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Lowicryl® Letters - No. 2

Trends and Developments in Low Temperature Embedding

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A. PRINCIPLES OF LOW TEMPERATURE EMBEDDING AND ITS VARIOUS PROCEDURES

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1. Principles

The basic notions of all the low temperature embedding procedures are the following:

a) All available knowledge points in the direction of a strong temperature dependence of denaturation of proteins in organic liquids and thus of possible concomitant conformational changes. Temperatures above 50°C, as used in curing of resins, are with certainty promoting denaturations. Studies of activities suggest that temperatures in the range of -20° to -70° should already provide substantial improvements.

b) Macromolecules in aqueous solutions precipitate in water-solvent mixtures. The size of the precipitates seems to be very temperature dependent. At lower temperatures it becomes much finer.

In conventional embedding, one tries to reduce the above mentioned phenomena by chemical cross-linking. In case of (b) one tries to produce a gel, which then is supposed to be more resistant to aggregation[1]. Whether or not fixation might stabilize the conformation of a protein molecule is completely unknown. Cross-linking, however, certainly stabilizes subunit assemblies against dissociation.

2. The different procedures

Three low temperature embedding procedures are in use:

a) Quick freezing an substitution of the ice by solvents and resin at low temperature. During substitution the presence of a fixative is in most cases necessary (freeze substitution embedding[2,3]).

b) Progressive lowering of the temperature during dehydration such as to prevent <u>any</u> formation of ice (progressive lowering temperature embedding, PLT[4,5]).

c) Quick freezing, sublimation of ice and infiltration of unpolymerized resin (freeze dry embedding[6]).

Each of the three procedures has potential advantages and disadvantages. Experimentally these have still to be explored, so as to make the right choices for each particular object of investigation. The events and actions involved in these procedures are purely physical in nature and thus understandable; on can predict what might happen, but one has no means to evaluate quantitatively each contribution and thus to predict the most likely outcome.

In "a" and "c" we have to face the events accompanying the freezing of a mixture of organic matter with water. The actions that might occur and the resulting events, in principle, are the same as those studied in <u>material sciences</u> on alloys.

In addition to these, we have to face in "a" the eutectic zone which is located between the still frozen part of the sample and the one soaked in the organic solvent. In the zone we have a gradient of <u>liquid</u> mixtures of water and solvent. The biological material becomes therefore reexposed to liquid water for some time before it is in pure solvent.

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B. A THEORETICAL COMPARISON OF THE EXPECTED POTENTIAL EFFICIENCIES OF ON-SECTION AND PRE-EMBEDDING LABELING PROCEDURES.

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As mentioned in the above note, only antigenic sites which are exposed on the surface of a section are available for cytochemical labeling. W. Villiger[1,2] has estimated the relief on the surfaces of sections made with different resins. He found Epon sections to be smoothest (±1 nm), while K4M showed ±2.5nm denivelations. Epoxy resins are glues and thus do not easily break at the interfaces between the antigenic site and the resin. This seems, however, to happen with K4M, explaining the high efficiency of these sections for lectin- and immunolabeling.

For a section of 40nm thickness, and labeling on one surface, ideally only 1/8 of the antigenic sites would be accessible among all others of a structure spanning the entire thickness of the section.

This has not been confirmed experimentally as yet: with B. Armbruster and M. Garavito we used a sample which could become easily labeled before embedding as alternative to the on-section labeling. This sample consisted of the microtubuli of the isolated membranes of Leichmannia tropica. Between preembedding and on-section label only a gain of about 2 was observed.[3] For both purposes the same polyclonal serum was used. That the theoretical value was not achieved might be explained by an insufficient concentration of the serum: Indeed, for preembedding the requirement towards the concentration of IgG's is much higher than for on-section labeling. Precise comparisons of this sort should be continued.

A second comparison which is much debated, concerns cryosection labeling versus on-section labeling. Cryosections are between 100 (at the best) and 300nm thick. The number of antigenic sites is thus in principle 20-60 times higher than those exposed on a K4M section. Practical comparisons reported in conferences, workshops and seminars have as yet not achieved these predicted values, but at the most factors far below 10.

