Carbodiimide Fixation for Electron Microscopy and Immunoelectron Cytochemistry

The potential value of carbodiimide (CDI) as a fixative for immunofluorescence has already been reported by KENDALL, POLAK and PEARSE[1]. In connection with our investigations into development of antibody-labelling methods for electron microscopy[2], and the demonstration of polypeptide hormones[3], we required a fixative which would a) preserve the ultrastructure of the tissue and the granular storage form of the polypeptide hormones and b) leave the antigenic site(s) of the polypeptide fully reactive to labelled antibody. In this communication we show that conditions of fixation by CDI which give excellent results by immunofluorescence also provide good preservation of ultrastructure at the electron microscope level.

Materials and methods. Canine tissues. Small samples of duodenal mucosa were taken for both light and electron microscopy to study the effects of fixation in varying concentrations of CDI on immunofluorescence and tissue ultrastructure.

Tissue was fixed at 4°C for 24 h in 2, 10, 15, 20, 30, 40 and 50% solutions of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAP-CDI) (Sigma) in 0.01 *M* phosphate buffered saline, pH 7.1. After fixation specimens were washed for 24 h (light microscopy) or 3 h (electron microscopy) in 0.1 *M* phosphate buffer pH 7.1 containing 0.1 *M* sucrose.

For immunofluorescence studies tissues were quenched in Arcton (Freon) 22. Cryostat (5 μm) sections were cut and an indirect technique for the localization of secretin was carried out[4], using fluorescein-labelled anti-guinea pig IgG (Hyland).

For electron microscopic studies tissues were post-fixed in 3% glutaraldehyde in 0.1 *M* phosphate buffer pH 7.1 for 2 h, at 4°C, subsequently washed in several changes of 0.1 *M* phosphate buffer pH 7.1 containing 0.1 *M* sucrose and post-fixed again in 1% osmium tetroxide in Millonig buffer for 1 h at 4°C. Tissues were then dehydrated through an ascending ethanol series, taken through epoxypropane and embedded in an Araldite mixture.

Ultrathin sections were stained by alcoholic uranyl acetate and lead citrate and viewed in an AEI EM 6B electron microscope.

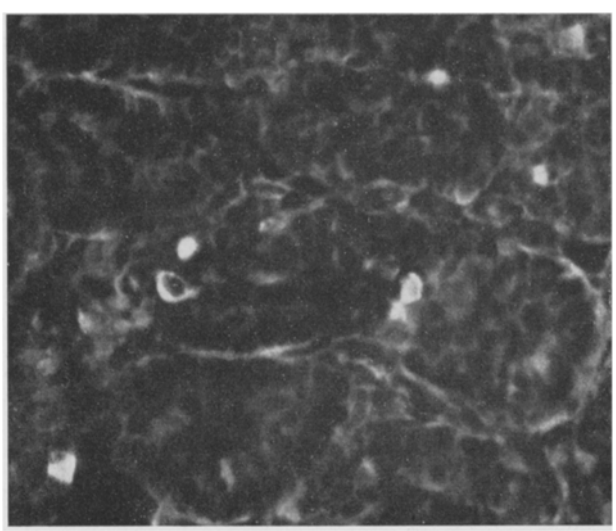

Fig. 1. Dog duodenum fixed in 10% EDAP-CDI. Immunofluorescent localization of secretin (in the S cells). ×990.

Murine tissues. Samples of pancreas were processed for electron microscopy in order to investigate ultrastructural features of exocrine and endocrine pancreas using CDI as a fixative.

Small pieces of tissue were fixed in 10% EDAP-CDI in 0.01 *M* PBS pH 7.1 for 1, 3, 7 or 24 h, and in 15% EDAP-CDI for 3 or 24 h at 4°C. For each of these experiments tissues were washed for 3 or 21 h, the longer periods of washing being to replicate conditions in which CDI fixation would be followed by several hours' exposure to antibodies as needed for immunological reactions at ultrastructural level.

As controls, small samples of tissue were fixed in 2% formaldehyde (freshly prepared from paraformaldehyde[5]) for 1 h at 4°C, then washed for 21 h, or 3% glutaraldehyde as described elsewhere[3].

All samples were post-fixed, after washing, in 3% glutaraldehyde in phosphate buffer pH 7.1 and post-fixed again in 1% osmium tetroxide in Millonig buffer, as described previously.

Ultrathin sections were stained by alcoholic uranyl acetate and lead citrate and viewed in an AEI EM 6B microscope.

