Resin embedment of organs and postembedment localization of antigens by immunoperoxidase methods

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Summary

Various procedures for nonpolar and polar resin embedment were applied to mouse and rat livers for the study of postembedment immunolocalization of alpha$_1$-fetoprotein, albumin and the microsomal enzyme epoxide hydrolase. Fixations with formaldehyde and with formaldehyde-glutaraldehyde mixtures were used for tissue stabilization. Both fixation schedules did not abolish immunoreactivity. Treatment of liver with inert compounds such as polyvinyl-pyrrolidones or chemical modification of antigens with ethyl acetimidate prior to embedment improved immuno-staining. Either the low-polarity solvent ethanol or the high polar ethylene glycol could be employed as dehydrating agents. Antigens were readily localized in sections from Epon 812 embedded livers. For this purpose, polymerized resin had to be partially removed. On the other hand, immunoreactivity of antigens was only faint after embedment in an epoxy resin based on diepoxide octane. Also, antigens reacted faintly in sections from livers which were embedded at 0° C in the polar acrylate-methacrylate based Lowicryl K4M resin. The indirect peroxidase labelled antibody method was as specific and sensitive as the PAP technique. Optimal antigen detection was attained with antibodies isolated by affinity chromatography and purified conjugates. Apart from purified immunological reagents, the addition of high molarity sodium chloride and bovine serum albumin to the wash solutions enhanced immunohistological specificity.

Introduction

Immunological ligand assays in histology enable relationships between biological structure and function to be readily discerned. For this purpose, distinct tissue stabilization and its subsequent processing must be reconciled. Paraffin embedment ist often a reliable method for immuno-staining of intracellular antigens at the light microscopic level. In the case of immuno-electron microscopy of antigens, the latter can be sometimes detected in sections from resin embedded tissue (postembedment procedure) or in ultrathin frozen sections (cryoultramicrotomy), but often special tissue processing and immuno-staining prior to resin embedment (preembedment procedure) is necessary for successful immunolocalization (Kuhlmann et al. 1974; Kuhlmann 1977; Kuhlmann 1980).

The logical way to render intracellular components accessible to immunocytological reagents would be to use sections prepared from embedded tissue. Thus, labelling of intra-

cellular substances can be regarded as independent of reagent penetration. This assumption is correct in principle, but successful antigen staining is only possible if antigenic determinants reach the section surface and if these are still able to bind specifically with antibodies. Some problems involved in postembedment immunohistology were discussed earlier (Kuhlmann et al. 1974; Vogt et al. 1976; Kuhlmann 1977). Typical electron microscopic resin embedment has not been used routinely up to now for antigen staining in semithin and ultrathin sections even if satisfactory immuno-staining is achieved in some models, e.g. peptide hormones (Moriarty 1973; Martin et al. 1979). The latter are now being studied in numerous laboratories.

Because of the general importance and advantage of resin embedment of cells for light and electron microscopic studies, we studied conditions of tissue embedment for a wider application of immunohistology. For this purpose, resin embedments with the widely used Epon 812, with a low-viscosity epoxy resin diepoxide octane (DEO) and the new polar methacrylate based Lowicryl K4M resin were tested. Apart from organ fixation, the influence on the quality of immunoreactivity of cellular components in semithin sections of tissue conditioning by inert filier, chemical modification of antigens, dehydration and resin curing were examined. Three intracellular antigens in rat and mouse hepatocytes served as models: alpha$_1$-fetoprotein (AFP), albumin and the microsomal enzyme epoxide hydrolase (EH). The indirect peroxidase labelled antibody method and the PAP technique (Sternberger 1974) were compared.

Materials and Methods

Animal Models. Experiments were performed with BALB/c/J mice and Wistar rats. Regenerating mouse livers after acute CCl_4 intoxication (Kuhlmann 1979) were removed for AFP and albumin localization. EH was stained in preneoplastic and neoplastic rat livers (Kuhlmann et al. 1981).

Immunological Reagents. Preparation of rabbit anti-AFP, anti-albumin, anti-EH immune sera and the isolation of the respective antibodies were described (Kuhlmann 1975; Kuhlmann 1976; Kuhlmann et al. 1981). Immunohistological control sera included normal rabbit IgG globulin, rabbit anti-glucose oxidase, rabbit anti-mouse IgG and rabbit anti-rat IgG immune sera which were prepared in the same manner. An anti-rabbit IgG immune serum was produced in sheep.

AFP, albumin and EH were stained by an indirect peroxidase labelled antibody method. For this purpose, the "sandwich" antibodies (sheep anti-rabbit IgG) were conjugated with horseradish peroxidase (HRP, RZ 3) (Kuhlmann et al. 1974; Kuhlmann 1975), and the antibody-HRP conjugates were purified in two steps by use of Sephacryl S-200 and Sepharose 4B-Concanavalin A columns (Lanner et al. 1978). Furthermore, the antigens were localized by the PAP technique (peroxidase-antiperoxidase antibody complexes) according to Sternberger (1974). PAP was purchased from DAKO-Immunoglobulins (Copenhagen, Denmark).

