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Simultaneous localization of type II collagen and core protein of chondroitin sulfate proteoglycan in individual chondrocytes

(differentiation/cartilage matrix/immunofluorescence)

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ABSTRACT **In order to investigate the coordinated synthesis of matrix components by individual chondrocytes, specific antibodies to type I collagen, type II collagen, and chondroitin sulfate proteoglycan core protein were used in simultaneous double immunofluorescence reactions. Extensive accumulation of core protein surrounding chondrocytes and the intracellular accumulation of type II collagen were observed. Extracellular core protein immunofluorescence obscured the intracellular reaction product, but the extracellular immunoreactive material could be removed by digestion with purified testicular hyaluronidase prior to fixation. Subsequent to digestion, core protein and type II collagen were observed in the same chondrocytes within discrete, sometimes identical, cytoplasmic regions, thus demonstrating the simultaneous localization of these two products characteristic of differentiating cartilage.**

Numerous studies are currently concerned with the mechanisms of gene expression in the course of differentiation. Of special interest is the problem of simultaneous expression of a number of genes characteristic of a given differentiated cell type. Transition of limb bud mesenchyme cells to chondrocytes results in the acquisition of the capacity to synthesize in large quantities and accumulate extracellularly (*i*) type II collagen (1, 2), (*ii*) chondroitin sulfate proteoglycan (1, 3, 4), and probably (*iii*) the link protein(s) necessary for stabilization of aggregates of chondroitin sulfate proteoglycan and hyaluronic acid (5). It is of considerable interest to determine whether the simultaneous synthesis of more than one structurally unrelated gene product occurs in the same cell. Although it has been inferred (6, 7), this phenomenon has not so far been directly demonstrated in cartilage.

Immunohistochemical methods have been applied successfully to determine the extent of coordination of biosynthesis for the different collagen types and fibronectin in fibroblasts and chondrocytes (8–13). In this report, we demonstrate the simultaneous intracellular localization of type II collagen and the core protein (CP) of chondroitin sulfate proteoglycan in individual chondrocytes by utilizing double immunofluorescence reactions with specific antibodies prepared against these two proteins.

MATERIALS AND METHODS

Cell Culture. Modifications (4) of the culture of embryonic chicken sternal chondrocytes (14) and stage 24 limb bud mesenchyme (15) have been described. In the appropriate experiments, freshly prepared ascorbate was added to the culture medium at a final concentration of 50 μM. When used, cycloheximide (Aldrich) was added to a final concentration of 2 μg/ml. Mesenchyme cultures were blocked from differentiation by BrdUrd as described (15).

Preparation of Antigens and Antibodies. Antibodies against cartilage CP from chicken embryo epiphyses were prepared in rabbits as described (4) and assayed by a radioimmunological method (3).

Type II collagen was pepsin extracted from adult chicken sternae and purified by a series of precipitations and extractions (16). Type I collagen was isolated by neutral salt extraction of calvaria of 17-day-old chicken embryos injected *in ovo* with β-aminoproprionitrile at 13 days (17) and further purified. The purity of collagen preparations was demonstrated by amino acid analyses and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (18). Type II collagen used in some experiments was a gift from E. J. Miller.

Antibodies to type I collagen and type II collagen were raised in rabbits and guinea pigs and purified by immunoadsorption (8). Crossreactive antibodies were removed by adsorption on heterologous collagen affinity columns (8). Guinea pig antibodies specific for type II collagen used in preliminary experiments were generously provided by K. and H. von der Mark.

A passive hemagglutination assay (8, 19) and a radioimmunoassay utilizing indirect immunoprecipitations with [^{3}H]-proline-labeled type I and type II procollagens (20) were used to determine titers and specificity of collagen antisera as well as to demonstrate the lack of crossreactivity of CP antibodies with either collagen. The direct radioimmunoassay with $^{35}SO_4$-antigen (3) was used to demonstrate lack of crossreactivity of collagen antibodies with CP.

Preparation of Link Protein. Link protein was purified from adult chicken sternae in collaboration with William Upholt and Louis Phillipson by a procedure described by Caterson and Baker (21) and was analyzed by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis (18).

