THE APPLICATION OF THE FREEZING-DRYING TECHNIQUE TO RETINAL HISTOCHEMISTRY

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THREE FIGURES

INTRODUCTION

In a quantitative histochemical study of retina, it is essential that serial sections containing predominantly one retinal layer be obtained. Due to the fact that the structure of the retina changes radically every 40–50 micra, the method of Linderstrøm-Lang and Mogensen ('38) which employs alternate sections for chemistry and histological control cannot be used. The slight crumbling which occurs during the sectioning of this frozen tissue also prohibits the use of this technique since a fundamental requirement of the Linderstrøm-Lang and Mogensen method is that all sections be of uniform area and weight. A procedure has consequently been evolved which embodies the main principles of this method and those of the Altman-Gersh freezing-drying technique (Gersh, '32), permitting the use of the same section of tissue both for identification of cell type present and for chemical determinations of certain tissue constituents. The method offers advantages over present freezing-drying techniques in that the process of dehydration can be carried out in a much shorter time and with simpler equipment and that paraffin embedding is avoided. The principle consists essentially of the following steps: the rapidly frozen tissue is mounted directly on the disc of an object holder; frozen sections are cut on the microtome in the constant temperature freezing chamber of Linderstrøm-Lang and Mogensen, and are dehydrated at atmospheric pressure over phosphorus pentoxide in the same chamber. Each section is then stained with a xylol solution of methyl violet, and finally, after histological observation has been made, the section is employed for the chemical determination.

Enzyme activity of stained frozen-dried sections. Experiments were carried out to test the effect of the procedure on the stability of various enzyme systems. As examples, peptidase activity and diphosphopyridine nucleotide assay of rat liver and choline esterase of rat brain cortex have been studied.

Peptidase was determined by the micro method of Linderstrøm-Lang and Holter ('35), diphosphopyridine nucleotide by a modification of the method of Jandorf, Klemperer and Hastings ('41), employing the Cartesian diver technique of Linderstrøm-Lang ('37), and choline esterase by the method of Glick ('38). In the case of peptidase, slight reduction in activity was noted, the greatest decrease in activity amounting to less than 10% when the determinations were carried out within 24 hours after dehydration. In the case of the other substances tested no diminution in activity was observed. Data showing the agreement between the choline esterase activity of frozen-dried, stained sections of rat brain cortex and fresh frozen tissue are presented in figure 1.

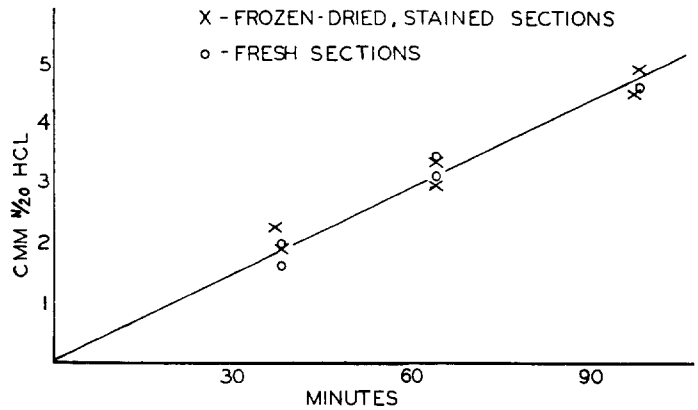

Fig. 1 Choline esterase activity of frozen-dried, methyl violet stained sections, and fresh frozen sections of rat brain cortex.

The incubation mixture in each vessel consisted of one 20-micra, circular section of rat brain cortex, 3 mm. in diameter, 10.6 mm^3. of 30% glycerine, and 13.2 mm^3. of substrate (Glick, '38).

It is observed that the drying and staining procedure involved has no effect on the choline esterase activity of the tissue. Alternate sections of the rapidly frozen liver or brain cortex were employed as controls in the case of peptidase and choline esterase respectively. In the case of diphosphopyridine nucleotide, the micro assays were compared with values obtained in this laboratory by the method of Jandorf et al., employing the Warburg manometric technique. The rate of CO_2 evolution per microgram of DPN as determined by the micro- and macro-methods was found to be identical.

GENERAL TECHNIQUE

Freezing. It has been shown by Hoerr ('36) that rapid freezing is essential in the prevention of ice crystal growth in the preparation of tissue for histological work. To prevent distortion from this cause,

small samples of tissue must be employed and rapid stirring of the freezing mixture is necessary. For accurate histological control, therefore, the following procedure has been used. As soon as possible after the death of the animal, a small piece of tissue is removed and dropped into isopentane previously chilled to a semi-liquid state by immersion in liquid nitrogen. The isopentane is immediately stirred vigorously.

Mounting. The tissue is then removed, trimmed to a cube roughly about 0.3 cm. on a side, and placed on a small object holder which has been moistened with a film of blood. (An ordinary flat-headed bolt may be used for this purpose.) The whole block is immediately immersed in a mixture of carbon dioxide snow (dry ice) and petroleum ether until its transfer to the microtome thermostat. An alternative procedure consists of placing the tissue block on the object holder, allowing the portion adjacent to the warm metal to thaw, and, before further thawing can take place, immersing the entire block in the freezing mixture at once.