Tissue Fixation. Livers were sliced into cubes of about 2 mm and fixed at 0° C in formaldehyde (6% for 6-8 h) or in formaldehyde-glutaraldehyde mixture (6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25% glutaraldehyde for 1 h) as described by Kuhlmann (1978 a). Tissues were then washed overnight at 0° C with several changes of the buffer solution.

Additives, Chemical Modification and Dehydration of Tissues. The general plan of all tissue treatments is shown in Table 1. Details on tissue dehydration are given in Table 2. Two procedures of ethanol dehydration (Methods A and B) were compared with inert dehydration (Method C) by ethylene glycol (Pease 1966; Pease 1973).

Prior to dehydration, a batch of aldehyde fixed tissue blocks was first conditioned with 5 and 10% polyvinylpyrrolidones (PVP, MW 10,000; Serva-Heidelberg, Germany) in buffer for 2 h. Dehydration was then performed in ethanol supplemented with 5 and 10% PVP. Another batch of aldehyde fixed tissue blocks was treated with 0.2 M ethyl acetimidate-HCl (EAI) in 0.2 M K_2HPO_4 (pH adjusted to 7.3) for 2 h at 0-4° C (modified from Hunter and Ludwig 1962) before dehydration was started.

Table 1. General design of experiments for postembedment immunohistology

Fixation	Buffer	Additives	Dehydration	Embedment
Formaldehyde	0.2 M cacodylate pH 7.3	–	Method A–C	Method I–III
Formaldehyde/ glutaraldehyde	0.2 M cacodylate pH 7.3	–	Method A–C	Method I–III
Formaldehyde	0.2 M cacodylate pH 7.3	5–10% PVP	Method A[a]	Method I[a]
Formaldehyde	0.2 M cacodylate pH 7.3	0.2 M ethyl acetimidate pH 7.3	Method A	Method I

[a] PVP added to ethanol; embedment in Epon via ethanol/PVP instead of propylene oxide as intermediate

Table 2. Schedules of tissue dehydration for resin embedment

Method A	Method B	Method C
Ethanol[a]	*Ethanol*[a]	*Ethylene glycol*
30%/30 min	70%/30 min	10%/30 min
50%/30 min	95%/2 × 30 min	gradually to 65%
70%/30 min	100%/3 × 20 min	(addition of pure
90%/60 min[b]	100%/3 × 20 min	ethylene glycol)
95%/30 min	prop. oxide[c]	65%/60 min
100%/3 × 20 min	prop. oxide/Epon	gradually to 100%
100%/3 × 20 min		100%/4 × 30 min
prop. oxide[c]		prop. oxide[c]
prop. oxide/Epon		prop. oxide/Epon

[a] all steps at 0° C until 100% ethanol was reached
[b] for Lowicryl K4M embedment cf. Table 3, Method III
[c] for intermediate solvents and embedment cf. Table 3

Resin Embedment. Three different resin embedments were examined (Methods I, II and III). Their formulas are given in Table 3. The epoxy embedment by Epon 812 (Luft 1961) was compared with an epoxy embedment by 1,2,7,8-diepoxy octane (DEO) (Luft 1973). The polar acrylate-methacrylate based resin Lowicryl K4M was also used (Carlemalm et al. 1980); dehydration and embedment at 0° C.

Epon 812 (epoxy equivalent 151) was purchased from C. Roth (Karlsruhe, Germany) and DEO from Merck (Darmstadt, Germany); dodecenyl succinic anhydride (DDSA), methyl nadic anhydride (MNA), nonenyl succinic anhydride (NSA), 2,4,6-tri (dimethylaminomethyl)

phenol (DMP-30) and propylene oxide were from Serva-Labor (Heidelberg, Germany). The desired anhydride :epoxy ratio (A:E) was calculated according to Luft (1973). Lowicryl K4M monomer, cross-linker and initiator were products from Chemische Werke Lowi (Waldkraiburg, Germany).