Immunofluorescent Staining. Cells cultured on coverslips were washed in Hanks' balanced salt solution (HBSS), fixed 10 min in 70% (vol/vol) ethanol, treated for 2 min with 98% ethanol/ether (1:1 vol/vol), and air dried as described by von der Mark *et al.* (9). Cells were allowed to react simultaneously with rabbit anti-CP (1:3000 dilution) and either guinea pig anti-type II collagen (1:2000) or guinea pig anti-type I collagen (1:2000) for 20 min at room temperature. After subsequent washes, cells were incubated in rhodamine-conjugated goat anti-rabbit IgG (Cappel, Cochranville, PA; 1:300 dilution) and fluorescein-conjugated goat anti-guinea pig IgG (Cappel; 1:100 dilution) for 20 min at room temperature. Coverslips were mounted in glycerol and observed and photographed by using a Leitz Orthoplan microscope with epifluorescence optics and with a Ploemopak containing specific filter combinations for fluorescein and rhodamine.

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Abbreviations: CP, core protein of chondroitin sulfate proteoglycan; HBSS, Hanks' balanced salt solution.

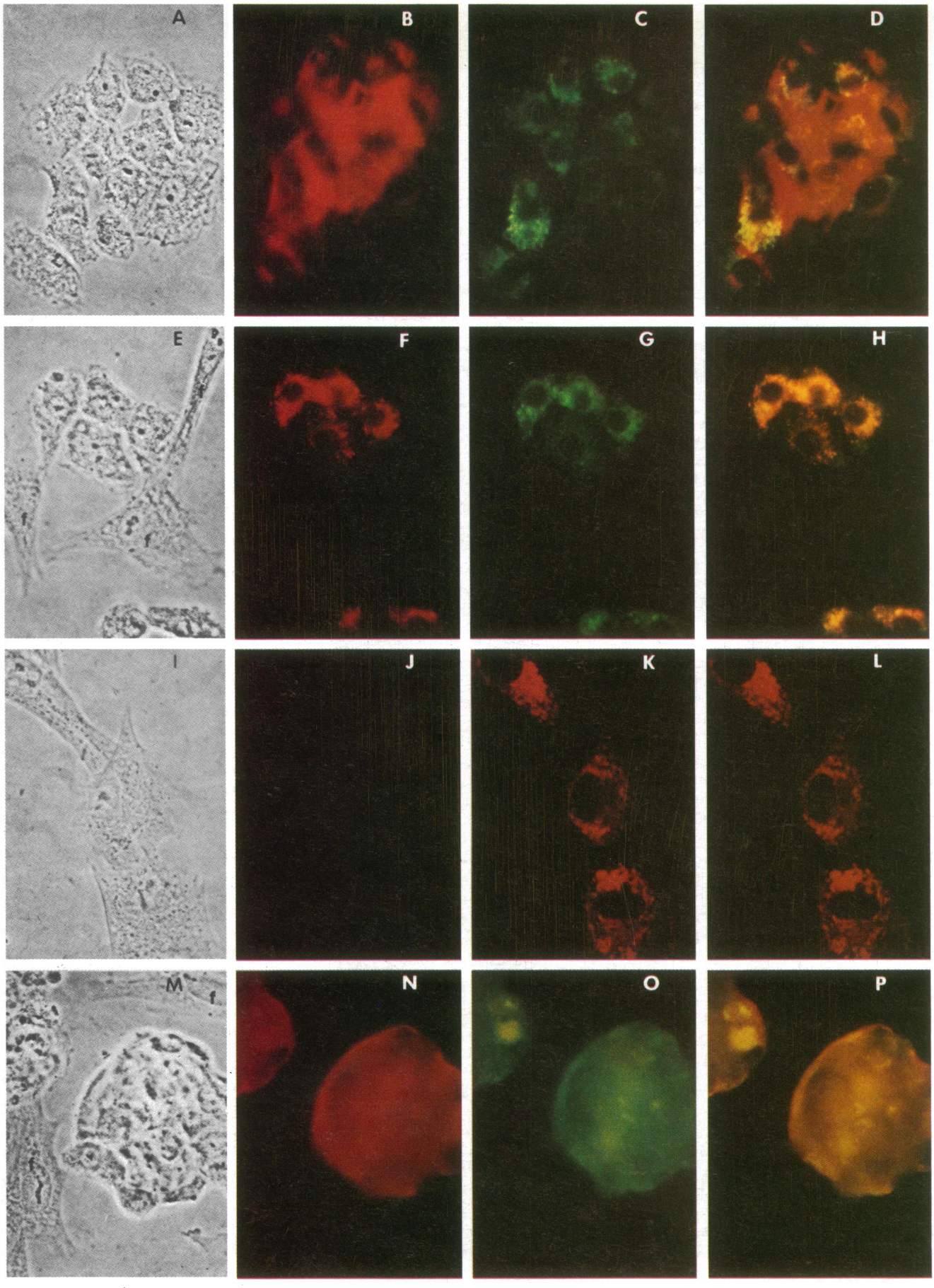

FIG. 1. (*Legend appears at the bottom of the next page.*)

