Electron microscopic studies on developing cartilage

II. Demonstration and distribution of glycogen in chick and mouse epiphyseal cartilage

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SUMMARY

A procedure for the study of glycogen in the same cell, under both light and electron microscopes is described. The synthesis and accumulation of glycogen is a feature of chondrogenesis in the mouse epiphyseal cartilage and the amount of glycogen increases with the progress of differentiation. In contrast, no glycogen was detected in differentiating epiphyseal cartilage of the chick at any stage; except for a small amount of glycogen in the chick cartilage cells of the diaphysis. Because of this difference it is suggested that presence of glycogen is not essential for cartilage differentiation.

INTRODUCTION

This communication reports a method for the study of glycogen in the same cell under both light and electron microscopes; and describes the pattern of glycogen distribution during the differentiation of cartilage cells in the mouse and the chick epiphyseal cartilages from hind-limb buds. The presence of glycogen in the cytoplasms of already differentiated cartilage cells has been histochemically demonstrated by several workers (Rouget, 1859; Harris, 1932; Follis & Berthrong, 1949; Montagna, 1949; Anderson, 1964). Montagna (1949), after studying hyaline, white, fibrous and elastic cartilages of man, dog, cat and rabbit stated that 'in adult mammalian tissues only liver and muscle contain more glycogen than cartilage' and that in cartilage cells glycogen is a 'normal and constant component'. The study of glycogen during chondrogenesis has not been done except for the brief remarks made by Godman & Porter (1960) in the case of rat. It was considered desirable to study the glycogen component of developing cartilage and to try to assess the role of glycogen in metabolism of differentiating cartilage cells.

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MATERIALS AND METHODS

Chick embryos of Brown Leghorn variety were incubated for varying times in a humid atmosphere at 38 °C and were staged according to Hamburger & Hamilton (1951). Mouse embryos were obtained from the inbred JBT/Jd strain (Jurand, 1965, 1968) by mating 6- to 9-week-old virgin females to their cousins. The embryo was considered 1-day-old on the day the vaginal plug was found. The embryos were dissected open in the uterus while the pregnant female was under slight ether anaesthesia.

The whole embryos, or in the case of large embryos only excised hind-limb buds, were immersed in the fixative chilled to 0-4 °C. The fixation of fully formed cartilage was improved by the prior removal of all surrounding tissues, including perichondrium. The cartilage was taken from the epiphyseal region of the third toe of the hind-limb bud, or its presumptive area, in both the chick and the mouse.

Fixation in 2 % osmium tetroxide in veronal buffer containing 1·8 % sucrose (Jurand, 1962) and 0·1 % dried CaCl₂, for 45–60 min gave the overall best results. With fully formed cartilage prefixation in 2·5 % glutaraldehyde in sodium phosphate buffer for 3·5–4 h improved the results. Araldite blocks were prepared for both light and electron microscopic observations.

For light microscopy, 1 μm thick sections were cut on Porter–Blum Ultramicrotome and were stained with periodic acid–Schiff reagent (PAS) (Schiff’s reagent according to Berger & DeLamater: Pearse, 1960). The staining was carried out at 38 °C (30 min in 0·5 % aqueous periodic acid and 15–30 min in Schiff’s reagent); treatment of sections with xylol, prior to staining, as suggested by Munger (1961) for staining at room temperature, was not found necessary at 38 °C. The sections were counterstained with 0·5 % toluidine blue buffered with sodium tetraborate.

In cytochemical work for glycogen the 1 μm thick sections were treated for 2–3 h at 38 °C with either ptyalin (saliva) or with 2 % α-amylase (× 2 crystallized from hog pancreas, minimum activity 500 units/mg; from Worthington Biochemical Company, Freehold, New Jersey).

For electron microscopy, thin sections, about 80 nm thick, were cut and double stained in uranyl nitrate and lead citrate. For identification of glycogen granules some were treated with 0·2 % periodic acid prepared from 50 % (w/w) HIO₄·2H₂O, BDH, for 20 min (Perry, 1967) before staining with lead citrate. The sections were observed on AEI EM6 electron microscope. For further details of various procedures see Goel & Jurand (1968) and Goel (1970).
In the light microscope glycogen appears occupying distinct areas of the cytoplasm (Fig. 1) which are bright red after PAS reagent staining and turn to magenta after counterstaining with toluidine blue. With toluidine blue alone it stains less intensely in contrast to deep blue of the ground cytoplasm (Fig. 2). In low-power electron micrographs the glycogen is seen in the cytoplasm as lumps of electron opaque material (Fig. 3), which at high power can be resolved into granular units. These units are characteristic of glycogen and are distinguishable from the ribosomes not only because of their larger size, 25–50 nm as compared to 20 nm, but also due to their substructure after oxidation with periodic acid. The substructure visible only after this treatment shows each granule to consist of 10–25 smaller subunits of approximately 5 nm in diameter (Fig. 4).

