Control of Chondrogenesis in Limb-Bud Cell Cultures by Bromodeoxyuridine

(differentiation/glycosaminoglycans/D-xylose/enzymes)

DANIEL LEVITT AND ALBERT DORFMAN

Departments of Pediatrics, Biochemistry, and Biology, Joseph P. Kennedy, Jr., Mental Retardation Research Center, La Rabida-University of Chicago Institute, Pritzker School of Medicine, University of Chicago, Chicago, Illinois 60637

Communicated by Hewson Swift, May 7, 1973

ABSTRACT Initial exposure of cultured limb-bud cells (stage 23-24) to 5-bromo-2'-deoxyuridine (BrdU) irreversibly inhibits differentiation to cartilage under three different culture conditions. The inhibition of chondroitin sulfate synthesis is partially reversed by D-xylose in limb-bud cells after treatment with BrdU. The activities of four enzymes involved in chondroitin sulfate production were reduced in BrdU-treated cultures, but the magnitude of decrease was far less than the decrease in glycosaminoglycan synthesis. The slight increase in the turnover rate of sulfated glycosaminoglycans in BrdU-treated mesenchyme was not sufficient to account for the marked decrease in chondroitin sulfate content. The results suggest that BrdU treatment interferes with normal synthesis of chondroitin sulfate core protein in cultured limb-bud cells, but does not greatly diminish enzyme activities or UDP-sugar levels necessary for production of polysaccharide chains.

Culture of several types of differentiated cells in the presence of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) results in decreased synthesis of macromolecules characteristic of cell specialization (1-4). Differentiated cell expression is resumed upon removal of BrdU. In contrast to this reversible effect, BrdU irreversibly inhibits differentiation of certain precursor cell populations to specific phenotypes (5, 6). Irreversibility apparently depends upon treatment of cells at stages of development before "determination" has been stabilized.

Differentiated chondrocytes are characterized by synthesis of large quantities of chondroitin sulfate proteoglycan. Production of chondroitin sulfate is sharply decreased in both differentiated chick chondrocytes growing in the presence of BrdU and cultures of chick limb-bud cells (stages 23-24) (7) initially exposed to BrdU then grown in its absence for 1-4 weeks (5).

Brett and Robinson (8), using minced chick epiphyseal cartilage, have shown that the inhibition of chondroitin sulfate synthesis by puromycin is partially reversed by addition of D-xylose. The pentose appears to substitute for core protein as initiator of chondroitin sulfate chains. The studies to be reported here show enhancement of chondroitin sulfate synthesis by D-xylose in limb-bud cells previously treated with BrdU. Activities of certain enzymes involved in chondroitin sulfate formation and turnover rates of sulfated mucopolysaccharides were also determined. The results suggest that BrdU treatment interferes with the onset of synthesis of a particular core protein characteristic of cartilage.

MATERIALS AND METHODS

Source of Materials. F-12 powdered medium, fetal-calf serum, horse serum, and 0.25% trypsin in ethylene diamine tetracetate (EDTA) were purchased from Grand Island Biological Co. UDP-glucose, glucose-6-phosphate, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Co. D-Xylose was manufactured by Pfanstiehl Laboratories, and puromycin dihydrochloride by Nutritional Biochemicals Co. $\text{H}_2^{35}\text{SO}_4$ (43 Ci/mg), sodium $[^3\text{H}]\text{acetate}$ 100 Ci/mol, sodium $[1\text{-}^{14}\text{C}]\text{acetate}$ (56 Ci/mol), UDP- $[^{14}\text{C}]\text{xylose}$ (190 Ci/mol), and UDP-N- $[^{14}\text{C}]\text{acetylgalactosamine}$ (43 Ci/mol) were obtained from New England Nuclear Corp. 4-Methyl-umbelliferyl- β -D-glucuronide trihydrate was purchased from Koch-Light Laboratories, Ltd., and 4-methyl-umbelliferyl- β -D-N-acetylglucosaminide was obtained from Sigma Chemical Co. Carrier chondroitin-4-sulfate was donated by Dr. J. A. Cifonelli.