Cutting and dehydration. Cutting is carried out as described by Linderstrøm-Lang and Mogensen ('38). The sections are then transferred to small desiccators where they are arranged serially on a clean slide. With phosphorus pentoxide as the desiccant, 20 micra sections are completely dehydrated within 1 to 1½ hours at −20°C. For more rapid dehydration, the sections are dried in vacuo over phosphorus pentoxide. The tissue sections are then quite dry within 15 to 20 minutes. Since fine histological detail has not been essential in our present applications of the method, extended experiments on vacuum dehydration have not been carried out.

After dehydration is complete, the desiccators are stored in the refrigerator until needed for chemical or histological examination.

Staining. To prevent the solution or displacement of the proteins and of other more soluble constituents, such as electrolytes, coenzymes, and small organic molecules, and at the same time to prevent the denaturation of enzymes from occurring, it is essential to use a non-aqueous solvent for staining which does not injure the enzymes to be studied. It has been found that xylol satisfies the above requirements. To stain the tissue, one part of a 40-mg. per cent solution of methyl violet in absolute alcohol to 50 parts of xylol are used. Under these conditions, the cytoplasm is stained violet leaving the nuclei completely unstained and appearing as clear vacuoles. When a sufficiently deep stain has been obtained (generally after 10 minutes), the section is washed with xylol to insure complete removal of the soluble lipoid material, transferred to a slide, flattened with a cover slip, and a photograph or camera lucida

drawing is made. A drop of xylol is then touched to the edge of the cover slip to diminish its adhesion to the slide, the slip is lifted off, and the excess xylol removed with filter paper. After drying in air, the section is ready for chemical or enzyme determinations. It is essential to remove the xylol-soluble lipids completely, since, otherwise, the section may adhere strongly to the slide.

Special technique for retina. The eye [1] is removed from the animal as soon as possible after death (5–10 minutes in our experiments), and trimmed free of adhering muscle and fat. It is then plunged, optic nerve down, into the freezing mixture consisting of dry ice and petroleum ether. This freezing medium has been found to be satisfactory for our present purposes since the greater histological detail obtained by more rapid freezing is not needed here (fig. 2).

The posterior one-third of the eye is separated for study with a chilled saw. With bone-cutting forceps, the frozen vitreous humor is removed with a few sharp cuts leaving the retina, attached to choroid and sclera, intact. The material is occasionally dipped into the freezing mixture to prevent thawing and to maintain a brittle condition. With the same forceps and a scalpel, the retina and adherent choroid can now be chipped off and immediately placed in the chilled petroleum ether. This can be done quite easily because of the natural cleavage plane existing between the choroid and sclera.

Well mashed liver which has been frozen to the object holder and slightly thawed at the surface by the application of a warm spatula is used as a base on which to mount the retina. A small piece of retina and its adhering layer of choroid is then removed from the freezing mixture, blown on gently to remove the film of petroleum ether, and rapidly placed, with the retinal side down, on the liver. To ensure subsequent adhesion of the retina-choroid sample to the liver during sectioning, the tissue is gently pressed against the liver base with the chilled blade of a small scalpel. The above operations must be carried out with considerable rapidity in order to preclude any thawing of the retina. The block is then immersed at once in the dry ice mixture, and finally transferred to the thermostat. The object holder is mounted on the microtome after equilibrating for 1 hour at the temperature of the thermostat.

In order to obtain sections containing primarily one retinal layer, the surface of the retina-choroid sample must be oriented as nearly parallel as possible to the cutting plane. This orientation can be greatly facilitated by the use of a small hand-mirror, held above or at the side

[1] Ox eyes have been used exclusively in our experiments.

of the tissue block during the manipulation of the adjusting screws in the microtome head.

Each section is removed as it is cut because of the great fragility of the retinal tissue. In doing this, one sacrifices some of the uniformity of thickness emphasized by Linderstrøm-Lang and Mogensen, but this uniformity is unnecessary in the present technique because each section is weighed and studied individually. The analytical results have been based on the dry weights or lipid-free weights as determined on a sensitive torsion balance similar in principle to the one described by Barrett et al. ('38), except that torsion is supplied by a quartz fiber suspension rather than by a tungsten wire, and the displacements are measured with a cathetometer.

After weighing and staining as described above, the sections are used directly for chemical study. In some cases, portions of the sections containing one retinal layer may be dissected out with a small scalpel under low power magnification. Such dissection is carried out under xylol.

The method is at present being applied to the distribution of choline esterase in retina. Reproducible correlations are found from experiment to experiment indicating the reliability of the histological control.

APPRAISAL OF THE SECTIONS OBTAINED

In order to demonstrate that the staining technique employed here permits adequate histological differentiation for the present purposes, the following comparison with hematoxylin staining was made. Figure 2 shows the results obtained by (a) staining a frozen-dried section of retina with xylol-methyl violet, and (b) by staining another section in the series fixed in 95% alcohol with hematoxylin. The section on the left illustrates the fact that methyl violet, dissolved in xylol, has the property of staining the cytoplasmic elements in the various layers of the retina. The stain does not, however, differentiate between one cytoplasmic layer and another except in the case of the layer of rods and cones which stains more deeply than the other zones and, in addition, contains pigment granules, particularly in light-adapted eyes. However, in the course of consecutive examination of a complete series of horizontal retinal sections, the alteration of stained and unstained layers is sufficient for their identification and correlation with enzyme content.