Work continues at different laboratories to explain this discrepancy between theory and experiment.

It is obvious, however, that the confection and handling of crytosection is still so difficult that it cannot yet be considered as a routine technique as is the on-section labeling with K4M.

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C. CYTOCHEMICAL LABELING ON THIN SECTIONS OF LOWICRYL K4M - EMBEDDED CELLS AND TISSUES

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When we first used Lowicryl K4M for embedding at low temperature (-35°C) to test its applicability for postembedding cytochemistry (Roth et al., 1981) the following principal observations were made. First, an overall improvement in the preservation of fine structural details especially of cellular membranes was evident. Organelles such as the Golgi apparatus could now unequivocally identified in only glutaraldehyde fixed pancreatic tissue. Second, the degree of background staining as compared to Epon embedding was drastically reduced. Therefore, a relative increase in specific labeling intensity was achieved due to the improved signal to noise ratio. Over the past years we could confirm these observations. The degree of fine structural preservation of cultured cells or various tissues (stomach, small and large intestine, liver, kidney, salivary glands, lung, myocardium, skeletal muscle) after fixation with 1% glutaraldehyde, with paraformaldehyde (2-4%)-glutaraldehyde (0.05%-0.2%) mixtures or with paraformaldehyde (4%) was excellent and compared well with the seen after conventional double fixation with aldehyde and osmium tetroxide. Therefore, one goal which is quite critical for successful postembedding cytochemical labeling was achieved. But the main goal which we hoped to reach was improvement in retention of what is generally names "antigenicity" or in practical terms spoken higher labeling intensity with the protein A-gold technique. From further studies it became clear that the theoretically expected protective effect of low temperature on proteins under denaturing conditions such as dehydration in organic solvents was effective. Although this statement is based only an indirect means of measurement, namely the quantification of the protein A-gold labeling we feel confident. Bendayan and Shore (1982) found with Lowicryl K4M as compared to Epon or glycolmethacrylate (4°C) embedding higher labeling intensities for mitochondrial carbamyl phosphate synthetase.

Galactosyltransferase could not be localized after Epon embedding but using the same fixative and Lowicryl K4M embedding highly specific labeling was found over trans-Golgi cisternae of Hela cells (Roth and Berger, 1982) which was as intense as labeling on ultra-thin frozen sections (Strous et al., 1983). As mentioned in another note, theoretically the number of exposed antigenic sites in ultra-thin frozen sections should be 20-60 times higher than those exposed on the surface of Lowicryl K4M sections. As for galactosyltransferase the labeling intensity for albumin on Lowicryl K4M section of rat hepatocyte compares very well with the one obtained on ultra-thin frozen sections. (Geuze et al., 1981; Keller et al., 1984). Other examples for the successful and improved

D. LIGHT MICROSCOPICAL CYTOCHEMICAL LABELING ON LOWICRYL K4M SEMI-THIN (0.5-1.5μM) SECTIONS.

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Particles of colloidal gold exhibit a red color in transmitted light and this property makes them a useful marker for light microscopy. The protein A-gold technique or lectin-gold complexes cannot be only applied to paraffin sections (Roth, 1982, 1983) or Epon semi-thin sections (Roth et al., Lucocq and Roth, 1984a) but also to semi-thin sections prepared from Lowicryl K4M-embedded tissues (Lucocq and Roth, 1984a,b). In contrast to Epon semi-thin sections where resin removal is performed before labeling no such treatment for resin removal of Lowicryl K4M semi-thin sections is known at present. However, in many cases the light microscopical signal as seen by transmitted light illumination is strong enough to be easily appreciated. When needed the staining can be intensified by a photochemical silver staining (Danscher, 1981; Danscher and Nörgaard, 1983) which makes even before undetectable staining clear-cut visible.