Cell Culture. Embryonic-chick limb buds (stage 23-24) were dissociated in 0.25% trypsin in EDTA. 25 Million single cells were plated on 60-mm Falcon tissue culture dishes in F-12 medium with 7% horse serum and 8% fetal-calf serum (complete medium). Cultures either remained untreated or were exposed to 32 μM BrdU for their first 2 days of growth. The BrdU was removed, and after two washes with complete medium, the cells were maintained for 7 additional days in complete medium without BrdU. Cells were fed every other day.

Glycosaminoglycan Synthesis and Turnover. On the ninth day of growth, cultures were labeled with 5 μCi of $\text{H}_2^{35}\text{SO}_4$, 10 μCi of $[^3\text{H}]\text{acetate}$, or 5 μCi of $[^{14}\text{C}]\text{acetate}$ per ml of medium for 6 hr. For analysis of total glycosaminoglycan synthesis, cells and media were pooled and total glycosaminoglycans were isolated as described (9). For turnover measurements, fresh medium was added after a 6-hr pulse with 5 μCi of $\text{H}_2^{35}\text{SO}_4$ per ml on the ninth day of culture. Glycosaminoglycans were purified from cells plus fresh medium in 9-day, 11-day, and 13-day cultures, and radioactivity was determined by liquid scintillation counting.

Enzyme Assays. Cells were scraped from culture dishes, washed twice with the appropriate buffer, and disrupted by 25 1-sec bursts with a Branson Sonifier at 70 W. Lysis and homogenization were monitored with trypan blue stain.

Xylosyltransferase (10) and N-acetylgalactosamine transferase assays (11) were performed on 10,000 $\times g$ supernatant

TABLE 1. *Effect of BrdU on chondroitin sulfate synthesis by limb-bud cells under various culture conditions*

Culture conditions	cpm/10 ⁶ cells	
	Control	BrdU
Agar, 2 days → plastic, 7 days*	64,900	9200
Agar, 2 days → agar, 5 days → plastic, 7 days*	58,400	9100
Plastic, 14 days†	16,600	2200

Cells were grown in the presence or absence of BrdU for the first 2 days. Subsequently all cells were grown for the remainder of the experiment in the absence of BrdU and incubated on the last day with 5 μ Ci of H₃³⁵SO₄ per ml for 6 hr. Radioactivity incorporated in glycosaminoglycans was measured by liquid scintillation counting.

* Freshly dissociated limb-bud cells were incubated over agar (10 × 10⁶ cells per 100-mm dish) and subcultured on plastic-tissue culture dishes at low density (0.5 × 10⁶ cells per 60-mm dish).

† Cells cultured directly on plastic-tissue culture dishes at high density (25 × 10⁶ cells per 60-mm dish).

fractions. UDP-Glucose dehydrogenase (EC 1.1.1.22) (12), UDP-N-acetylhexosamine-4-epimerase (13) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (14) activities were measured on 20,000 × *g* supernatant solutions. β -N-Acetylhexosaminidase (EC 3.2.1.30) (15) and β -glucuronidase (EC 3.2.1.31) concentrations (15) were determined on the 10,000 × *g* supernatant solutions after sonic disruption and three rounds of freezing and thawing of the scraped cells.

Protein was measured by the method of Lowry *et al.* (16), with bovine-serum albumin as standard. Cell counts were performed with a Coulter Counter model B after results were corroborated with multiple hemocytometer determinations. Liquid scintillation counting was performed in a Packard Tri-Carb Scintillation Counter (9).

