**LOCALIZATION OF ANTERIOR PITUITARY HORMONES ON
EPON SECTIONS BY PEROXIDASE-LABELED ANTIBODY
METHOD—LIGHT AND ELECTRON
MICROSCOPIC OBSERVATIONS**

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In order to improve immunohistochemical staining of standard paraffin sections, and to explore the possibility of a single procedure for both light and electron microscopic observations, the plastic embedding method was investigated. The rat pituitary glands fixed with Acrolein and embedded in Epon showed excellent light microscopic staining with the peroxidase-labeled antibody method on 1 μ m sections cut by a glass knife. Antigenicity of both peptide hormones (ACTH, prolactin, GH) and glycoprotein hormone (LH) was preserved even after osmification. Compared to the Zamboni fixed or Bouin fixed paraffin sections, this method resulted in much more sharply defined and better preserved positively stained cells. Furthermore, this method is especially useful in the neoplastic condition, i.e. pituitary adenomas, where fixation is extremely difficult.

Ultrastructural observations of cells positively stained with this method revealed the presence of the immunoreactive hormones on the secretory granules and probably on the adjacent membranous structures. In considering these advantages, it seems probable that this method will replace paraffin sections in localizing pituitary hormones, and possibly other peptide or glycoprotein hormones with the peroxidase-labeled antibody method.

In order to localize anterior pituitary hormones with the peroxidase-labeled antibody method in normal and neoplastic conditions, paraffin sections have been standardly used. It has been reported that paraffin sections give fair results in staining at the light microscopic level with proper fixatives, such as Bouin's and Zamboni's fixative. However, the members of the present study have found that in localizing anterior pituitary hormones, paraffin sections have been unsatisfactory in certain respects even though they give selective immunologically specific staining. Inadequate cytological details of positively stained cells occurred when Zamboni's fixative was used, as well as frequent artifactually positive staining on nuclei with

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Bouin's fixatives. This problem has also been encountered in pituitary adenomas. The present study describes a recently attempted method of utilizing Epon sections of the pituitary tissue fixed with Acrolein, in order to resolve the described difficulties resultant of paraffin sections, as well as to extend the same procedure to ultrastructural observations of the immunologically stained material.

MATERIALS AND METHODS

Male and female Wistar rats of approximately 300 g were sacrificed by decapitation. The pituitary glands were fixed in 10% Acrolein (Merck)/phosphate buffer for 4 hr and postfixed in 1% osmic acid in phosphate buffer for 1–2 hours. Then, the pituitary glands were dehydrated by a graded alcohols and embedded in Epon at 40°C for 3 days. 1–2 μ m sections were cut with a glass knife and attached to albumin coated slides. Sections were subsequently incubated in a NaOH-alcohol solution for 3 min in order to remove the plastic, then in a 10% peroxide solution for 10 min. For light microscopy, Nakane's indirect peroxidase-labeled antibody method (2) was performed by using antibodies against adenocorticotrophic hormone (ACTH), growth hormone (GH), leuteinizing hormone (LH) and prolactin (PRL) supplied by NIAMDD. The sections were reacted with each antiserum for one hr. Histochemical reaction for labeled peroxidase was performed with 3,3'-diaminobenzidine-HCl (DAB, DOTITE). For light microscopy, sections were stained after the reaction with hematoxylin, then dehydrated by graded alcohols and mounted with Eukitt. For electron microscopy, the sections were fixed in 2% glutaraldehyde in phosphate buffer after the immunologic reaction, and incubated in DAB (40 mg/100 ml tris buffer) for 30 min followed by DAB with H₂O₂ (0.005%) solution for 2 min. After postfixation with 2% osmic acid in phosphate buffer for 20 min, the sections were dehydrated by a graded alcohol series and embedded in Epon by the inverted gelatin capsule method. Unstained and stained ultrathin sections were observed by JOEL 100-C electron microscopy.

Tissue fragments of PRL secreting human pituitary adenomas were also processed by a similar procedure. The above staining was compared with that obtained by paraffin sections of Zamboni or Bouin fixed tissue.

RESULTS

The above procedure resulted in excellent preservation of the cytologic details of positively stained cells. Antigenicity of both peptide hormones (ACTH, GH, PRL) and glycoprotein (LH) was preserved even after osmification. By this method ACTH cells appeared in general as angulated or stellate shapes with slender elongated cytoplasmic processes (Fig. 1). The elongated cytoplasmic processes were frequently extended toward capillaries, while ending on the capillary basement

FIGS. 1 and 2. ACTH cells localized on Acrolein fixed, Epon embedded rat pituitary sections. ACTH cells are stellate in shape and most of the elongated slender cytoplasmic processes end on the capillaries (Fig. 2 arrows). ACTH is localized in the cytoplasm as fine granules. Peroxidase-labeled antibody method. Fig. 1 $\times 300$, Fig. 2 $\times 600$