The following procedure was adopted to confirm that the PAS-positive material in the light microscope is identical with the material identified in electron microscopy after periodic acid–lead citrate treatment as glycogen. Thin Araldite sections for electron microscopy were followed by cutting approximately 1 μm thick sections for light microscopy; this permits observation of the same cell under both electron and light microscopes. The comparative study of these thin and thick sections, after appropriate treatments and staining, showed that the PAS-positive regions correspond precisely with regions containing material reactive to periodic acid–lead citrate treatment in thin sections (Figs 5, 6).

The cytochemical confirmation of the presence of glycogen was done by digesting the PAS-positive material in thick sections with α-amylase. In the glutaraldehyde–osmium tetroxide fixed material, the sections digested with the enzyme showed a considerably weaker PAS-positive reaction compared with those without enzyme digestion, while the sections of the material fixed with osmium tetroxide only became completely PAS-negative after the enzyme digestion (Fig. 7). This indicates that the PAS-positive material is glycogen. It may be noted that ptyalin, which is supposed to remove entirely glycogen from paraffin embedded sections (Pearse, 1960), is ineffective in digesting glycogen from Araldite sections. The above procedure enables obtaining unequivocal evidence of the glycogenic nature of the granular electron opaque material in the electron micrographs. In quite a few cases, however, the areas that contained glycogen, as judged by the PAS-positive reaction of the adjacent 1 μm thick light microscopic sections, are seen to be translucent rather than opaque. An observation on two adjacent thin sections of the same cell on the same grid showed that the electron opaque material is present in one section while it appears extracted in the other (Figs. 8, 9).
(B) Distribution of glycogen during chondrogenesis

(i) Mouse. The glycogen can be detected in the developing epiphyseal cartilage cells of the hind-limb toes as early as the chondroblast stage. In the light microscope the 1 μm sections of the cytoplasm of chondroblast from 18-day-old mouse embryos show one or two sharply defined areas which appear bright red after PAS staining. The glycogen may be present even in the cells undergoing mitotic division (Fig. 1). In the electron micrographs the main mass of the glycogen is seen in the cortical cytoplasm as one or two large accumulations unbound by any membrane. It is also present in smaller amounts either as single granules or as granule-groups scattered throughout the cytoplasm. Sometimes the glycogen granules like the ribosomes seem to be arranged on the endoplasmic reticulum or outer nuclear membrane (Fig. 10); this arrangement is probably a mere spatial phenomenon and is not a reflexion of any functional association as suggested by Manasek (1968). The glycogen granules are never seen in the Golgi vacuoles or cisternae and only once was a granule seen in the extracellular space (Fig. 4).

The amount of glycogen in the epiphyseal cartilage from newborn mouse is increased as compared with that in the cartilage of the 18-day-old mouse embryo. In the light microscopic sections the glycogen is extensive in amount, and in addition to one or two large lumps it is also present in a few smaller accumulations in the cytoplasm. In the electron micrographs the distribution of

**Figures 1-3**

Symbols referring to the techniques used:

M1 = Osmium tetroxide fixation, Araldite embedding, digestion with α-amylase and staining with PAS-reagent and toluidine blue.

M2 = Glutaraldehyde-osmium tetroxide fixation, Araldite embedding, staining with uranyl acetate and lead citrate.

M3 = Osmium tetroxide fixation, Araldite embedding, staining with PAS and toluidine blue.

M4 = Glutaraldehyde-osmium tetroxide fixation, Araldite embedding, sections treated with periodic acid and stained with Schiff's reagent for light microscopy and lead citrate for electron microscopy.

Fig. 1. Light micrographs of late chondrogenic tissue (mouse, 18-day-old embryo). A characteristic appearance of the mouse epiphyseal cartilage can be seen. Glycogen-laden chondroblasts with small eccentric nuclei are interspersed in an extensive matrix. The glycogen can be seen even in a mitotic cell (arrow). This tissue differs from corresponding tissue in chick (Fig. 12) where glycogen is absent. M4, ×1000.

Fig. 2. Light micrograph of a section from the same Araldite block as used in Fig. 1 but only with toluidine blue stain to show the light staining of the glycogen containing areas. ×1800.

Fig. 3. Electron micrograph of the epiphyseal cartilage (mouse, newborn). Note the widespread distribution of glycogen, which appears opaque in the low power of electron microscope. M2, ×3750.
the glycogen is similar to that in the 18-day-old mouse embryo except that the amount of glycogen is increased. In the cells undergoing hypertrophy the increasing amount of the glycogen occupies considerably more space in the ground cytoplasm (Fig. 11).

(ii) Chick. The presence of glycogen in the epiphyseal cartilage from toes of the chick hind-limb buds was not detected in the present study at any time during chondrogenesis up to Hamilton & Hamburger stage 39, regardless of the fixative used (Fig. 12). Some chondrocytes from or near that part of the diaphysis where periosteal ossification has started, show slight amounts of glycogen distributed more or less evenly in the cytoplasm.