RESULTS

Irreversibility of BrdU Effect with Several Different Culture Conditions. Culture of limb-bud cells at low density on plastic-tissue culture dishes does not lead to chondrogenesis (5). If such cells are first cultured over agar for 2 days, differentiation to chondrocytes ensues upon subculture to plastic dishes at low density. However, exposure to BrdU during the period

TABLE 2. *Effect of xylose on glycosaminoglycan synthesis in control and BrdU-treated limb-bud cells*

Xylose (mM)	H ₂ ³⁵ SO ₄ (cpm/10 ⁶ cells)		[³ H]Acetate (cpm/10 ⁶ cells)	
	Control	BrdU	Control	BrdU
0	10,000	2220	5050	970
10.6	14,400	5060	7820	2250
21.2	13,050	5880	7150	2330
42.4	13,700	6000	7180	2210

Both 5 μ Ci of H₂³⁵SO₄ and 10 μ Ci of [³H]acetate per ml of medium, plus the indicated amount of xylose, were added to high-density limb-bud cell cultures (initial density 25 × 10⁶ cells per 60-mm dish) on the ninth day of growth, 6 hr before harvest. Glycosaminoglycans were isolated as described (9).

over agar results in irreversible inhibition of differentiation on subculture. Since growing limb-bud cells at high density on plastic dishes without previous exposure to agar also results in chondrogenesis, it was necessary to examine the effect of BrdU on such high-density cultures. Table 1 shows that when limb-bud cells are cultured at high densities on plastic dishes in the presence of 32 μ M BrdU for the first 2 days, they do not differentiate into cartilage upon removal of the drug and continued culture in its absence for 14 days. Likewise, if freshly dissociated limb-bud mesenchyme is exposed to BrdU for 2 days while growing over agar and then permitted to remain over agar in the absence of BrdU for 5 more days, subculture onto plastic dishes for 7 additional days does not result in differentiation into chondrocytes, as judged by meta-chromatic staining and incorporation of radioactive sulfate into glycosaminoglycans (Table 1). The permanent suppression of chondrogenesis by BrdU appears to be independent of several culture techniques.

Effect of BrdU on Chondroitin Sulfate Chain Initiation. A direct measure of the capacity of mesenchyme cells previously treated with BrdU to synthesize chondroitin sulfate chains was obtained by the use of xylose as a chain initiator. For this purpose, limb-bud cells at high density were exposed to 32 μ M BrdU for 2 days, then grown for 7 days in the absence of BrdU. On the ninth day, cultures previously treated with BrdU and control cultures were incubated for 6 hr with concentrations of D-xylose ranging from 0.1 to 42.6 mM together with 5 μ Ci of H₂³⁵SO₄ and 10 μ Ci of [³H]acetate per ml of medium. Glycosaminoglycans were isolated from pooled cells and media. Table 2 shows a stimulation of glycosaminoglycan synthesis of about 250% in BrdU-treated cultures incubated with 10.6–42.4 mM xylose, whereas control cells increased their polysaccharide production by only 40%. A concentration of 1.1 mM xylose did not stimulate glycosaminoglycan synthesis in either BrdU-treated or control cultures, nor did 30 mM D-glucose increase glycosaminoglycan production in progeny of BrdU-treated mesenchyme cells. Radioactive xylose was incorporated into polysaccharide chains that were not precipitable with cold 5% Cl₃CCOOH and, thus, were not linked to a protein core. These chains cochromatographed

TABLE 3. *Effect of xylose on glycosaminoglycan synthesis in control and BrdU-treated cultures in the presence of puromycin*

Xylose (mM)	H ₂ ³⁵ SO ₄ (cpm/10 ⁶ cells)		[³ H]Acetate (cpm/10 ⁶ cells)	
	Control	BrdU	Control	BrdU
0	840	340	260	160
10.6	3100	2720	920	700
21.2	4250	2730	1320	850
42.4	4380	2780	1320	890

High-density cultures were first incubated for 20 min with 10 μ g of puromycin per ml of medium on the ninth day of growth. Both 5 μ Ci of H₂³⁵SO₄ and 10 μ Ci of [³H]acetate per ml of medium, plus the indicated amounts of xylose, were added to each dish 6 hr before harvest. Puromycin was present throughout the entire 6-hr period. The above cultures were from the same experiment as those in Table 2.

