at 4 2/31, 10% NEUTRAL BUFFERED FORMALIN, 3 HOURS FIXATION, ABC IMMUNOSTAIN ANTI-MOUSE IgG PRIMARY ANTISERUM, 1:500 DILUTION. THE PICTURE SHOWS BROWN MEMBRANOUS DEPOSITS ON INTERSTITIAL VESSELS ALSO SHOWS NONSPECIFIC BACKGROUND STAIN.