# THE SYNTHESIS OF HYALURONIC ACID BY CELL-FREE EXTRACTS OF EMBRYONIC SKIN*

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An enzyme catalyzing the synthesis of hyaluronic acid from uridine diphospho-N-acetylglucosamine and uridine diphosphoglucuronic acid has been found in Rous sarcoma (Glaser and Brown, 1955) and Group A streptococcus (Markovitz, Cifonelli and Dorfman, 1959). Whereas the former investigators reported loss of radioactivity on electrodialysis and reprecipitation, the latter demonstrated net synthesis of high molecular weight material. The present communication describes the synthesis of hyaluronic acid in cell-free preparations of skin from rat fetuses which have been shown to contain relatively large quantities of hyaluronic acid (Schiller, unpublished).

Late pregnancy (approximately 19 days) rat fetuses were obtained by Caesarian section following exsanguination of the mothers. Subsequent manipulations were performed at $0^{\circ}$ to $4^{\circ}$ C. In most experiments the skin was minced and homogenized in 4 volumes of 0.015 M phosphate - 0.14 M NaCl - 0.010 M $MgCl_2$ - 0.003 M cysteine buffer, pH 7.5, by means of a Kontes Duall homogenizer. A portion of the homogenate was centrifuged at 20,000 x g for 1 hour and, after removal of the supernatant fluid, the pellet was suspended in the same buffer by homogenization. Incubation with uridine nucleotides was carried out at $38^{\circ}$ for 3 hours, except as indicated. After incubation, the reaction was stopped by heating at $100^{\circ}$ for 1 minute. Zero time controls were immersed in boiling water for 1 minute before incubation.

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Two and one-half to three mg of carrier hyaluronic acid were added to each vessel and the contents dialyzed for 24 hours against running tap water. Hyaluronic acid was isolated by the method of Schiller, Slover and Dorfman (1961) and converted to the acid form by passage through a column of Dowex-50 ($H^+$). Prior to precipitation of the mucopolysaccharide with cetylpyridinium chloride the extracts were passed over columns (1x2 cm) of acid-washed Norit-A - 20% stearic acid (Markovitz, Cifonelli and Dorfman, 1959). The samples were analyzed for uronic acid (Dische, 1947) and aliquots were removed for counting in the Tri-Carb liquid scintillation spectrometer.

Tritiated uridine diphospho-N-acetylglucosamine was prepared enzymatically by action of a uridine diphospho-N-acetylglucosamine pyrophosphorylase from Group A streptococcus on uridine triphosphate and N-acetylglucosamine-1-$PO_4$ (Markovitz, Cifonelli and Dorfman, 1959) previously tritiated by the Wilzbach (1957) procedure.[1]

Tables I and II illustrate the results of 2 experiments which demonstrate incorporation of radioactivity into hyaluronic acid. In both experiments the 0 time controls were free of radioactivity and both experiments demonstrated the requirement for uridine diphosphoglucuronic acid. Experiment 2 (Table II) established the need for $Mg^{++}$. Preliminary fractionation of homogenates showed the activity to be sedimentable at 20,000 x g for 1 hour, although there was an indication that addition of the supernatant to the pellet resulted in enhanced activity. Two experiments which have not been tabulated showed that centrifugation of the homogenate at 1000 x g for 10 minutes resulted in a distribution of the activity between the supernatant and sedimentable fractions. No loss of radioactivity occurred on repurification by precipitation with glacial acetic acid.

The hyaluronic acid fraction with specific activity of 220 cpm, isolated from the reaction mixture (Table I) was subjected to paper electrophoresis (Fig. 1). A single spot that migrated at the same rate as a purified sample

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TABLE I

Formation of Hyaluronic Acid-$H^3$ from UDPGNAc-$H^3$
by Cell-Free Extracts of Embryonic Rat Skin

| Preparation | UDPGA | Incubation Time | Specific Activity | Total Activity |
|---|---|---|---|---|
| | µM/ml | hrs. | cpm/mg U.A. | cpm |
| Homogenate | 2.5 | 0 | 0 | 0 |
| " | 2.5 | 3 | 220 | 250 |
| " | 0 | 3 | 18 | 20 |
| 20,000 x g supernatant | 2.5 | 0 | 3 | 3 |
| " | 2.5 | 3 | 0 | 0 |
| 20,000 x g pellet | 2.5 | 0 | 2 | 2 |
| " | 2.5 | 3 | 60 | 68 |

Abbreviations used: UDPGNAc, uridine diphospho-N-acetylglucosamine; UDPGA, uridine diphosphoglucuronic acid; U.A., uronic acid.

Each tube contained $2.41 \times 10^5$ cpm of UDPGNAc-$H^3$ which was diluted with inactive UDPGNAc to give a final concentration of 2.11 µmoles/ml.