Table 3. Formulas of resin embedments

Procedure	Embedding media		Intermediate solvents	Embedment polymerization
	resin	hardener accelerator		
Method I	Epon 812 87 ml (A:E=0.70)	DDSA 90.0 ml MNA 23.0 ml DMP-30 3.0 ml	prop. oxide 2×30 min followed by prop. oxide/Epon ratio 1:1/60 min	(a) 35° C/24 h 45° C/24 h 60° C/24 h (b) 60° C/24 h
Method II	DEO 72 ml (A:E=0.60)	NSA 128.0 ml DMP-30 2.6 ml	prop. oxide 2×30 min followed by prop. oxide/DEO ratio 1:1/60 min	(a) 35° C/24 h 45° C/24 h 60° C/24 h (b) 60° C/24 h
Method III	Lowicryl K4M monomer 260 g	cross-linker 40.0 g initiator 1.5 g	K4M/90% ethanol ratio 1:1/60 min followed by K4M/90% ethanol ratio 2:1/60 min followed by pure K4M overnight	indirect U.V. (360 nm) 12 h at 0° C

Postembedment Immunohistology. 1 to 1.5 μm thick sections were deposited with a drop of water on acetone cleaned slides and dried at 90° C for 20 min. Resin was partially removed with sodium methoxide (Merck-Darmstadt, Germany): 7.5 g in 225 ml of absolute methanol/benzene mixture (1:1). Reaction time (3 min) and subsequent washings were according to Mayor et al. (1961). Endogenous peroxidases were then inhibited by treatment with 10% H_2O_2 in 0.01 M phosphate-0.15 M NaCl pH 7.4 (PBS) for 10 min, followed by rinsings with PBS. Prior to incubation in antibodies, sections were washed with PBS supplemented with 1% bovine serum albumin plus 0.5 M NaCl (BSA/PBS) and with normal sheep serum (5%) in BSA/PBS.

All antibodies and sera were diluted in BSA/PBS. In the HRP labelled antibody method, sections were first incubated with unlabelled antibodies (0.001 mg/ml) for 24 h at 4° C, then with HRP labelled sheep anti-rabbit IgG (0.1 mg/ml) for 15 min at room temperature. In the PAP technique, sections were first incubated with the respective antibodies (0.0005-0.001 mg/ml) for 24 h at 4° C, then at laboratory ambient temperature with sheep anti-rabbit IgG (0.05-0.1 mg/ml) for 15 min followed by PAP (diluted 1:50 to give ca. 0.05 mg antibody/ml) for 15 min. Unreacted antibodies were rinsed off with BSA/PBS; normal sheep serum was used between first and second step antibodies.

Peroxidase activity was revealed by incubation in 3,3'-diaminobenzidine and H_2O_2 (Graham and Karnovsky 1966). After washings in PBS, sections were treated with 0.1% OsO_4/PBS for 1 min, dehydrated and mounted under coverglass.

Controls. Parallel to aldehyde fixed tissues, liver blocks were also fixed in 99% ethanol-1% acetic acid for 12-15 h at 0° C, embedded in paraffin. They served as controls. With the latter technique, AFP, albumin and EH can be specifically stained as documented earlier (Kuhlmann 1975; Kuhlmann et al. 1981).

Immunohistological specificity was proved on serial sections by incubation in (1) normal rabbit IgG globulins; (2) rabbit antibodies absorbed with homologous antigens; (3) rabbit anti-glucose oxidase antibodies; (4) rabbit anti-mouse and anti-rat IgG antibodies, respectively. Each procedure was followed by HRP conjugates or by the PAP schedule and the enzyme substrate.

For routine histology, resin sections were stained by Azur II (Richardson et al. 1960).

Results

Histological preparations from liver fixed in formaldehyde or in formaldehyde-glutaraldehyde mixtures and processed by the paraffin method gave specific localizations for AFP, albumin and EH. The same antigens were also detected in sections when aldehyde fixed liver blocks were submitted to resin embedment. Certain treatments after aldehyde fixation and certain resin embedments im-proved the immunohistological reactions. Thus, conditioning of tissue blocks with 5 and 10% PVP or amidination of amino groups by EAI prior to resin embedment gave reliable and strong antigen staining (Figs. 1-3). All results from postembedment immuno-staining of AFP are summarized in Table 4. Localizations of albumin and EH followed the same rules.
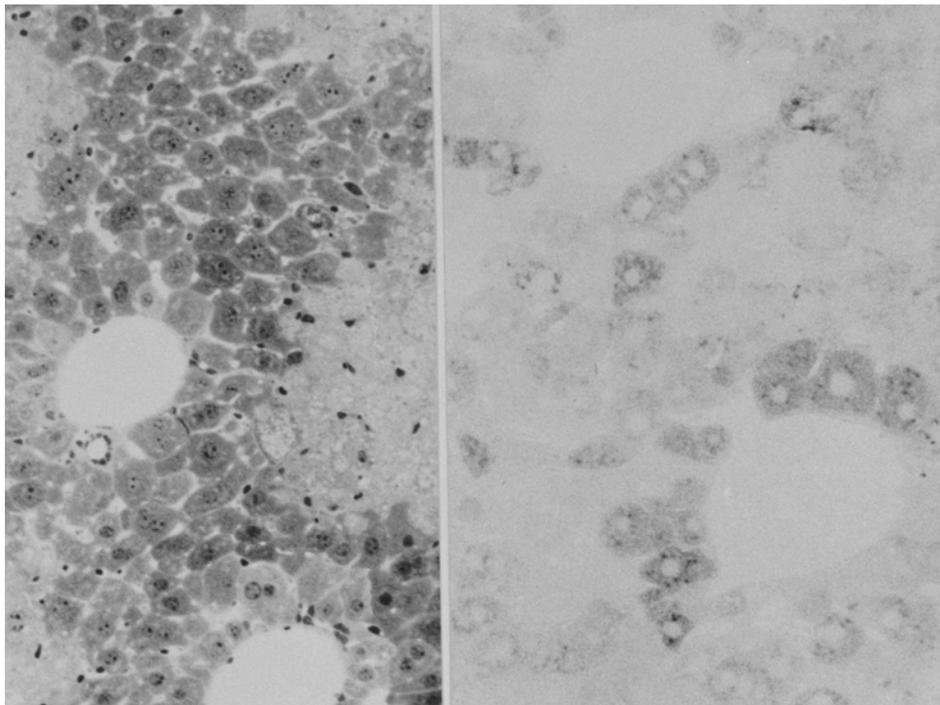
Fig. 1a, b. Regenerating mouse liver on day 3 after CCl$_4$ intoxication. Formaldehyde fixed and cacodylate washed liver blocks were dehydrated by Method A and embedded in Epon 812 according to Method I. **a** Azur II stain; necrotic area. Original x 160; **b** Same preparation in higher magnification and immunostained for AFP. AFP is seen in non-damaged hepatocytes. Original x 250.

