APPLICATION OF METAL LABELED ANTIBODY METHOD AND A CONSIDERATION OF THE FIXATIVES SUITABLE FOR IMMUNOHISTOCHEMICAL STUDY

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1) The preparation of metal labeling antibody;

Since Singer (1959) introduced the immunoferritin method, many metals have been used for labeling antibody. Metal labeled antibody has an advantage over other labeled antibodies both in its smaller molecular weight and in producing a sharp image under the electron microscope at high magnification. However, the number of metal atoms capable of conjugating with the antibody without losing antibody reactivity is quite limited, and this restriction leads to the production of insufficient electron opacity. In order to both minimize the loss of antibody titer caused by the direct labeling of heavy metal to antibody and to label as many metal atoms as possible to the antibody molecule to obtain high contrast, an indirect label of 1-(chloromercuri) ferrocene (CFM) to antibody was introduced, with thiolated egg albumin (EA) employed as an intermediate material. The method was reported in detail elsewhere (Yasuda and Yamamoto, 1975).

Fig. 1 shows the location of pancreatic amylase in the zymogen granule of the acinal cell of mouse pancreas stained by CMF-labeled antibody. Many tiny grains of almost uniform size representing the location of amylase are recognized in the zymogen granules, while the grains were scarcely observed in the area outside of the zymogen granules. The concentration of the grains within the zymogen granule demonstrated by this method was heavier than that demonstrated by use of the ferritin-labelled antibody. This difference may be due to the difference in the molecular weight of the labelling materials; The molecular weight of CMF-EA (MW. of CMF; 421; EA MW. of 45,000) is much smaller than that of ferritin (MW. 650,000 on the average). Judging from this result, this new labelling method would seem to be useful for immunohistochemical study, especially for the observation of the localization of antigens at high magnification in electron microscopy.

2) Preparation of specimens for immunohistochemistry;

It is a question whether or not protein molecules such as γ -globulin or its Fab fragments labeled by any kind of substance can penetrate to the cell membrane of fixed cells. As a test examination conducted in order to determine the ratio of protein penetration into the tissue block, horseradish peroxidase (MW. 39,000) was chosen as the test material in this study. Mouse liver was cut into small blocks of approximately $1\text{ mm}^2 \times 5\text{ mm}$ in size and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 hr, and then washed overnight with the same buffer containing 5% sucrose. Non-frozen sections, about $40\text{--}50\text{ }\mu$ in thickness, were prepared with a Smith-Farquhar tissue sectioner (Iran Sorvall). The

FIG. 1. The localization of pancreatic amylase in the zymogen granule of the acinar cell of mouse pancreas stained by CMF-labeled antibody. Lots of tiny grains of almost uniform size, representing the location of amylase, are recognized in the zymogen granules, while the grains were scarcely observed in the area outside of the zymogen granules. $\times 73,000$

slices of the tissue were then divided into 2 parts. One part was frozen in liquid nitrogen and then thawed. Both the non-frozen slices and frozen slices were immersed in the peroxidase solution (10 mg/ml dissolved in phosphate buffered saline) and allowed to stand for 48 hr with a gentle shaking at 4°C. After incubation for 48 hr, the slices were immediately fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 30 min, and then washed well with phosphate buffered saline. After 30 min preincubation in Karnovsky's diaminobenzidine solution minus H_2O_2 , the slices were then stained in Karnovsky's solution containing H_2O_2 for 10 min. The stained slices were post-fixed with 1% OsO_4 and embedded in epoxy resin. Fig. 2 shows the location of peroxidase penetration into the fixed tissue. The deposits of the polymerized 3,3'-diaminobenzidine were seen in only 2 layers of the cells from the surface of the tissue blocks (upper, right) and not observed in any cell organellae (below). This result shows that the peroxidase molecules can not penetrate freely beyond the cell membrane and into the cell organellae in fixed slices of 40–50 μ in thickness. It also suggests that the penetration of γ -globulin molecule (MW. 160,000) or its Fab fragment into the fixed tissue may be more limited. Therefore, other methods are required in order to

FIG. 2. The location of penetrating peroxidase into the fixed tissue stained by diaminobenzidine solution. The deposits of the polymerized diaminobenzidine were seen only 2 layers of the cells from the surface of the tissue blocks and scarcely observed in any cell organellae. Upper-left; non-frozen tissue, upper-right; frozen tissue, below; frozen tissue.

facilitate the penetration of the proteins into the specimens for immunohistochemical study. The following methods might help in demonstrating better localization of antigens in immunohistochemistry on the electron microscopic level;

- a) Isolated cell organellae (Hackensbrock et al., 1975; Olsen et al., 1974; Morimoto et al., 1976);

Tissue or cells were broken by mild conditions, and the cell organellae were collected by use of the appropriate centrifugation. The collected cell organellae were briefly fixed with the suitable fixatives and stained with the labelled antibody. This method may be required for the careful check to prevent the release of antigen from the cell organellae.

FIG. 3. The fine structure of hepatocyte (1, $\times 5,500$), the acinar cell of mouse pancreas (2, $\times 13,000$), the intestinal epithelial cell (3, $\times 11,000$) and goblet cell (4, $\times 6,500$) obtained by using cyanuric chloride containing 0.05% glutaraldehyde as a fixative.

- b) Single cell or monolayer cell culture (Gonatas et al., 1974; Rufener et al., 1975; McKeever et al., (1976);
- c) Ultrathin sections;
 - 1) Embedded in the appropriate resins (Kawarai et al., 1970; Parsons et al., Erlandsen et al., 1974);

After the tissue was embedded in epoxy resin or methacrylate resin, the ultrathin sections were prepared and stained with the labeled antibody. However, most of the antigens lost their antigenicity after embedding in the resins. Therefore, the application of this method in immunohistochemical staining is restricted to some special antigens.