Hyaluronidase Digestion. Cultures were washed three times in HBSS and incubated for 20 min at 37°C in 1.5 ml of HBSS containing 250 international units of purified testicular hyaluronidase (Leo, Helsingborg, Sweden). Cells were subsequently washed in HBSS before fixation, in 70% ethanol and further preparation for immunofluorescence reactions.

Collagenase Digestion. After fixation, treatment with ethanol/ether, and air drying, cells were incubated for 1 hr at 37°C in 1.5 ml of HBSS containing 75 international units of purified bacterial collagenase (form III, Advance Biofactures, Lynbrook, NY). Cells were washed in HBSS prior to immunofluorescence reactions.

RESULTS

Chondrocytes were cultured either from limb bud mesenchyme that had been previously grown over agar for 48 hr (15) or from embryonic sternae. Cells were fixed 3–7 days after plating at low density and stained simultaneously for type II collagen and CP. As shown in Fig. 1 *A–D*, type II collagen was localized intracellularly, whereas CP was demonstrated extracellularly. When the fixed and ethanol/ether-treated material was digested with bacterial collagenase prior to immunohistochemical reactions, chondrocytes still reacted with antibodies against CP, but no longer reacted with antibodies against type II collagen.

Extracellular CP immunofluorescence was so abundant that the presence of intracellular reaction product could not be detected clearly (Fig. 1*B*). However, extracellular immunoreactive material was removed by digestion with purified testicular hyaluronidase prior to fixation. Intracellular localization of both type II collagen and CP could then be demonstrated in chondrocytes (Fig. 1 *E–H*). Cells with chondrocyte morphology stained intracellularly for both cartilage products, sometimes in the same cytoplasmic compartments. The similarities and differences in intracellular distribution of the two cartilage products within the same chondrocytes are clearly demonstrated in the double-exposed micrograph (1*D*). In a few cases, (<15%), individual chondrocytes stained more intensely for one or the other of these products. Less than 5% of morphologically defined chondrocytes stained simultaneously for type I collagen and CP. Most of such cells stained intensely for one or the other product; less than 2% stained moderately for both.

Flattened fibroblast-like cells in the same cultures did not react with type II collagen or CP antibodies. Instead, they reacted with antibodies directed against type I collagen (Fig. 1 *I–L*). Mesenchyme cells that were blocked from differentiation by treatment with BrdUrd reacted similarly to fibroblast-like cells.

The following results were obtained in control experiments. Preimmune rabbit and guinea pig serum controls showed no reaction. Anti-rabbit IgG and anti-guinea pig IgG coupled to fluorescent dyes did not crossreact with the heterologous IgG. Prior incubation of antibodies with purified cartilage CP completely blocked the immunofluorescence reaction with CP, but had no effect on the immunofluorescence localization of type I or type II collagen. Preincubation of antibodies with purified link protein had no effect on any immunofluorescence reaction. Treatment of cells with cycloheximide reversibly blocked intracellular localization of both CP and type II collagen.

When cells were grown in the presence of 50 μM ascorbate for several days, type II collagen accumulated in an extracellular matrix. Extracellular immunofluorescence for CP was more intense. Under these conditions, digestion with hyaluronidase did not result in complete removal of extracellular immunoreactive CP. After enzyme digestion, both CP and type II collagen immunofluorescence were localized in the same extracellular matrix (Fig. 1 *M–P*).

DISCUSSION

These results demonstrate that expression of the cartilage phenotype in chondrocytes usually involves the simultaneous accumulation of the two characteristic cell products, CP and type II collagen. Although accumulation of type II collagen and CP was observed intracellularly in the same chondrocytes, the extracellular deposition of type II collagen fibers was not observed despite the presence of extracellular CP. Digestion with purified hyaluronidase of such an extracellular matrix, containing CP but no collagen, resulted in the removal of all extracellular immunoreactive material (see also ref. 22), thereby permitting visualization of intracellular CP. However, if chondrocytes were grown in 50 μM ascorbate, type II collagen was deposited in an extracellular matrix and increased extracellular CP immunofluorescence was observed. Under these conditions, hyaluronidase digestion no longer completely removed extracellular CP. Instead, both type II collagen and CP remained localized in the same extracellular matrix, suggesting an interaction between these two proteins.

