Ester Wax as a Medium for Embedding Tissue for the Histological Demonstration of Glycogen

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INTRODUCTION

IT is generally recognized that glycogen is a very labile substance which disappears rapidly from tissues unless treated by special methods of fixation and embedding. The recognized and widely used techniques, advocated by the standard microtomical treatises, call for fixation in a glycogen-precipitating fixative (absolute alcohol; picro-formol-alcohol; picro-dioxane) followed by celloidin embedding.

Since glycogen is considered to be readily soluble in water, if paraffin sections are to be used, special precautions are advocated: sections must be flattened on slides with 70 per cent. alcohol instead of albumen-water; mounted sections, after removal of wax, must be brought into absolute alcohol and then into 1 per cent. celloidin in absolute ether. This latter process covers the section with a thin film of celloidin which 'seems to prevent the diffusion of the glycogen from the section into the water' (Carleton, 1938). Most workers further emphasize that water must be avoided during the subsequent staining processes. On the other hand, Bensley (1939) in a recent account of staining methods for glycogen makes no mention of the necessity for the celloidin film, but merely states that 'tissues may be embedded in paraffin or celloidin'; and when staining 'sections may be brought down to water'.

In recent work on cestode physiology we have had available a considerable quantity of material rich in glycogen, namely, the plerocercoid larvae of the cestode *Ligula intestinalis* which contain seldom less than 30 per cent. glycogen (dry weight; Markov, 1939). It has thus been possible to test the embedding methods on this material on an extensive scale. Confirmatory experiments were carried out with rabbit liver.

EXPERIMENTAL RESULTS

Ligula intestinalis

Paraffin wax. Pieces of fresh larvae removed from a fish immediately after pithing were fixed in hot (60° C.) picro-formol-alcohol (absolute alcohol saturated with picric acid—90 c.c.; neutral formol—10 c.c.), embedded in paraffin wax, sectioned at 5μ, and stained with iodine or Best's

carmine using the modifications of Bensley (1939). Results were briefly as follows:

(i) Some blocks gave sections rich in glycogen, whereas others gave sections poor in glycogen.

(ii) Blocks which gave sections poor in glycogen, and which had only been sectioned about half-way through, when re-embedded in pure wax for long periods (at least overnight) now gave sections rich in glycogen!

(iii) When a 'short-embedded' block, partly sectioned, was immersed in aqueous iodine, the *centre* of the tissue at the block face stained immediately, whereas the periphery stained only after some minutes' immersion.

(iv) Sections from 'long-embedded' blocks or re-embedded blocks, provided they cut properly (which they rarely did), were as rich in glycogen if floated on albumen-water as on 70 per cent. alcohol.

Ester wax. Prolonged embedding in paraffin wax had the effect of making material very refractive and sections were usually crumbly and difficult to cut. This latter difficulty was overcome satisfactorily by using the very hard ester wax recently introduced by Steedman (1947). The procedure adopted was as follows:

Fix in picro-formol-alcohol: 2 hrs.
Absolute alcohol (3 changes): 12–24 hrs.
Absolute alcohol/ester wax (1:1): 2 hrs.
Pure ester wax: 3 hrs.
Pure ester wax: 12 hrs.—overnight.

Sections were cut with ease at 5μ, flattened on albumen-water in the usual way, and stained as before. All sections from blocks embedded in this manner contained great quantities of glycogen filling every available inter-cellular space in the cestode tissue.

In order to test the lability of glycogen, a number of sections were brought down to water, left standing in water, and removed and stained at intervals of one day to determine how rapidly glycogen disappeared from the sections. In contrast to expectations, it was found that *such sections could remain in water for periods of 5–7 days without any appreciable glycogen loss.*

Liver

The results outlined above—though agreeing with those obtained from similar but much-less-detailed experiments on larval *Diphyllobothrium* (Smyth, 1947)—were so contrary to the accepted views on the lability of glycogen, that it was considered possible that glycogen in such cestode tissue might exist in a form different from that in mammalian tissue, although results of chemical analysis suggests that glycogen in cestodes does not differ significantly from mammalian glycogen (Brand and Oesterlin, 1933; Wardle, 1937; Salisbury and Anderson, 1939).

In view of this possibility, a confirmatory series of experiments was therefore carried out using as a test material pieces of liver from a rabbit previously fed on carrots for 3 days—following the usual practice. Pieces 2–3 mm. thick were fixed in picro-formol-alcohol and embedded in (*a*) paraffin wax, using normal embedding times, i.e. 'short embedding'; (*b*) paraffin wax, using overnight embedding, i.e. 'long embedding'; (*c*) ester wax.

It was found that there was no significant difference between (*a*) and (*b*), but that on the whole sections from these two series were somewhat inferior to ester wax sections as regards the discreteness of the globules and the brightness of the staining. All series of sections could remain standing in water for very considerable periods without glycogen loss. Paraffin wax sections showed some loss after 5 days, but ester wax sections showed not the slightest trace of loss even after 7 days' immersion in water, and it was impossible to distinguish sections that had been soaked in water for this period from those which had been stained without immersion.

Discussion

The main results in these experiments that require explanation are: (*a*) pieces of *Ligula* require 'long embedding' in paraffin wax in order to retain glycogen, whereas 'short embedding' is sufficient for thin pieces of liver; (*b*) ester wax sections are superior to paraffin wax sections; (*c*) sections of properly embedded material when brought down to water can remain in water for long periods without appreciable glycogen loss.