Dehydrations by ethanol with *Method A* and by ethylene glycol (*Method C*) impaired anti-genicity less than dehydration with ethanol *Method B*. In sections from Epon 812 (*Method I*) embedded liver, immunolocalization of antigens was more intense than in sections from DEO

embedment (*Method II*). The curing temperature had no significant effect on subsequent immuno-staining. Immunoreactivity of antigens was very faint after Lowicryl K4M embedment (*Method III*).

Table 4. Summary of results from postembedment immuno-staining

Basis[a]	Dehydration	Resin embedment	Immunohistology[b]
(1) cacod.	Method A	Method I	+
		Method II	(+)
		Method III	−/(+)
(2) PVP	Method A	Method I	++
(3) EAI	Method A	Method I	++
(4) cacod.	Method B	Method I	(+)
		Method II	(+)
		Method III	n.d.
(5) cacod.	Method C	Method I	+
		Method II	(+)
		Method III	n.d.

[a] treatment of aldehyde fixed tissue
[b] intensity of reactions: − No; (+) faint; + clear; ++ strong; n.d. not done
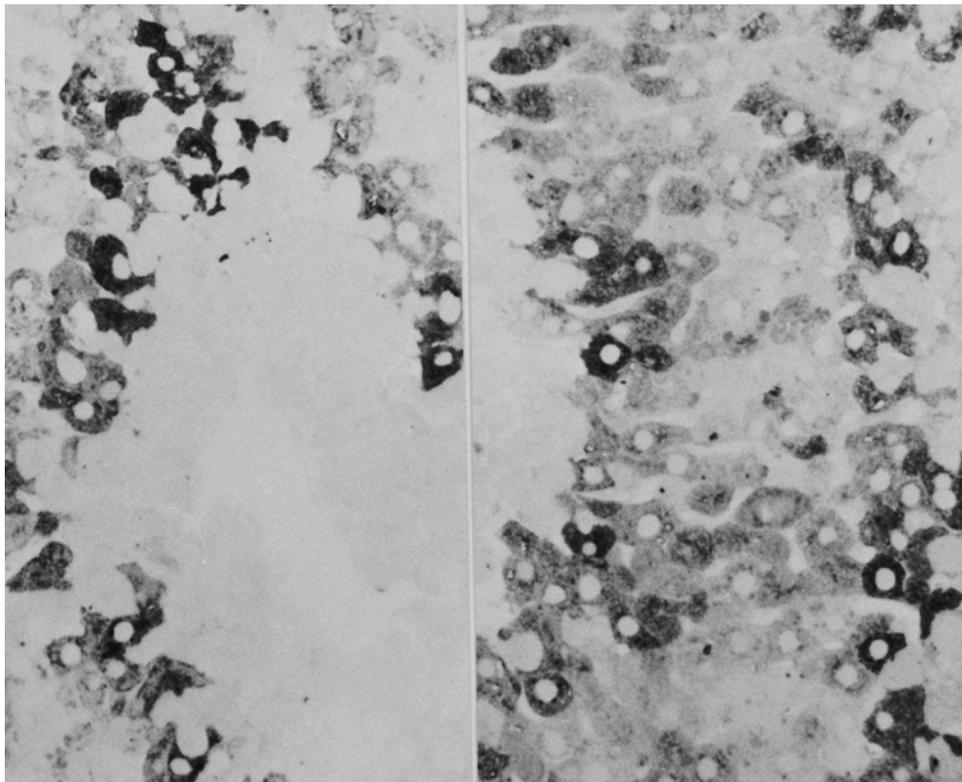
Fig. 2. Strong AFP reaction in hepatocytes on day 3 after CCl_4 intoxication. Liver blocks were fixed in formaldehyde, washed in cacodylate buffer and conditioned with 10% PVP; dehydration by Method A and resin embedment by Method I. Original x 250.