FIG. 1 (*on preceding page*). Simultaneous immunofluorescence localization of CP and type I or type II collagen in sternal chondrocyte cultures. All horizontal rows represent the same field. Vertical columns from left to right are phase contrast micrographs, cells stained for CP, cells stained for collagen, type I or type II, and double-exposed micrographs of fluorescein and rhodamine presented in the previous two columns. (Each micrograph ×1770.) (*A–D*) Chondrocytes were fixed directly and incubated simultaneously with rabbit anti-CP and guinea pig anti-type II collagen followed by incubation with rhodamine-coupled goat anti-rabbit IgG and fluorescein-coupled goat anti-guinea pig IgG. (*A*) Phase micrograph of chondrocytes in a nodule. (*B*) Localization of CP antibodies primarily in extracellular matrix. (*C*) Localization of type II collagen antibodies exclusively within discrete regions of chondrocyte cytoplasm. (*D*) Double-exposed micrograph emphasizes the differences in distribution of the predominantly extracellular CP and intracellular type II collagen. Some areas of possible intracellular CP (e.g., in the bottommost chondrocyte) become evident in the double exposure. (*E–H*) Chondrocyte cultures were digested for 15 min with testicular hyaluronidase prior to fixation and incubation with antibodies as in *A–D*. (*E*) Phase micrograph of two groups of chondrocytes and three fibroblast-like cells (f). (*F*) Localization of CP antibodies within chondrocyte cytoplasm. (*G*) Localization of type II collagen antibodies within the same chondrocytes. (*H*) Double-exposed micrograph reveals that most of the CP and type II collagen antibodies are localized in the same intracellular areas of these chondrocytes (yellow), while some discrete cytoplasmic regions contain primarily CP (red) or type II collagen (green). Fibroblast-like cells in the same field did not react with anti-CP or anti-type II collagen. (*I–L*) Chondrocyte cultures were fixed and incubated in rabbit anti-CP and guinea pig anti-type I collagen followed by incubation in fluorescein-coupled goat anti-rabbit IgG and rhodamine-coupled goat anti-guinea pig IgG. (*I*) Phase micrograph of noncartilage cells. (*J*) Negative reaction with CP antibodies. (*K*) Intracellular localization of type I collagen antibodies. (*L*) Double-exposed micrograph appears equivalent to *K*. (*M–P*) Cultures were grown for 3 days in 50 μM ascorbate and digested for 15 min in testicular hyaluronidase prior to fixation and simultaneous incubation in antibodies as described for *A–D*. (*M*) Phase micrograph of chondrocytes in nodules and several fibroblast-like cells (f). (*N*) Localization of CP antibodies in extracellular matrix. Enzyme digestion no longer removes all extracellular immunoreactive material. (*O*) Localization of type II collagen antibodies in extracellular matrix. Growth in ascorbate results in the deposition of type II collagen in extracellular matrix. Visualization of intracellular type II collagen is obscured under these conditions. (*P*) Double-exposed micrograph reveals that both CP and type II collagen antibodies are localized in the same extracellular matrix. Fibroblast-like cells in the same field did not react with anti-CP or anti-type II collagen.

Conclusions concerning biosynthesis and extracellular matrix deposition drawn from immunohistochemical data depend on the demonstration that antibodies used react with the components in question and do not react with other cellular components. The composition and structure of CP is incompletely understood. Therefore, characterization of the specific antigenic determinants for CP antibodies is somewhat limited. However, reactivity of the serum was demonstrated by the fact that greater than 90% of a $^{35}SO_4$-labeled CP antigen was precipitated by the anti-CP serum, whereas antisera to the collagens did not precipitate this labeled antigen (unpublished data). In contrast, types I and II procollagens labeled with [^{3}H]proline were specifically precipitated by the appropriate homologous antisera, but not by heterologous antisera or by antibodies to CP. Passive hemagglutination assays were also used to demonstrate lack of crossreactivity of antibodies to CP and types I and II collagen.