FIG. 1. Turnover of sulfated glycosaminoglycans in control and BrdU-treated limb-bud cell cultures. Cultures were labeled with $5\mu\text{Ci}$ of $\text{H}_2^{35}\text{SO}_4$ per ml of medium for 6 hr on the ninth day of culture. Each dish was washed twice with complete medium. Then cells plus fresh medium were assayed for sulfate-labeled glycosaminoglycans on the ninth, tenth, eleventh, and thirteenth days of growth (0, 1, 2, and 4 days of chase).

on Sephadex G-200 identically with chondroitin sulfate obtained from alkali-treated chondroitin sulfate proteoglycan.

To prove that the presence of core protein was not a prerequisite for chondroitin sulfate synthesis in the presence of xylose, we first incubated cultures for 20 min with $10\mu\text{g}$ of puromycin per ml, after which $10.6\text{--}42.4\text{ mM}$ xylose were added to each plate, in addition to $^{35}\text{SO}_4^{2-}$ and ^3H acetate. Puromycin was present throughout the 6-hr labeling period. The results (Table 3) indicate that even when synthesis of chondroitin sulfate proteoglycan is inhibited more than 90% by puromycin, addition of xylose partially overcomes this suppression in both control and BrdU-exposed cultures. This observation confirms the previous findings of Brett and Robinson (8). We may conclude that BrdU-treated limb-bud cells possess the potential to synthesize chondroitin sulfate.

Activities of Enzymes Involved in Chondroitin Sulfate Synthesis. Further proof of the capacity of BrdU-treated limb-bud cells to synthesize chondroitin sulfate was provided by measurement of the activities of certain enzymes involved in

TABLE 4. Chondroitin sulfate synthesis and transferase activities

	Control	BrdU
Chondroitin sulfate synthesis (cpm/ 10^6 cells)		
$\text{H}_2^{35}\text{SO}_4$ incorporation	13,600	1,680
^{14}C Acetate incorporation	7,840	1,160
Transferase activities (cpm/mg of protein per hr)		
Xylosyl transferase	2,060	1,390
<i>N</i> -Acetylgalactosamine transferase	24,200	13,800

Chondroitin sulfate synthesis was determined as described. Xylosyltransferase activity was assayed by measurement of the transfer of xylose from UDP- ^{14}C xylose to Smith-degraded proteoglycan according to Stoolmiller *et al.* (10). *N*-Acetylhexosaminetransferase activity was determined by measurement of the addition of *N*-acetylgalactosamine from UDP- ^{14}C -acetylgalactosamine to a hexasaccharide acceptor possessing a terminal β -glucuronic acid residue (11).

TABLE 5. Activity of nucleotide sugar synthetic enzymes

Assay	Control	BrdU
UDP- <i>N</i> -acetylhexosamine-4-epimerase*	27,650	15,600
UDP-glucose dehydrogenase†	56.5	51.0
Glucose-6-phosphate dehydrogenase‡	5.4	5.0

UDP-*N*-acetylhexosamine epimerase activity was determined by measurement of the conversion of UDP- ^{14}C acetylgalactosamine to UDP- ^{14}C acetylglucosamine by the $20,000 \times g$ supernatant. The UDP sugars were then hydrolyzed in 0.2 N HCl at 100° for 30 min and neutralized with 0.4 N NaOH. *N*-acetylgalactosamine was separated from *N*-acetylglucosamine by descending paper chromatography (13). Dehydrogenase activities were determined as the reduction of either NAD or NADP, and were measured spectrophotometrically as increased absorbance at 340 nm.

* cpm of N - ^{14}C acetylglucosamine per mg of protein per hr.

† μmol of NADH per mg of protein per min.