The reaction mixture also included 4 µmoles of ATP, 5 µmoles of DPN, 20 µmoles of $MgCl_2$ and 1.5 ml of enzyme in a final volume of 2 ml.

of hyaluronic acid was found after staining with an aqueous alcoholic solution of toluidine blue. All the radioactivity in the hyaluronic acid coincided with the position of the stained spot and eliminated the possibility that the results were due to contamination with the tritiated precursor, uridine diphospho-N-acetylglucosamine, which migrates at a faster rate than hyaluronic acid (Fig. 1).

These results are compatible with the conclusion that mucopolysaccharide synthesis in a mammalian tissue follows a pathway similar to that demonstrated in streptococci. Similar results have been indicated by Altshuler, Kinsman and Bareta (1961).

TABLE II

Effect of UDPGA and $MgCl_2$ on Synthesis of Hyaluronic Acid from UDPGNAc

| Preparation | UDPGA | $MgCl_2$ | Incubation Time | Specific Activity | Total Activity |
|---|---|---|---|---|---|
| | µM/ml | µM/ml | hrs. | cpm/mg U.A. | cpm |
| Homogenate | 1.0 | 12 | 0 | 0 | 0 |
| " | 1.0 | 12 | 3 | 108 | 147 |
| " | 1.0 | 0 | 3 | 31 | 42 |
| " | 0 | 12 | 3 | 31 | 42 |
| 20,000 x g supernatant | 1.0 | 12 | 0 | 0 | 0 |
| " | 1.0 | 12 | 3 | 0 | 0 |
| 20,000 x g pellet | 1.0 | 12 | 0 | 0 | 0 |
| " | 1.0 | 12 | 3 | 55 | 75 |
| Combined supernatant & pellet | 1.0 | 12 | 0 | 0 | 0 |
| " | 1.0 | 12 | 3 | 73 | 99 |

Abbreviations used: UDPGNAc, uridine diphospho-N-acetylglucosamine; UDPGA, uridine diphosphoglucuronic acid; U.A., uronic acid.

Each tube contained $2.41 \times 10^5$ cpm of UDPGNAc-$H^3$ which was diluted with inactive UDPGNAc to give a final concentration of 1.0 µmoles/ml. The reaction mixture also included 4.1 µmoles of ATP, 6 µmoles of DPN and 2.0 ml of enzyme in a final volume of 2.4 ml.

The presence of hyaluronic acid synthesizing activity in the sedimentable fraction is of interest in view of the demonstration by Markovitz and Dorfman (1960) that the streptococcus enzyme is on the protoplast membrane.

## References

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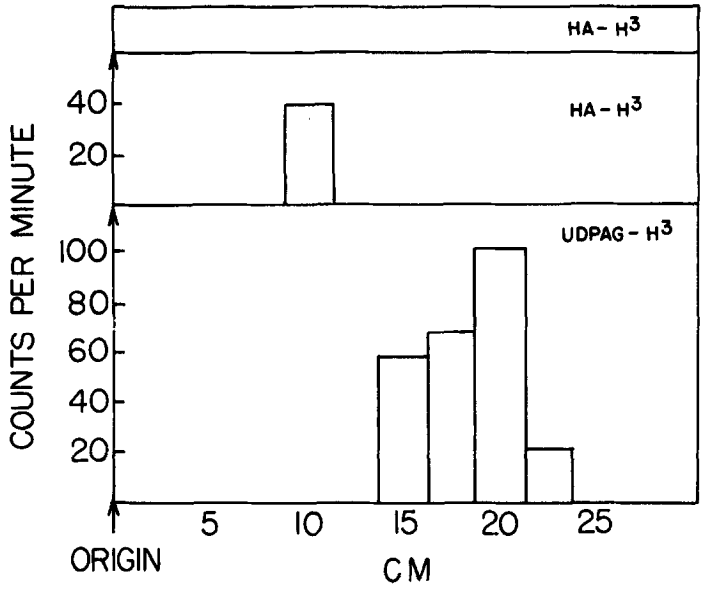


Figure 1. Electrophoretic patterns of tritiated uridine diphospho-N-acetyl-glucosamine (UDPAG-$H^3$) and radioactive hyaluronic acid (HA-$H^3$) isolated from the reaction mixture. Electrophoresis was carried out on 1 inch Whatman No. 3 filter paper strips in 0.1 M ammonium formate buffer, pH 3.06, for 15.5 hours at 1.5 volts per cm. The uppermost strip is a reproduction of the electrophoretic pattern of the isolated HA-$H^3$ stained with a toluidine blue solution. The middle strip illustrates the site of radioactivity following electrophoresis of 184 µg of HA-$H^3$ as uronic acid. The bottom strip represents the radioactivity of 0.16 µg of UDPAG-$H^3$ following electrophoresis.

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