Results. Immunofluorescence studies. Specific immunofluorescence was obtained when tissues were fixed in 2, 10, 15 and 20% EDAP-CDI. Further increase in concentration of the fixative led to a decrease of the specific staining.

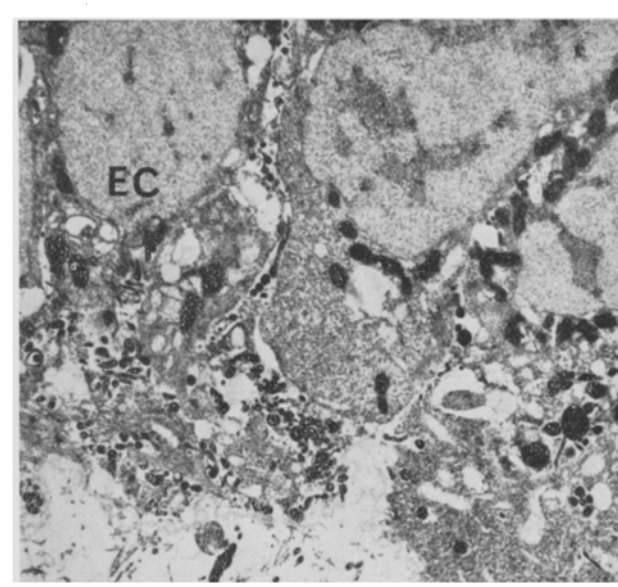

Fig. 2. Dog duodenum fixed in 10% EDAP-CDI for 24 h, washed for 3 h. The polymorphic granules of an enterochromaffin (EC) cell are well preserved. Nuclear chromatin distribution is unusual. ×7.500.

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[4] JULIA M. POLAK, S. BLOOM, I. COULLING and A. G. E. PEARSE, Gut *12*, 605 (1971).
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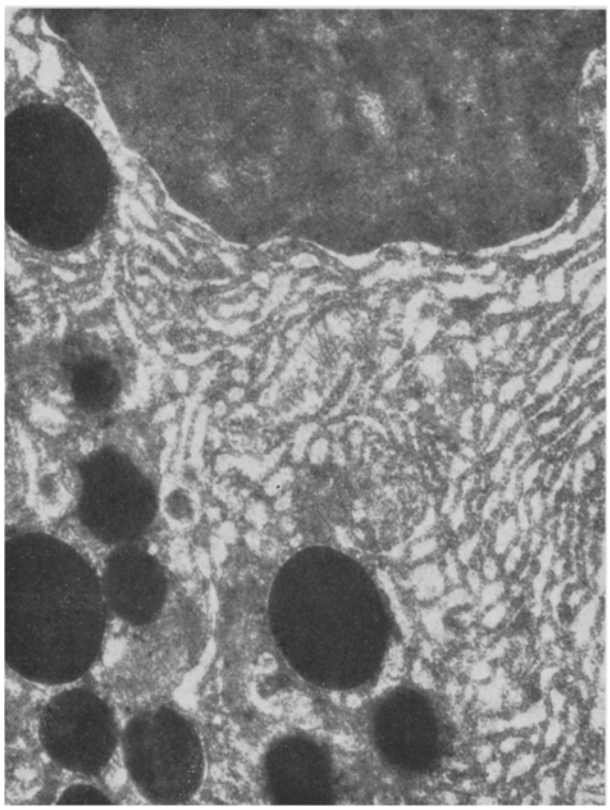

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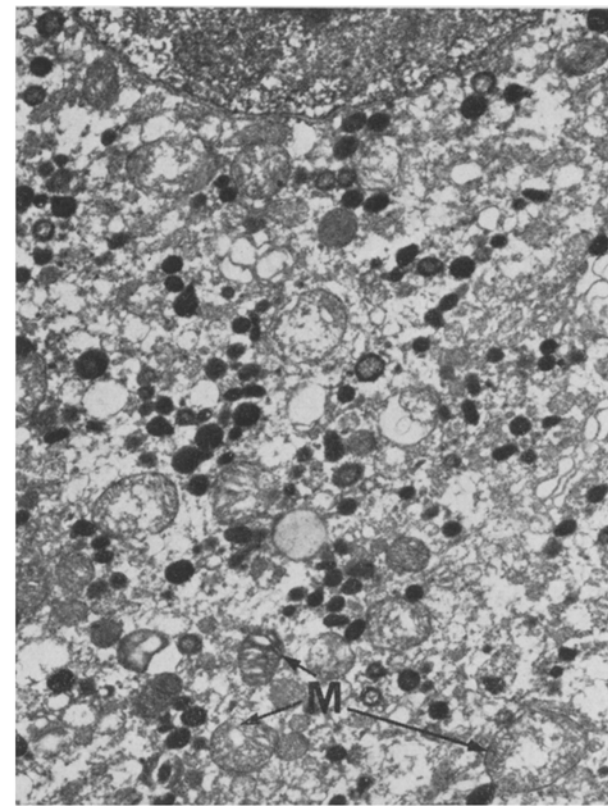