Fig. 3. Strong immuno-reaction of AFP in hepatocytes on day 3 after CCl_4. Formaldehyde fixed liver blocks were treated by EAI, then dehydrated by Method A and embedded by Method I. Original x 250

The indirect HRP labelled antibody method was as effective and specific as the PAP technique. Treatment of sections with H_2O_2 was necessary in order to inhibit endogenous peroxidase activities which otherwise interfered with immunohistological antigen localization. Background reactions due to interaction of immunocytochemical reagents with tissue components and resin did not occur when isolated antibodies instead of whole immune sera were employed. Moreover, optimal immuno-staining was observed when in the course of incubation sections were washed in PBS supplemented with bovine serum albumin and 0.5 M NaCl.

Liver sections did not stain with normal rabbit IgG globulins or in absorbed and unrelated antibodies (anti-glucose oxidase). Anti-mouse or anti-rat IgG antibodies did not react with hepatocytes except with those in necrotic liver zones e.g. due to acute CCl_4 intoxication. Such hepatocytes have ingested plasma IgG. Upon incubation with anti-mouse or anti-rat IgG antibodies, respectively, extracellular sinusoidal staining of IgG was regularly observed and was the result of synthesis and excretion of this protein by immunocompetent cells in the host animals.

Discussion

Application of immunological methods to sections from resin embedded organs is a goal in immunohistology which, however, is not always reached. The present study is a contribution to improvements in postembedment immuno-staining of antigens. For this purpose, several aspects of tissue processing were explored for the localization of three different intracellular antigens in hepatocytes, i.e. alpha$_1$-fetoprotein (AFP), albumin and epoxide hydrolase (EH).

Difficulties in postembedment immunohistology are due to several factors. Loss of immunoreactivity of antigens can occur at various stages of resin embedment which is often a denaturation and implies irreversibility. Here, tissue fixation, dehydration and resin embedment are the most important factors. Fixation with aldehydes may alter immunological reactivity by modification of one or several amino groups of antigenic reactive regions as well as by cross-linkage of the latter and by consequent conformational changes. Moreover, nature and extent of conformational changes (inside and outside antigenic reactive sites) due to dehydration of organs play an important role. Finally, further modifications and cross-linkages (inside and outside antigenic reactive sites) and both concomitant with conformational changes can occur by reaction of resin monomers or oligomers and curing agents in the final tissue embedment. The extent of all denaturation steps in tissue embedment is difficult to predict. However, it is clear that these should be reduced to a minimum in order to optimize postembedment immuno-staining.

Fixation. A prerequisite for immunohistological studies of the proposed type was the establishment of appropriate tissue stabilization by fixation. Aldehydes are of primary value for that purpose, and in a first set of experiments we examined the effect of formaldehyde and glutaraldehyde on the immunoreactivity of antigens via a paraffin technique. Such control experiments already proved useful for the establishment of tissue fixation in preembedment immunohistology and technical details are not repeated here (Kuhlmann 1977; Kuhlmann 1978 a). General descriptions and data on the reaction of aldehydes with tissue antigens are given in Kuhlmann (1977), Kuhlmann (1980) and references cited in these papers.

Following aldehyde fixation, we obtained strong and specific immunolocalization for the three liver antigens examined. Hence, fixation per se did not alter our model antigens significantly. However, we bear in mind that fixation schedules must always be adapted to each biological material.

Dehydration and Antigen Protection Steps. In classical resin embedment, aldehyde fixation is followed by dehydration. Usually lowpolarity solvents such as ethanol are employed. Apart from possible extraction of tissue proteins by ethanol-water mixtures (Luft and Wood 1963), a change of hydration shells of protein antigens which involves denaturation is known to occur. In contrast, the native conformation of proteins is less affected by the more polar ethylene glycol (Tanford et al. 1962), and this agent proved to be suitable for the electron microscopic preparation of biological specimens (Pease 1966; Sjöstrand and Barajas 1968; Sjöstrand and Kretzer 1975). Due to its two hydroxyl groups ethylene glycol fits well into water shells of proteins so that these are not seriously disturbed and denatured.

We conclude from our postembedment immuno-stainings that low polar ethanol can be employed for dehydration. An interesting finding was that a distinct procedure (Tables 2 and 4) gave comparable results to those obtained by inert dehydration with ethylene glycol. However, we are aware that other antigens will behave quite differently. For these, alternative procedures must be elaborated.