Evidence for lack of reactivity of these antisera with other cell components also derives from observations of immunofluorescence. Antibodies to the characteristic cartilage products, type II collagen and CP, reacted specifically with cells identified by morphological criteria as chondrocytes and did not react with fibroblasts. In contrast, antibodies to type I collagen reacted intensely with chicken embryo fibroblasts and not with chondrocytes except during the process of switching from the cartilage phenotype (9). In cultures not digested with hyaluronidase, antibodies to type II collagen were localized intracellularly within chondrocytes, whereas CP antibodies were localized extracellularly surrounding the same chondrocytes, thereby demonstrating that these antibodies react with different cartilage molecules. The localization of CP antibodies corresponded to the pattern of alcian blue stain in developing cartilage cultures (4) and could be completely blocked by prior incubation of antibodies with purified cartilage CP. Similar preincubation of antibodies to type II collagen had no effect on their localization. In contrast, collagenase digestion of fixed samples prior to immunofluorescence reactions completely abolished the localization of type II collagen antibodies but did not affect reactivity with CP antibodies. Incubation of antibodies to CP and type II collagen with purified sternal cartilage link proteins prior to immunofluorescence reactions had no effect on the intensity of staining or localization of the specific antibodies, thus demonstrating directly that our antibody preparations contain no measurable anti-link protein determinants.

The recent immunochemical results of Dessau *et al.* (12) demonstrated synthesis and the extracellular deposition of fibronectin between chicken sternal chondrocytes *in vitro* and suggested that the extracellular accumulation of cartilage matrix might involve interactions with yet another component. Dessau *et al.* observed a strandlike pattern of localized immunofluorescence for fibronectin quite different from the immunofluorescent localization described in this report for type II collagen (see also refs. 9 and 12) and CP, which argues against the presence of fibronectin determinants in CP or collagen antisera.

The experimental approach utilized in this report can be used to further examine the relationships between intracellular synthesis and processing and the extracellular deposition in matrix for several components in order to understand coordinate gene expression during cartilage differentiation.

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1. Levitt, D. & Dorfman, A. (1974) *Curr. Top. Dev. Biol.* **8**, 102–149.
2. von der Mark, K. & von der Mark, H. (1977) *J. Cell Biol.* **73**, 736–747.
3. Ho, P.-L., Levitt, D. & Dorfman, A. (1977) *Dev. Biol.* **55**, 233–243.
4. Vertel, B. & Dorfman, A. (1978) *Dev. Biol.* **62**, 1–12.
5. Vasan, N. S. & Lash, J. W. (1977) *Biochem. J.* **164**, 179–183.
6. Smith, P. H. (1972) *Connect. Tissue Res.* **1**, 181–188.
7. Prockop, D. J., Pettingill, O. & Holtzer, H. (1964) *Biochim. Biophys. Acta* **83**, 189–196.
8. von der Mark, H., von der Mark, K. & Gay, S. (1976) *Dev. Biol.* **48**, 237–249.
9. von der Mark, K., Gauss, V., von der Mark, H. & Müller, P. (1977) *Nature (London)* **267**, 531–532.
10. Gay, S., Martin, G. R., Müller, P. K., Timpl, R. & Kühn, K. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4037–4040.
11. Müller, P. K., Lemmen, C., Gay, S., Gauss, V. & Kühn, K. (1977) *Exp. Cell Res.* **108**, 47–55.
12. Dessau, W., Sasse, J., Timpl, R., Jilek, F. & von der Mark, K. (1978) *J. Cell Biol.* **79**, 342–355.
13. Vaheri, A., Kurkinen, M., Lehto, V.-P., Linder, E. & Timpl, R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4944–4948.
14. Cahn, R. D., Coon, H. G. & Cahn, M. B. (1967) in *Methods in Developmental Biology*, eds. Wilt, F. H. & Wessels, N. K. (Crowell, New York), pp. 493–530.
15. Levitt, D. & Dorfman, A. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1253–1257.
16. Miller, E. J. (1971) *Biochemistry* **10**, 1652–1659.
17. Pawlowski, P. J., Gillette, M. T., Martinell, J., Lukens, L. N. & Furthmayr, H. (1975) *J. Biol. Chem.* **250**, 2135–2142.
18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
19. Beil, W., Furthmayr, H. & Timpl, R. (1972) *Immunochemistry* **9**, 779–788.
20. Uitto, J. (1977) *Biochemistry* **16**, 3421–3429.
21. Caterson, B. & Baker, J. (1978) *Biochem. Biophys. Res. Commun.* **80**, 496–503.
22. Meier, S. & Solursh, M. (1978) *J. Ultrastruct. Res.* **65**, 48–59.