(*a*) The fact that by prolonged embedding of *Ligula* in paraffin wax the glycogen is held, whereas by short embedding it is not, suggests that with short embedding the wax molecules do not completely permeate into the great amorphous masses of glycogen present in the inter-cellular spaces. This view is substantiated by the fact that when a short-embedded block, partly sectioned, is immersed in iodine the tissue stains instantly in the middle region yet only slowly at the periphery. This we interpret as indicating that the wax, which slows up the movement inwards of the aqueous iodine, has never reached the centre. If the glycogen in such a block was not firmly held by the wax, on sectioning some would fall out as a powder and other masses become loosened to such an extent that they could be lost by mechanical means in later mounting and flattening. This is exactly what happens to sections cut from the block just mentioned; although the face of the block after standing in iodine takes up the iodine intensely—thus showing glycogen to be present—yet sections from this block when stained contain little glycogen in the middle region, i.e. the region where the wax had not properly permeated. If such a block be re-embedded in wax for a long period, it gives sections uniformly rich in glycogen.

It thus seems reasonable to conclude that the glycogen is lost in cutting sections and in the subsequent flattening processes. This must be considered to be purely a *mechanical* effect due to the fact that glycogen is not completely permeated in wax in short-embedded blocks and is thus only loosely held.

If this hypothesis be true, the question immediately arises—why is short embedding sufficient for pieces of liver? This result can be accounted for when the amount of glycogen in liver cells is compared with that present in *Ligula*. In the former, glycogen is present only as small globules which can be penetrated on all sides by wax, whereas in the latter enormous masses of glycogen fill the parenchymal and muscular inter-cellular spaces. It seems self-evident that the time required to permeate the dense masses in *Ligula* will be considerably longer than that required to permeate the small globules in liver cells.

Previous workers have drawn attention to the flattening procedure as being a critical one, as the aqueous albumen would seem a likely place for the generally supposed highly labile glycogen to diffuse out. In one detailed experiment 10 sections of liver were mounted on water, and the same number on 70 per cent. alcohol; it was impossible to distinguish any difference in the amount of glycogen in the slides of the two series. Since this original experiment we have mounted many hundreds of sections flattened on albumen-water without ever getting results suggestive of a loss at this stage, provided material was properly embedded.

(*b*) If good results depend only on getting the paraffin wax right into the glycogen, it is to be expected that long-embedded paraffin wax blocks should give as good results as ester wax. The reason why ester wax gives better results we have already indicated, i.e. prolonged embedding makes tissues exceedingly hard and refractive with the result that sections are frequently difficult to cut and often crumbly, torn, or wrinkled, thus giving glycogen a chance to be lost at a later stage. Ester wax, due to its celloidin-like toughness and strength, permits sectioning of very hard material with great ease and does not introduce the difficulties resultant of paraffin wax embedding. We do not consider, therefore, that *per se* ester wax has any intrinsic property for retaining glycogen, but that like celloidin it merely overcomes, due to its toughness, the technical difficulties introduced by alternative methods.

(*c*) It has long been accepted that glycogen in sections is a highly labile substance, and for that reason most previous techniques have emphasized that sections should be handled without taking them below 60 per cent. alcohol—the strength at which alcohol precipitates glycogen from aqueous solutions. When, for example, aqueous reagents—or reagents with a low alcohol content—were used in the staining technique (i.e. as in counterstaining in the Best carmine method), the celloidin film technique was introduced to prevent diffusion of the labile molecules into the surrounding medium.

Basing our hypothesis on the above results, we believe that—providing material has been properly fixed, thoroughly dehydrated, cleared and embedded, sections not damaged in cutting, and smoothly flattened—when wax is removed and slides brought down to water, no glycogen is lost by dissolving in water. The fact that slides can be stored in water for 7 days and still be packed with glycogen must force us to revise prevalent ideas concerning the

lability of glycogen, and points to the inevitable conclusion that glycogen is either in an insoluble form at this stage or is 'held' in some way.

It is at once apparent that the answer to this problem lies in a greater knowledge of the structure and properties of glycogen. Recent work has shown that glycogen is a mixture of two complex polymers with highly branched molecules: *lyo*-glycogen, whose molecule is weakly linked to proteins and is *soluble*, and *desmo*-glycogen, whose molecule is strongly linked to proteins and is *insoluble* (Myer, 1942; Carter and Record, 1939). Since liver-glycogen contains only about 15 per cent. of the *desmo*- form (Genkin, 1946) it cannot possibly account for the amount present in our sections; we resort therefore to the alternative conclusion that the *lyo*- form has at least been partly—if not entirely—retained. We believe that the *lyo*- form does not diffuse from sections immersed in water because its large branched molecules are held by the associated protein network which has been precipitated around them. Lison (1936) has already put forward this hypothesis in more general terms, and, as he has pointed out, it adequately explains why a protein-coagulating fixative such as picric acid—while not precipitating glycogen *in vitro*—is an excellent fixative for glycogen in tissues.

Summary

1. The effect of paraffin wax embedding on the lability of glycogen in sections has been tested using as material (*a*) plerocercoid larvae of *Ligula intestinalis*; (*b*) rabbit liver.

2. It was found that glycogen in tissues was very impermeable to wax, and where large masses of glycogen occur prolonged embedding is essential.

3. Improperly embedded material lost glycogen easily; it was concluded that this loss takes place during the processes of cutting and flattening sections.

4. The effect of prolonged embedding was to make tissue hard and refractive. This difficulty was overcome by embedding in Steedman's ester wax.

5. Ester wax blocks allowed thin sections of very hard material to be cut with ease.

6. Ester wax sections brought down to water did not lose glycogen even after standing in water for 7 days.

7. It is emphasized that glycogen exists in two forms: an insoluble *desmo*-form and a soluble *lyo*- form. It is suggested that the latter does not dissolve from sections in water because its highly branched molecule is held by the coagulated protein network.

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