In view of the various immunohistological results, we became interested in improvement of immuno-staining by some measures which might protect antigens from denaturing effects in the course of dehydration and resin embedment. One approach was the use of PVP, an inert product of pharmaceutical relevance (Reppe 1954). PVP forms micelles with proteins by adsorption, so that stabilization is achieved. Addition of PVP to wash buffer, ethanol and Epon (via ethanol/Epon mixture as intermediate prior to embedment) was tried with two objectives: (a) antigen protection by micellar distribution of PVP; (b) incorporation of PVP in tissue as ready-for-use wash-off filler material.

We postulate that micellar distribution of PVP may help antigens to remain nearer to their native states. On the one hand, dehydration would have less deleterious effects on the conformation. On the other hand, antigenic determinants would be protected from reactive resin and copolymerization would be prevented to a large extent. Finally, the inert filler material might be readily washed off from sections after partial removal of resin in order to enhance the penetration of immunohistological reagents to reach antigenic sites. All such effects of PVP are difficult to measure quantitatively, but can be assessed from the above experiments. Indeed, tissue conditioning with 5 or 10% PVP gave improved immunoreactivity of antigens in the sections.

In another approach we used EAI as a protective agent for antigenically reactive regions. This monofunctional imidate was applied after aldehyde fixation and prior to dehydration of tissues for the conversion of free amino groups into unreactive amidines. Technical and theoretical aspects of chemical modification of proteins were reviewed by Means and Feeney (1971). Our idea was that EAI would protect antigens inasmuch as blocked amino groups could no longer react with epoxy groups in the course of resin curing. Thus, antigenic reactive sites would be more readily exposed upon partial removal of resin in sections when submitted to postembedment immunohistology. This assumption was supported by the observation that EAI-reacted livers showed particularly strong and specific immuno-staining. Apart from partial removal of Epon from sections, sodium methoxide will also displace acetimidyl groups from the antigens. Interestingly, further removal of acetimidyl groups by ammonia-acetic acid (Ludwig and Byrne 1962) and regeneration of the original amino groups seemed not to be important because equally strong immunohistological reactions were seen in reversed and non-reversed sections. Finally, no difference was observed between PVP- and EAI-treated livers.

It is thus clear that dehydration with low-polarity solvent will alter the immunoreactivity of tissue antigens, but excessive impairment can be reduced by appropriate schedules. Alternatively, an inert dehydration e.g. with ethylene glycol may be employed. PVP or EAI treatments of aldehyde fixed tissues prior to dehydration enables antigen protection in the

course of dehydration and resin embedment. However, the protective effect probably will vary from one antigen to another.

Resin Embedments. In previous studies, we were unable to label protein antigens in sections from resin embedded tissues. The reason for this failure was not readily comprehensible, and our efforts in immuno-electron microscopy focused on preembedment procedures instead of postembedment techniques (Kuhlmann et al. 1974; Kuhlmann 1977; Kuhlmann 1980). Because of the advantage of immunological ligand assays applied to tissue sections after resin embedment and since peptide hormones can be reasonably studied by such techniques, we continued to reinvestigate postembedment immunohistology. Several resins are known for electron microscopy of cells, and three types are usually employed: epoxy, polyester and methacrylate resins. From the available nonpolar epoxy resins, Epon 812 is the most common one. Here, the usefulness of Epon 812 and a rarely used diepoxide (DEO) were examined for postembedment immunohistology. Also, the polar Lowicryl K4M resin was tried.

In the course of experimentation we realized that immuno-staining of our model antigens was possible in sections of Epon 812 embedded liver blocks. One reason for this was the adoption of appropriate incubation schedules with optimally prepared immunocytochemical reagents. Another reason was the partial removal of Epon from the section. This step was probably not always well executed in previous experiments so that antigenic determinants became insufficiently exposed.

When epoxy monomers are cured, three-dimensional structures are formed and cross-linkings are principally based on di-ester bridges and ether bridges (Fisch et al. 1956; Lee and Neville 1967). With sodium methoxide in a mixture of methanol and benzene, at least di-ester bridges are broken up. Then, subsequent washings of sections in methanol/benzene mixture and acetone will also remove unreacted monomers and oligomers: not fully reacted compounds occur especially when Epon is cured in steps of increasing temperatures (Luft 1961). In view of this, we preferred the curing of our tissues at 35° C/24 h, then at 45° C/24 h and finally at 60° C/24 h even if direct curing at 60° C/24 h also yielded sections in which immuno-staining was well performed. Further treatment of sections with concentrated H_2O_2 was necessary to prevent interference of endogenous peroxidase activities with specific immuno-staining. We have no evidence that H_2O_2 could break up and remove polymerized Epon from sections since immuno-staining was not possible after this measure alone.