‡ μmol of NADPH per mg of protein per min.

formation of this polysaccharide. Xylosyltransferase initiates polysaccharide chain formation by transfer of *D*-xylose from UDP-xylose to serine residues of the core protein. *N*-Acetylgalactosamine transferase extends the polysaccharide chain by transferring *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine to terminal β -glucuronic acid residues. The activities of xylosyltransferase and *N*-acetylgalactosamine transferase were diminished by only 33% and 43%, respectively, in BrdU-treated cultures, whereas incorporation of $^{35}\text{SO}_4^{2-}$ and ^{14}C acetate into glycosaminoglycans was inhibited by 85–90% (Table 4). Although transferase activities measured were somewhat diminished, the magnitude of decrease did not appear to be sufficient to account for the striking decrease of glycosaminoglycan synthesis.

As a possible measure of the capacity of BrdU-treated mesenchyme cells to synthesize the requisite nucleotide precursors, the activities of UDP-glucose dehydrogenase and UDP-*N*-acetylhexosamine-4-epimerase were determined. The data in Table 5 showed that these activities were somewhat diminished in cultures initially exposed to BrdU. Assays of glucose-6-phosphate dehydrogenase, an enzyme not involved in the pathway of glycosaminoglycan synthesis, also demonstrated a minimal decrease of activity in BrdU-treated cells. Accordingly, the sizes of UDP-glucuronic acid, UDP-*N*-acetylhexosamine, and UDP-xylose pools were measured and found not to be reduced in BrdU-treated cultures (Levitt and Dorfman, unpublished).

TABLE 6. Activity of glycosaminoglycan degradative enzymes

Assay	μmol of umbelliferone per mg of protein per hr	
	Control	BrdU
β -D-Glucuronidase	1.09	1.75
β -D- <i>N</i> -Acetylhexosaminidase	0.95	1.31

Both assays involved cleavage of the sugar from 4-methylumbelliferyl glycosides, resulting in fluorescence that was determined in an Aminco-Bowman Spectrophotofluorometer. The excitation and emission wave lengths were 448 nm and 360 nm, respectively.

Turnover of Chondroitin Sulfate. It remained possible that BrdU-treated limb mesenchyme contained less chondroitin sulfate than control cells because of an elevation in the rate of turnover of this glycosaminoglycan. An increased rate of degradation might be due to augmented activities of certain lysosomal glycosidases involved in mucopolysaccharide destruction. Fig. 1 indicates that over a period of 4 days, sulfated glycosaminoglycans are degraded at only a slightly greater rate in BrdU-treated cells than in control cultures, a finding that does not account for the large differences in quantities of chondroitin sulfate contained in the two cultures. The activities of the lysosomal enzymes, β -glucuronidase and β -N-acetylhexosaminidase, were somewhat higher in cells that had been previously exposed to BrdU (Table 6). No significant hyaluronidase activity was found either in BrdU-treated or control cells as measured by the viscosity reduction method (17).

DISCUSSION

The chondrocyte phenotype is distinguished by a high rate of synthesis of chondroitin sulfate proteoglycan and cartilage-specific collagen. Treatment of stage 23-24 limb-bud cells with BrdU causes an irreversible inhibition of formation of chondroitin sulfate proteoglycan at a rate characteristic of mature chondrocytes.

Synthesis of chondroitin sulfate proteoglycan is thought to be initiated with formation of core protein, followed by addition of covalently linked sugar residues as a result of the sequential action of six microsomal glycosyl transferases and appropriate sulfotransferases (18). The reduced content of chondroitin sulfate proteoglycan in limb-bud cell cultures previously treated with BrdU could result from: (a) decreased synthesis of a specific core protein; (b) synthesis of an aberrant core protein that is not glycosylated; (c) deficient synthesis of nucleotide sugar precursors; (d) deficient glycosyl transferase activity; or (e) augmented rate of degradation of proteoglycan. Turnover studies indicate that the low levels of chondroitin sulfate in BrdU-treated limb-bud cells cannot be accounted for by an increased rate of degradation.