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Fig. 3. Exocrine pancreas (mouse) fixed in 10% EDAP-CDI for 1 h, washed for 21 h. Cytoplasmic membranes, nuclear chromatin and zymogen granules are all well preserved. ×17.500.

Fig. 4. Endocrine pancreas (mouse) fixed in 10% EDAP-CDI for 24 h. washed for 21 h. The prominent endocrine (β) granules are mainly spherical without halo or dense cores. The nucleus (above) is well fixed, as is the nuclear membrane. Mitochondria (M) are less well preserved. ×12.000.

Optimal staining was obtained with 10–15% EDAP-CDI (Figure 1). Tissue preservation also seemed better at this concentration.

Ultrastructural studies. Duodenal mucosa. Reasonably good preservation was obtained when tissues were fixed with 10% EDAP-CDI. With lower or higher concentrations the quality of the fixation decreased. As shown in Figure 2, the tissue components such as cytoplasmic membranes, organelles and endocrine polypeptide granules were generally well-preserved. Nuclei, however, were poorly fixed, especially at high and low concentrations of CDI, and showed an unusual chromatin distribution.

Pancreas. 10% CDI. Best results were obtained when tissues were fixed for no longer than 1 h. Cytoplasmic membranes, organelles and general structure were well preserved and the nuclei showed a chromatin distribution similar to that found in glutaraldehyde-fixed material (Figure 3). A short washing time after fixation also gave better tissue preservation, and thus the optimum conditions appeared to be 1 h fixation followed by a 3-h wash. However a 1-h fixation and a 21-h wash also gave better preservation than a 3-h fixation and a 3-h wash.

Endocrine polypeptide granules were well-preserved even in blocks with poor fixation, being numerous and showing good electron-density (Figure 4). Exocrine (zymogen) granules, on the contrary, were present in well-preserved specimens, but almost entirely absent in the poorly-fixed material.

15% CDI. Tissue preservation was not as good as with 10% CDI and very few zymogen granules were retained in either the long or the short fixation.

Formaldehyde. Formaldehyde-fixed material showed good preservation of zymogen granules, nuclei and cytoplasmic membranes, but organelles and endoplasmic reticulum appeared distorted (Figure 5). Endocrine granules were poorly preserved, demonstrably less well than with CDI.

Glutaraldehyde. Glutaraldehyde-fixed material showed, as expected, excellent preservation of both endocrine and exocrine pancreas including zymogen granules.

Discussion. Our results show that water-soluble carbodiimide is a good fixative for immunofluorescence, which also preserves ultrastructure reasonably well. It is therefore potentially a valuable fixative for immunoelectroncytochemical studies. Control experiments with aldehyde fixatives indicate that these, and especially glutaraldehyde, are better for the preservation of structural integrity. Glutaraldehyde can very seldom be used for immunoelectroncytochemistry, however, since it inhibits the subsequent binding of labelled antibodies. Formaldehyde has often been used as a fixative for investigations with labelled antibodies and some polypeptide hormones, such as ACTH, continue to react readily. The majority become unreactive, after short periods of fixation.

In the present work we observed that brief (1-h) fixation in CDI gave better structural preservation than a

longer fixation (up to 24 h), even when it was followed by an extended washing period. The deleterious effects of long fixation were much more evident in pancreas, which is rich in autolytic enzymes, than in duodenum, where such enzymes are less active. This observation suggests that, after the initial rapid fixation reaction of CDI, further exposure leads to a breakdown of the zymogen granules, with a release of active proteolytic enzymes. Hence it would appear that the action of CDI on tissues is biphasic, the second phase being responsible for disruption of the zymogen granules, nuclei and other constituents. The chemical or physical basis of this biphasic effect remains a matter for speculation since the known side-reactions of CDI [1] could scarcely be responsible for any significant disruption of structure. In the first phase it is considered that cross-links form between tissue carboxyl and amino groups which are in close proximity and that these stabilize and fix the structure. In the second phase it is conceivable that, under the influence of the CDI, condensations occur between tissue groups which necessitate spatial rearrangement. The latter then brings about a degradation of structural integrity.