The Lowicryl K4M semi-thin sections are mounted on glass slides which were treated with polylysine to prevent section detachment during the incubations. The grids are dipped in the polylysine solution (0.5mg/ml; MW300,000) afterwards rinsed with water and dried.

For observation in the light microscope we combine bright field transmitted light illumination with phase contrast and Nomarski differential interference contrast.

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E. ENZYME HYDROLYSIS ON THIN SECTIONS OF LOWICRYL-EMBEDDED TISSUES

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Enzymic hydrolysis, directly applied on thin sections of biological samples as a cytochemical tool, is routinely performed in many laboratories for many years - especially thanks to the pioneer work of W. Bernhard's team in Villejuif. However, technical difficulties reduce the general use of these techniques, particularly in cases where nucleases are used.

For a special problem - a more precise ultrastructural analysis of Dinoflagellate nuclear components - we now have combined low-temperature embedding procedures in Lowicryl HM20 and K4M with protease and nuclease extractions.

In Dinoflagellate arch-shaped chromosomes, DNA is not associated with histones, as in higher organisms, but only with low-molecular-weight proteins in very low ratio. Consequently, after only aldehyde fixation followed by the usual embedding at 60°C, these protist nuclei, and especially the archshaped chromosomes, present drastic signs of structural alterations provoked by dehydration and embedding. However, if the same fixation is followed by LT-embedding procedure, the preservation of these nuclear components was shown to be adequate for fine structural and cytochemical analyses.

We then applied to these preparations direct treatments with enzymes, floating thin sections on Marinozzi rings on enzyme solutions diluted in their usual dilution media, at pH 6.8-7.0. Pronase, RNase, and DNase were tested in this manner at concentrations varying from 0.1 to 1% (w/v), at 37°C, for 4 to 24 hours, alone or in successive treatments (for instances: pronase followed by RNase).

According to our predictions, these hydrolyses displayed very efficient gradual and specific extractions, thus allowing us to obtain new information concerning these protist's nuclear and cytoplasmic ultrastructure.

DNase action on Dinoflagellate chromosomal thin fibrils was especially shown to be clearly reproducible and efficient, either after only formaldehyde or after Karnovsky-Soyer formaldehyde+glutaraldehyde fixation, and with both embedding media tested (HM20 & K4M).

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antigen localization with the use of low temperature Lowicryl K4M embedding include the demonstration of actin in various tissues (Bendayan 1981, 1983, Bendayan et al., 1982), of vitamin D-induced calcium binding protein in chick duodenum (Thorens et al., 1982), of keratin in human skin and intestine (done in collaboration with Dr. M. Warhol Brigham and Women's Hospital, Harvard Medical School, Boston) of actin, tubulin and histone 2B in CHO cells (Armbruster et al., 1983a). From these and other unpublished data it appears that Lowicryl K4M when used at low temperature for embedding provides improved conditions for immunolabeling with the protein A-gold technique. The importance of low temperature in achieving such results has been recently demonstrated in one system (Armbruster et al., 1983b).

Our recent studies with lectin-gold complexes have clearly shown the high usefulness of Lowicryl K4M embedding for postembedding localization of lectin-binding sites (Roth 1982, 1983). By application of a battery of lectins various hexoses, hexosamines and sialic acid could be visualized along plasma membrane, extracellular matrix and various intracellular compartments (Roth 1984; Roth et al., 1983). An important observation in this context is that glycosidases can act on Lowicryl K4M thin sections which provides one means of cytochemical control reaction.

Finally, a few comments about practical aspects. We store the Lowicryl K4M blocks at room temperature in the laboratory. Thin sections mounted on nickel grids having a Parlodion-cardon film are used either immediately for labeling or are stored in LKB grid boxes. We found no difference in labeling of antigens or lectin-binding sites with freshly prepared sections stored up to 2 years. We are also still using blocks of the pancreatic tissue embedded in 1979 and get the same good labeling for amylase. We never perform any of the so-called etching procedures on Lowicryl K4M thin sections for antigen localization with the protein A-gold technique or glycoconjugate localization with lectin-gold complexes.

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