AFP, albumin and EH were better stained in sodium methoxide treated Epon 812 than in DEO sections. The reason for that is not yet understood. Neither in Epon 812 nor in DEO embedments were distinct measurements possible in order to calculate the amount of antigenic determinants which could still react after all embedding steps (i.e. fixation, dehydration and resin curing). Because the above mentioned resins may react with amino groups of antigens, a further loss of immunoreactivity will be added to that due to fixation and dehydration. The extent of such a loss is still unknown. At all events, we must expect that substantial quantities of antigen reactive sites/regions are no longer reactive. Yet, sufficient numbers can remain for subsequent postembedment immunohistology.

In order to reduce the reaction of epoxy resins with antigens we introduced by EAI treatment acetimidyl groups into tissue antigens or we employed PVP as an unreactive filler (see above). Both measures resulted in protection of antigens to a certain yet undefined extent. Such treatments were useful for clear-cut immunolocalization of the antigens we studied. The above observations are supported by experiments on different antigens in a variety of other organs (unpublished observations).

Apart from nonpolar resins we also used a polar low-temperature embedding medium, i.e. Lowicryl K4M which is based on cross-linked acrylate-methacrylate (Carlemalm et al. 1980). Polar media and low-temperature embedding procedures may be applied in order to reduce denaturation and conformational changes usually associated with dehydration and resin curing

(Tanford et al. 1962; Sjöstrand and Kretzer 1975; Kellenberger 1979). The above K4M resin was developed for this purpose (Kellenberger et al. 1980).

In contrast to epoxy embedment, only very faint immuno-staining was obtained in sections from K4M embedded liver. Dehydration per se at 0° C was certainly not the reason for that pitfall because dehydration of tissue for Epon embedment followed the same schedule. One objection may be that in our experiments K4M was cured at 0° C. Apart from a different type of cross-linking in K4M than in Epon 812 embedment, the influence of curing temperature on tissue antigens must be reconciled. Lowering the temperature will probably reduce denaturation. In view of a publication with Protein A - gold complex as marker where K4M was reported to be a suitable embedding medium for postembedment immunohistology (Roth et al. 1980), low-temperature dehydration and embedment in polar resin are of great promise for future postembedment immunohistology.

Immunohistology. For detailed histological descriptions of the model antigens see Kuhlmann (1979), Kuhlmann (1981), Kuhlmann et al. (1981) and references therein. Here, the applicability of immunohistology to resin embedded preparations was considered. A prerequisite was a partial removal of the cured resin which was discussed above. Furthermore, the application of highly purified immunocytochemical reagents in appropriate concentrations (determined by titration) proved to be essential. Generally, antibodies purified by affinity chromatography appear to be more reliable in immunohistological techniques than fractions from immune sera (Kuhlmann 1977; Kuhlmann 1978 b). In the present material, best results were also obtained with specific antibodies isolated from high titer hyperimmune sera even if IgG fractions gave specific reactions, too. So-called background reactions did not occur when wash buffers were supplemented with bovine serum albumin and 0.5 M NaCl which already proved very effective for antigen localization in sections from paraffin embedded livers (Kuhlmann 1978 b). Otherwise, PBS might be also supplemented with 1% bovine serum albumin alone. Addition of NaCl in high molarity to the wash buffer will give elution of low affinity antibodies, thus high affinity antibodies must be used.

In our hands, the indirect peroxidase labelled antibody method worked at least as specifically and sensitively as the PAP technique. We prefer the indirect peroxidase labelled antibody over the PAP technique: reagents are prepared more rapidly and incubations are shorter than in the PAP technique. Best results are obtained with immune sera and conjugates prepared in our laboratory. Commercially available immune sera, PAP and HRP labelled reagents were often not safe because considerable background reactions occurred.

In conclusion, postembedment immunohistology with sections from Epon 812 embedded organs can be applied to a broader spectrum of antigens than expected. Alternatively, preparation of organs according to some of the modifications described here will be useful.

References

Carlemalm E, Villiger W, Acetarin JD (1980) Advances in specimen preparation for electron microscopy. I. Novel low-temperature embedding resins and a reformulated Vestopal. Experientia 36:740

Fisch W, Hofmann W, Koskikallio J (1956) The curing mechanism of epoxy resins. J Appl Chem 6:429-441

Graham RC, Karnovsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291-302

Hunter MJ, Ludwig ML (1962) The reaction of imidoesters with proteins and related small molecules. J Am Chem Soc 84:3491-3504

Kellenberger E (1979) Progress and new approaches in obtaining finer significant details in the electron microscopy of biological material. J Electron Microsc 28 (Suppl): 49-56