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

FIG. 9. Ultrastructural appearances of ACH cells localized in Acrolein fixed, Epon embedded sections by peroxidase-labeled antibody method. ACTH is observed mainly on the secretory granules (long arrows), and probably on some membranous structures in the cytoplasm (arrow heads). "Inset" reveals positive reaction products of DAB on the secretory granules (short arrows). Adjacent negative cells with larger secretory granules are probably GH cells. Unstained. $\times 10,000$

FIGS. 3 and 4. LH cells localized on Acrolein fixed, Epon embedded sections. LH cells are round, with some of them arranged near the capillaries (Fig. 4 arrows). LH was observed as rod shaped coarse granules. Peroxidase-labeled antibody method. Fig. 3 $\times 300$, Fig. 4 $\times 600$

FIG. 5. ACTH cells localized on Zamboni fixed, paraffin embedded rat pituitary sections. Note blurred outline of the positively stained cells. Peroxidase-labeled antibody method. $\times 600$

FIG. 6. ACTH cells localized on Bouin fixed, paraffin embedded rat pituitary sections. Note anti-factual positive staining on the nuclei (arrows).

FIGS. 7 and 8. Human prolactin secreting pituitary adenoma fixed by Acrolein and embedded in Epon shows well preserved prolactin and cytologic structures, using the peroxidase-labeled antibody method. Note prolactin is localized as perinuclear round or rod-shaped masses (Fig. 8 arrows). Fig. 7 $\times 300$, Fig. 8 $\times 600$

membrane in some cases. Positively stained ACTH was observed as fine round granules (Fig. 2). The LH cells appeared as round or ovoid cells, some of which were arranged adjacent to the capillaries (Fig. 3). No apparent cytoplasmic processes were present. Some LH cell were lying on or near the capillary basement membrane (Fig. 4). Positively stained LH were observed as larger rod-like granules in the cytoplasm (Fig. 4). No nuclear staining was obtained. Compared to Zamboni fixed or Bouin fixed paraffin sections (Figs. 5 and 6), the positively stained cells were clearly defined cytologically as well as structurally.

Sections of the PRL secreting human pituitary adenomas showed positively stained PRL as perinuclear homogenous irregular masses within the relatively well preserved cytoplasm. Occasionally, perinuclear rod-like staining was obtained (Figs. 7 and 8).

Fig. 10. Ultrastructural appearance of rat pituitary ACTH cells without immunohistochemical reaction. The tissues were fixed with Acrolein and embedded in Epon. The section shows excellent ultrastructural preservation of the cytoplasm. Lead and uranyl stains. $\times 3,300$

Electron microscopically, an incubation period in NaOH-alcohol for 3 minutes in order to remove the plastic resulted in the best staining. The rat anterior pituitary glands showed ACTH localized selectively not only on the secretory granules but probably on some adjacent membranous structures (Fig. 9). Negative control performed by utilizing normal rabbit serum instead of antihormone antibodies did not show any deposition of DAB reaction products. Electron microscopic features of the rat pituitary without the above immunohistochemical reaction showed well preserved intracytoplasmic organelles as well as membrane bound dense cored secretory granules, with Acrolein fixation (Fig. 10).

COMMENTS

With the method described herein, it was found that ACTH, GH, PRL, LH could be localized in the rat anterior pituitary glands much more clearly and sharply than those in the paraffin sections after Zamboni's or Bouin's fixatives. Antigenicity of these peptide or glycoprotein hormones was preserved as well as, or better than the paraffin sections, even after osmification. Other protein antigens, such as the basement membrane, were not localized by this method. This method, which successfully gave cytologic and structural details of the histochemically stained cells, was considered to be suitable for the study of the interrelationship of the hormone producing cells and the structure of the pituitary gland. Furthermore, this method was considered to be especially useful in localizing various hormones in the pituitary adenomas, where the fixation and preservation of hormone producing cells is extremely difficult.

Electron microscopic staining with this method needs to be tested further, but results suggest that this procedure may be successful in ultrastructural observations. Ultrastructural features were damaged in accordance with the time necessary for the removal of Epon by NaOH-alcohol. Removal of Epon by NaOH-alcohol for 3 minutes gave the best results. Similar methods have been attempted by several investigators, including polyethylene glycol (PEG) (3), but the advantage of the present method lies clearly in its use of easily available Epon as an embedding media, and in the possibility of selecting a certain area in large well stained light microscopic sections 1) to be processed for electron microscopy, after an immunohistochemical reaction described here, or 2) to stain adjacent ultrathin sections immunohistochemically after selecting a proper area of the same Epon block.

The above advantages may warrant this method's replacement of the standard paraffin sections in observation of anterior pituitary hormones and, most likely, the other peptide or glycoprotein hormones with the peroxidase-labeled antibody method.

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