DISCUSSION

The method of fixation in the case of glycogen is considered to be very important since unsuitable fixation leads to polarization and clumping of glycogen (Mancini, 1948; Pearse, 1960). In the present study the fixation of the tissue with glutaraldehyde and osmium tetroxide did not lead to polarization or artificial clumping. In none of the electron micrographs does the glycogen appear to have displaced any of the cytoplasmic organelles and it is distributed in unpolarized masses of various sizes as well as individual granules around 25–50 nm in diameter. Consequently it is not necessary to resort to the technique of freeze drying, which is considered suitable for preserving glycogen in its in vivo state and results in the even distribution of glycogen throughout the cell (Mancini, 1948).

The translucent rather than opaque appearance of the glycogen containing areas in the electron microscope, as observed sometimes in the present study, is the normal appearance in the micrographs of several workers (Godman & Porter, 1960; Silberberg, 1968 inter alia). The cause for the two different appearances of glycogen-containing areas is uncertain but the following interpretation is plausible. Some amount of glycogen from ultra-thin sections occasionally can be washed away during staining and washing of the sections. However, in the

FIGURES 4–7

Fig. 4. Electron micrograph of the late chondrogenic tissue (mouse, 18-day-old embryo) from the same Araldite block as Figs. 1 and 2. The substructure of glycogen granules, after periodic acid–lead citrate treatment, is visible. One of the granules is in the extracellular space (arrow). M4, ×46000.

Figs. 5, 6. Epiphyseal cartilage (mouse, newborn). Adjacent sections from the same block, showing the similarity in the intracellular distribution of PAS-positive material in the light micrograph and the periodic acid–lead citrate reactive material in the electron micrograph. M4, ×2500 and ×9000 respectively.

Fig. 7. Light micrograph of late chondrogenic tissue (mouse, 18-day-old embryo). The glycogen from the cells has been removed by digestion with amylase (arrows). M1, ×1500.
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In the present study the adjacent 1 μm thick sections invariably produce the PAS-positive reaction. Perry (1967) working on amphibian embryos also reports an extraction effect on glycogen particles, if thin sections are incubated for 3–4 h in any medium, including water; but not if incubated for 30 min only. In any event the observation of electron opaque as well as electron translucent appearance of glycogen-containing areas in the two adjacent sections of the same cell mounted on the same grid is worth noting.

The present observations extend the method of glycogen detection described by Perry (1967) in two important aspects. First, the tissue can be embedded in the widely used epoxy resin Araldite in place of the water soluble glycol methacrylate even for enzymic digestion of glycogen. Secondly, the method is designed to observe the same cell rather than different cells from the same block of tissue, under both light and electron microscopes. In accord with other workers (Harris, 1932; Follis & Berthrong, 1949; Silberberg, 1968), in the present work it has been confirmed that the amount of glycogen increases in direct proportion to the age of cartilage cells even when the cells undergo hypertrophy in the case of mouse. This led Harris (1932) to suggest that a possible relationship exists between the distribution of glycogen in cartilage cells and the degree of ossification. Follis & Berthrong (1949) also remarked that 'there is a rough inverse relationship between the presence of glycogen and the presence of lime salts deposition in the zone of provisional calcification', and suggested the glycogen is the initial substance in a series of reactions needed for calcification.

In our studies on the differentiating epiphyseal cartilage of the chick, no glycogen could be seen at any stage; but some glycogen is seen in cells of diaphysis where cartilage ossification is proceeding. Hay (1958) working on regenerating limbs of Ambystoma also did not report any glycogen in the cartilage. Anderson (1964), while reporting the presence of glycogen in most cells from head of rat femur, also remarked on its absence from some cartilage cells near the articular surface as well as from those in the deeper layers of the zone of proliferation. Matukas, Panner & Orbison (1967), working on the tibial cartilage of chick, also did not report the presence of glycogen. Moreover, Davies, Barnett, Cochrane & Palfrey (1962) reported the absence of glycogen from the

**Figures 8-12**

Figs. 8, 9. Late chondrogenic tissue (mouse, 18-day-old embryo). These two adjacent sections of the same cell mounted on the same grid, show the difference in the electron density of the glycogen-containing area after exactly the same treatment. M2, ×20700.

Fig. 10. Late chondrogenic tissue (mouse, 18-day-old embryo). M2 × 31500.

Fig. 11. Light micrograph of epiphyseal cartilage newborn mouse. Note the extensive amount of glycogen. M3, ×1350.

Fig. 12. Light micrograph of the late chondrogenic tissue (chick, stage 37). Note the absence of glycogen in the cells, although the development of cartilage is virtually complete. M3, ×1200.
articular cartilage of rabbit. The reason for this discrepancy in the distribution of glycogen in the mouse and the chick in this study, as well as in other cases, is unknown. It is feasible to assume that nutritional conditions in which mouse embryos develop (availability of carbohydrates) are more favourable for glycogen storage than those in which chick embryos develop. This difference in any case suggests that it is premature to assign to glycogen any essential role in process of chondrogenesis.

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REFERENCES


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