Earlier studies in this laboratory by Franco-Browder *et al.* (19) had shown that chondroitin sulfate was synthesized by embryonic-chick tissues before the morphological appearance of cartilage. Subsequently, Medoff (20) demonstrated that predifferentiated limb-bud mesenchyme contained some of the enzymes necessary for chondroitin sulfate synthesis. The results presented in this paper indicate that cultured limb-bud cells, previously treated with BrdU, contain two of the transferases required for chondroitin sulfate chain formation, as well as two of the enzymes involved in synthesis of sugar nucleotide precursors of galactosaminoglycans. Although some diminution in activity of these enzymes was observed, the extent of decrease was far less than the degree of inhibition of chondroitin sulfate synthesis. Direct measurement showed no diminution of UDP-N-acetylgalactosamine, UDP-glucuronic acid, and UDP-xylose pools in BrdU-treated cells (Levitt and Dorfman, unpublished results). These data suggest that the reduced chondroitin sulfate synthesis is not primarily due to diminution of the enzymic machinery responsible for formation of chondroitin sulfate chains. This conclusion is supported by the finding that xylose significantly restores the rate of chondroitin sulfate synthesis in cells previously treated with BrdU.

In total, the results imply that BrdU treatment at stage 23-24 prevents limb-bud cells from acquiring the ability to form a specific core protein. The possibility that an abnormal protein is formed cannot be ruled out. Palmoski and Goetinck (21) have recently proposed that chondrocytes form two types of chondroitin sulfate proteoglycan, one of which is either absent or severely depressed in both cultured nanomelic chick cartilage and BrdU-treated chondrocytes.

Chondroitin sulfate proteoglycan produced by limb-bud mesenchyme can be separated into two peaks by gel filtration with Bio-Gel A-50M (unpublished data). An excluded peak is present in differentiated cells but is markedly reduced in prechondrogenic limb-bud mesenchyme and the progeny of BrdU-treated limb-bud cells. An included fraction is detectable at comparable levels in both the differentiated and undifferentiated tissues. Chondroitin sulfate chain lengths are similar in the two cell types. We postulate that the first proteoglycan peak represents a cartilage-specific protein-polysaccharide, while the second peak contains a nonspecific chondroitin sulfate proteoglycan. Differentiation of mesenchyme to cartilage would involve the appearance of a cartilage-specific core protein as a prerequisite for production of large quantities of chondroitin sulfate proteoglycan characteristic of mature chondrocytes. If limb mesenchyme is exposed to BrdU at stage 23-24, this cartilage-specific core protein does not appear, or it is altered in such a manner that it is unable to act as an acceptor for xylose. Other direct or indirect effects on the biosynthesis of chondroitin sulfate proteoglycan have not been ruled out. In collaboration with Drs. B. D. Smith and G. R. Martin, unpublished studies show that stage 23-24 limb-bud cells produce collagen with an $\alpha_1:\alpha_2$ chain ratio characteristic of fibroblasts. After differentiation to chondrocytes in culture, the ratio of $\alpha_1:\alpha_2$ becomes characteristic of cartilage. When these limb-bud cells are treated with BrdU, the collagen manufactured has an $\alpha_1:\alpha_2$ ratio typical of mesenchyme or fibroblast cells. Thus, BrdU treatment appears to prevent acquisition of the capacity to synthesize both cartilage-specific collagen and core protein.

The mechanism of action of BrdU in differentiated cells remains unknown. Information obtained from several systems indicates that exposure to BrdU results in reversible inhibition of synthesis of specialized products in differentiated cells, while precursor cell populations never attain the ability to create those macromolecules associated with the differentiated state (1-6). These findings predict a unique control mechanism for synthesis of specialized products and even the process of differentiation itself.