- 2) Cryo-ultrathin sections (Painter et al., 1973; Kyte, 1976);

Recently, it has been possible to prepare cryo-ultrathin sections of various

organs. Hopefully, cryo-ultramicrotomy will become one of the most useful tools in immunohistochemical study.

3) Fixatives suitable for histochemical as well as immunohistochemical studies;

The successful use of aldehyde (Sabatini et al., 1963; Karnovsky, 1965; McLean and Nakane, 1974) as a primary fixative for electron microscopy suggests that the cross-linking of cellular components is an important factor for the preservation of fine structure. Although cross-linking of proteins is very tight with the use of aldehydes, most of the proteins in the tissue can not preserve enzymatic activity as well as antigenicity. Therefore, from the standpoint of the preservation of enzymatic activity and the antigenicity, aldehyde is not always a suitable fixative for histochemical and immunohistochemical study.

Supposing that the active site of enzymatic activity or antigenicity is determined by some definite groups, it may be possible to select the bifunctional reagent which does not damage the definite groups. By using 3 different cross-linking reagents, diimidoesters, water soluble carbodiimide (1-Ethyl-3(3-dimethyl-aminopropyl)-carbodiimide-HCl), and cyanuric chloride as a primary fixative, it is possible to preserve fine structure on the electron microscopic level. The electron microscopic photograph of liver, pancreas and small intestine taken from the tissue fixed with cyanuric chloride is shown in Fig. 3. The fine structure of the hepatocyte (Fig. 3-1) and the acinal cell of mouse pancreas (Fig. 3-2) was well preserved and the general morphology was not that different from observed after glutaraldehyde fixation. The fine structure of the intestinal epithelial cell fixed with cyanuric chloride (Figs. 3-3,4) was also as good as that obtained with glutaraldehyde fixation. Still, differences were noticed in some places upon closer observation; the electron density in absorbing epithelial cell (Fig. 3-3) and the secretory granules of the goblet cell (Fig. 3-4) was higher than that observed after glutaraldehyde fixation. By using two other reagents as a fixative (diimidoesters and water soluble carbodiimide) the fine structure was as well preserved as when glutaraldehyde was employed.

TABLE 1. *The preservation of enzymatic activities after fixation with aldehyde, diimidoester (DMS), water soluble carbodiimide (WSC) and cyanuric chloride*

Enzyme	Fixative					
	Non-fixed	2% Glutaraldehyde	4% Paraformaldehyde	1.5% DMS	4% WSC	Cyanuric chloride
Lacate dehydrogenase	100	negligible	50	79	68	52
Alcohol dehydrogenase	100	negligible	negligible	negligible	15	—
Glutamate dehydrogenase	100	negligible	negligible	55	negligible	—
Glucose-6-phosphatase	100	8	10	63	75	42
ATPase	100	16	61	76	73	45
Fructose-1,6-diphosphatase	100	14	20	—	61	—
Acid phosphatase	100	23	25	—	50	—
Glutamate-oxaloacetate transaminase	100	19	7	58	50	—
Glutamate-pyruvate transaminase	100	11	negligible	45	30	—

(Yamamoto, 1975; Ono et al., 1976; Yamamoto et al., 1976). These results suggest that these three cross-linking reagents are useful as the primary fixative in electron microscopic study.

Mouse liver blocks approximately 1–2 mm on a side were fixed for 2 hr with various fixatives. For the control, non-fixed liver blocks were frozen in liquid nitrogen and stocked in a deep freezer at -20°C until use. After fixation, the blocks were well washed with cold buffer overnight with gentle shaking. After washing the blocks were frozen, thawed and then homogenized. The enzyme assays were carried out according to methods described elsewhere (Yamamoto et al., 1977). The enzyme activities which remained in the tissue after the fixation with various fixatives are compared and shown in Table 1. Most of the enzymes tested in this study maintained higher activities after fixation with water soluble carbodiimide or diimidoester than after aldehyde fixation. It is especially noteworthy that alcohol dehydrogenase retained 15% of enzyme activity after water soluble carbodiimide fixation and that glutamate dehydrogenase retained 50% of enzyme activity after diimidoester fixation, although both enzymes were completely inactivated after fixation with either glutaraldehyde or paraformaldehyde. These results suggest that the histochemical localization of these enzymes on the electron microscopic level may become possible by using water soluble carbodiimide or diimidoester as a fixative. Higher activities of glucose-6-phosphatase, ATPase and other enzymes remained after fixation with water soluble carbodiimide, diimidoester or cyanuric chloride than after fixation with glutaraldehyde or paraformaldehyde. A large part of the enzyme activities remaining in the fixed tissue belong to the category of "insoluble enzyme proteins" (see Yamamoto et al., 1977).

Since with the use of water soluble carbodiimide, diimidoester or cyvic chloride the fine structure was well preserved and in most of the enzymes examined more enzyme activity remained in the insoluble fraction of the tissue homogenate than with the use of glutaraldehyde or paraformaldehyde treatment, these three cross-linking reagents may prove useful as fixatives for enzyme histochemistry. These cross-linking reagents may also prove useful as fixatives for immunohistochemical study, even though the good preservation of enzyme activities after fixation with these reagents is not directly closed to the good preservation of antigenicity. Recently, we have succeeded in showing the location of hyaluronidase in sheep testis only by using water soluble carbodiimide fixation by immunohistochemical technique on the electron microscopic level (Sakai et al., 1976), though it could not be demonstrated in the tissue after fixation with any other fixatives.

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