Kellenberger E, Carlemalm E, Villiger W, Roth J, Garavito RM (1980) Low denaturation embedding for electron microscopy of thin sections. Chemische Werke Lowi, D-8264 Waldkraiburg

Kuhlmann WD (1975) Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. Histochemistry 44:155-167

Kuhlmann WD (1976) Untersuchungen zur zellulären Lokalisierung von alpha$_1$-Fetoprotein unter normalen und pathologischen Bedingungen. In: Lehmann FG (Hrsg) Tumorantigene in der Gastroenterologie. Demeter, München, S 27-32

Kuhlmann WD (1977) Ultrastructural immunoperoxidase cytochemistry. Prog Histochem Cytochem 10/1:1-57

Kuhlmann WD (1978 a) Ultrastructural detection of alpha$_1$-fetoprotein in hepatomas by use of peroxidase-labelled antibodies. Int J Cancer 22:335-343

Kuhlmann WD (1978b) Localization of alpha$_1$-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats. Int J Cancer 21:368-380

Kuhlmann WD (1979) Immunoperoxidase labelling of alpha$_1$-fetoprotein (AFP) in normal and regenerating livers of a low and a high AFP producing mouse strain. Histochemistry 64:67-75

Kuhlmann WD (1980) Electron microscopy of intracellular antigens by peroxidase labelled antibodies. Biol Cell 39:261-264

Kuhlmann WD (1981) Alpha-fetoprotein: cellular origin of a biological marker in rat liver under various experimental conditions. Virchows Arch (Pathol Anat) 393:9-26

Kuhlmann WD, Avrameas S, Ternynck T (1974) A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. J Immunol Methods 5:33-48

Kuhlmann WD, Krischan R, Kunz W, Guenthner TM, Oesch F (1981) Focal elevation of liver microsomal epoxide hydrolase in early preneoplastic stages and its behaviour in the further course of hepatocarcinogenesis. Biochem Biophys Res Commun 98:417-423

Lanner M, Bergquist R, Carlsson J, Huldt G (1978) Purification of enzyme-labelled conjugate by affinity chromatography. In: Hoffmann-Ostenhof O et al. (eds) Affinity chromatography. Pergamon Press, Oxford, pp 237-241

Lee H, Neville K (1967) Handbook of epoxy resins. McGraw-Hill, New York

Ludwig ML, Byrne R (1962) Reversible blocking of protein amino groups by the acetimidyl group. J Am Chem Soc 84:4160-4162

Luft JH (1961) Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409-414

Luft JH (1973) Embedding media - old and new. In: Koehler JK (ed) Advanced techniques in biological electron microscopy. Springer, Berlin Heidelberg New York, pp 1-34

Luft JH, Wood RL (1963) The extraction of tissue protein during and after fixation with osmium tetroxide in various buffer systems. J Cell Biol 19:46A

Martin R, Weber E, Voigt KH (1979) Localization of corticotropin- and endorphin-related peptides in the intermediate lobe of the rat pituitary. Cell Tissue Res 196:307-319

Mayor HD, Hampton JC, Rosario B (1961) A simple method for removing the resin from epoxy-embedded tissue. J Biophys Biochem Cytol 9:909-910

Means GE, Feeney RE (1971) Chemical modification of proteins. Holden-Day, San Francisco Cambridge London Amsterdam

Moriarty GC (1973) Adenohypophysis: ultrastructural cytochemistry, a review. J Histochem Cytochem 21:855-894

Pease DC (1966) The preservation of unfixed cytological detail by dehydration with "inert" agents. J Ultrastruct Res 14:356-378

Pease DC (1973) Substitution techniques. In: Koehler JK (ed) Advanced techniques in biological electron microscopy. Springer, Berlin Heidelberg New York, pp 35-66

Reppe W (1954) Polyvinylpyrrolidon. Verlag Chemie, Weinheim

Richardson KC, Jarett L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313-323

Roth J, Bendayan M, Carlemalm E, Villiger W (1980) Advances in specimen preparation for electron microscopy. IV. Immunocytochemistry on thin sections with the protein A-gold (pAg) technique. Experientia 36:757

Sjöstrand FS, Barajas L (1968) Effect of modifications in conformation of protein molecules on structure of mitochondrial membranes. J Ultrastruct Res 25:121-155

Sjöstrand FS, Kretzer F (1975) A new freeze-drying technique applied to the analysis of the molecular structure of mitochondrial and chloroplast membranes. J Ultrastruct Res 53:1-28

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall, Englewood Cliffs, NJ

Tanford C, Buckley CE, De PK, Lively EP (1962) Effect of ethylene glycol on the conformation of γ-globulin and β-lactoglobulin. J Biol Chem 237:1168-1171

Vogt A, Takamiya H, Kim WA (1976) Some problems involved in postembedding staining. In: Avrameas S et al. (eds) Immunoenzyme techniques. North-Holland, Amsterdam, pp 109-115