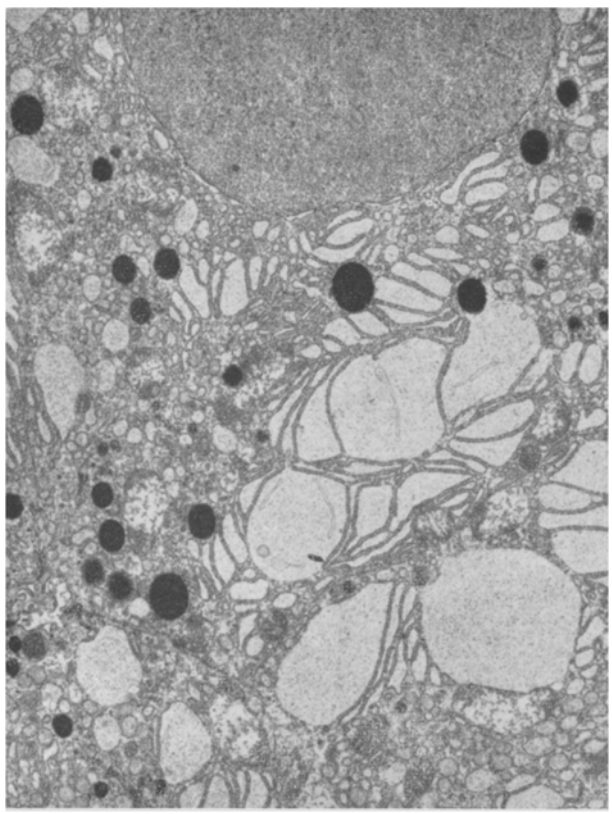

Fig. 5. Exocrine pancreas (mouse) fixed in freshly prepared formaldehyde for 1 h, washed for 21 h. Nuclei, zymogen granules and cytoplasmic membranes are well preserved but there is considerable distortion of the endoplasmic reticulum. ×5.250.

Zusammenfassung. In mit wasserlöslichen Carbodiimiden unter bestimmten Bedingungen fixiertem Gewebe können Polypeptidhormone immunfluoreszenzoptisch-nachgewiesen werden. Unter optimalen Bedingungen fixiertes Material ist auch zur Herstellung elektronenmikroskopischer Präparate vorzüglich geeignet. Carbodiimide werden deshalb als Fixierungsmittel in elektronenoptischen, immunzytochemischen Studien vorgeschlagen.

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The Use of Incident Light for Carbon Localization in Studies of the Reticulo-Endothelial System

Cells of the reticulo-endothelial system (RES) are morphologically heterogeneous and as such must be identified by the functional criterion of phagocytic activity. Colloidal carbon is usually used to identify these cells since the particles persist for a considerable period of time after injection, are of a convenient size and are easily recognized in some histological material. However, the use of certain staining techniques and the heavy deposition of pigment in some tissues may make carbon localization tedious when a conventional transmitted light microscope is used, and may demand a more efficient means of visualizing the injected particles.

Such problems were encountered in our laboratory during an investigation of the development and extent of the RES in an amphibian species, *Xenopus laevis* [1]. Animals of several stages were injected with colloidal carbon ('Dag 554', Acheson Colloids, Plymouth, England; or 'Pelikan C11/1431a', Günther Wagner, Hannover, Germany) then killed and fixed whole in Bouin's fluid at various time intervals thereafter. Paraffin-embedded sections of carbon-injected and normal animals were cut at 6 μm, stained routinely in haematoxylin and eosin and examined for carbon uptake. Haematoxylin and eosin was used since it gives excellent histological definition, but it obscures carbon uptake where darkly staining, closely packed cells are located, as in lymphocytic foci. Furthermore, pigment in the form of brown-black melanin granules was found widely distributed in tissues from both larval and post-metamorphic *Xenopus*: this pigment was present not only in the epidermis but also over all coelomic membranes, most blood vessels and in numerous glands, including the thymus, liver, spleen and kidney. The melanin granules are not restricted to melanophores, where they can be readily identified, but may also be found in extracellular locations or in phagocytes [2, 3].

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