Figure 3a shows the appearance of rat liver carried through the freezing-drying procedure, unstained, but cleared with xylol. This photograph illustrates that the procedure employed in this study does

not materially obscure the normal histological picture. It will be observed that the hepatic cells and their nuclei are well defined and that undistorted erythrocytes appear in typical hepatic sinusoids.

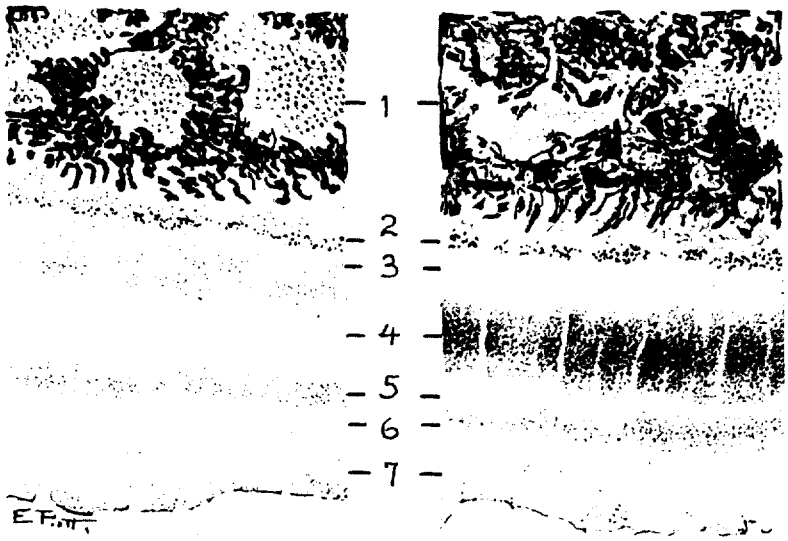

Fig. 2 Camera lucida drawing of the layers of the retina of an ox as stained by (a) xylol-methyl violet and (b) hematoxylin; 1, choroid; 2, pigment granules; 3, rods and cones; 4, outer nuclear layer; 5, outer plexiform layer; 6, inner nuclear layer; 7, inner plexiform layer. × 120

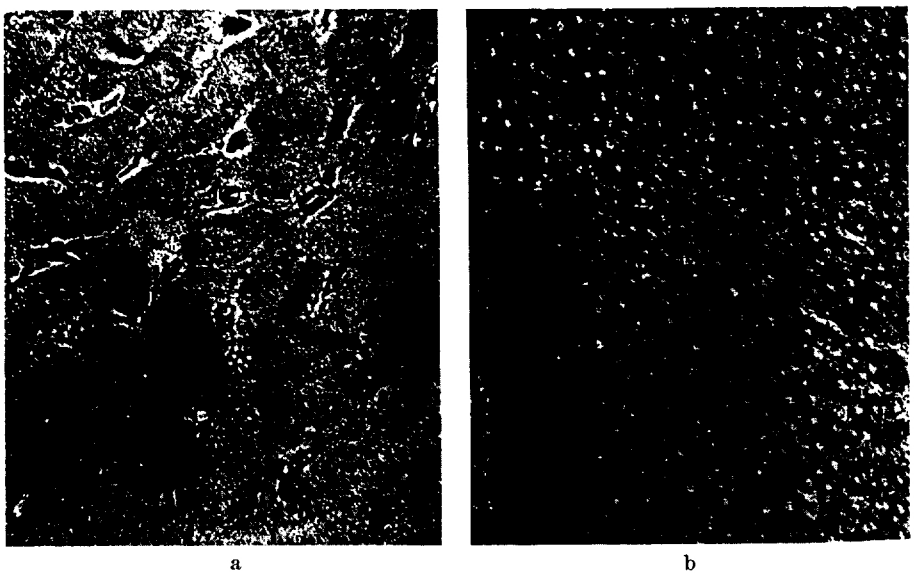

Fig. 3 (a) Frozen-dried, xylol cleared rat liver. (b) Frozen-dried, xylol-methyl violet stained rat liver.

Figure 3b is a frozen-dried section of a rat's liver treated with xylol-methyl violet, to illustrate that the cytoplasm of the hepatic cords is deeply stained, whereas the hepatic nuclei are unstained.

The authors wish to thank Dr. Philip L. Pillsbury for his valuable assistance in many of the earlier experiments.

SUMMARY

A method is presented which permits histological examination and quantitative enzymatic study of individual frozen-dried sections of tissue. Staining with methyl violet is carried out in xylol to prevent the solution and displacement of non-lipoid tissue constituents. The manipulations involved have a very slight or no effect on the enzyme systems tested. By this method, it has been possible to study quantitatively enzymatic localization in the various layers of the retina.

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