It seems unlikely that the specific effects of BrdU stem from a random substitution for thymidine in all structural genes. In fact, BrdU need substitute for less than 10% of the available thymidine residues in chick limb-bud cell DNA in order to exert its inhibitory influences (ref. 5, and unpublished results). This observation has been confirmed with chick hematocytoblasts (22). Therefore, the more likely possibility would be that BrdU is preferentially incorporated into a piece of DNA involved in control of synthesis of specialized products. The existence of such distinctive types of DNA that regulate the genes responsible for differentiated function has been proposed by Britten and Davidson (23). Such a regulatory mechanism may control the expression of both cartilage-specific collagen and cartilage-specific core protein.

The findings of Schulte-Holthausen *et al.* (24) and Marzullo (25) of strikingly decreased activities of UDP-glucose dehydrogenase, UDP-*N*-acetylgalactosamine-4-epimerase, and UDP-glucose epimerase in BrdU-treated chondrocytes are at variance with our data for limb-bud cells. Experiments in this laboratory indicate that xylose overcomes the inhibition of chondroitin sulfate synthesis in BrdU-treated chondrocytes as well as in limb-bud cells.

We thank Ms. Pei-Lee Ho for her excellent technical assistance. This study was supported by USPHS Grants AM-05996, HD-04583, and HD-00001.

1. Turkington, R. W., Majumder, G. C. & Riddle, M. (1971) *J. Biol. Chem.* **246**, 1814-1819.
2. Coleman, A. W., Coleman, J. R., Kankel, D. & Werner, I. (1970) *Exp. Cell Res.* **59**, 319-328.
3. Bischoff, R. (1971) *Exp. Cell Res.* **66**, 224-236.
4. Abbott, J. & Holtzer, H. (1968) *Proc. Nat. Acad. Sci. USA* **59**, 1144-1151.
5. Levitt, D. & Dorfman, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1253-1257.
6. Abbott, J., Mayne, R. & Holtzer, H. (1972) *Develop. Biol.* **28**, 432-442.
7. Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88**, 49-92.
8. Brett, M. J. & Robinson, H. C. (1971) *Proc. Aust. Biochem. Soc.* **4**, 92 abstr.
9. Dorfman, A. & Ho, P.-L. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 495-499.
10. Stoolmiller, A. C., Horwitz, A. L. & Dorfman, A. (1972) *J. Biol. Chem.* **247**, 3525-3532.
11. Horwitz, A. L. & Dorfman, A. (1968) *J. Cell Biol.* **38**, 358-368.
12. Gainey, P. A. & Phelps, C. F. (1972) *Biochem. J.* **128**, 215-227.
13. Davidson, E. A. (1966) in *Methods in Enzymology*, eds. Neufeld, E. F. & Ginsburg, V. (Academic Press, New York), Vol. 8, pp. 277-281.
14. Holliday, R. & Tarrent, G. M. (1972) *Nature* **238**, 26-30.
15. Van Hoof, F. & Hers, H. G. (1968) *Eur. J. Biochem.* **7**, 34-44.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-295.
17. Dorfman, A. & Ott, M. L. (1948) *J. Biol. Chem.* **172**, 367-375.
18. Dorfman, A. (1970) in *Advances in Biology of the Skin*, eds. Montagna, W., Bentley, J. P. & Dobson, R. L. (Appleton-Century-Crofts, New York), Vol. 10, pp. 123-147.
19. Franco-Browder, S., DeRydt, J. & Dorfman, A. (1963) *Proc. Nat. Acad. Sci. USA* **49**, 643-647.
20. Medoff, J. (1967) *Develop. Biol.* **16**, 118-143.
21. Palmoski, M. J. & Goetinck, P. F. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3385-3388.
22. Weintraub, H., Campbell, G. L. & Holtzer, H. (1972) *J. Mol. Biol.* **70**, 337-350.
23. Britten, R. J. & Davidson, E. H. (1969) *Science* **165**, 349-357.
24. Schulte-Holthausen, H., Chacko, S., Davidson, E. A. & Holtzer, H. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 864-870.
25. Marzullo, G. (1972) *Develop. Biol.* **27**, 20-26.