Organ-culture studies of achondroplastic rabbit cartilage: evidence for a metabolic defect in glucose utilization

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SUMMARY

Organ-culture studies were made using cartilage from achondroplastic (dwarf) rabbits (ac/ac) and their phenotypically normal litter-mates. A significantly higher incorporation of $^{14}$C from glucose and galactose was measured in the dwarf; incorporation of $^{35}$S from sulfate and $^3$H from thymidine was equal in the two types of explant. Also, $^{14}$CO$_2$ and [$^{14}$C]lactate production from glucose by the dwarf cartilage was increased. No difference in the ratio of $^{14}$CO$_2$ evolution from [1-$^{14}$C]- and [6-$^{14}$C]glucose was found between the two cartilage types. Explants of dwarf cartilage utilized more glucose from the medium than did the controls.

Radioautographs of tissue sections from the explants showed an increased number of grains from [$^{14}$C]glucose overlying the dwarf cartilage, and this difference was particularly great over the central portions of cartilage. The proportion of $^{14}$C grains from glucose was greater over the dwarf nuclei, less over the dwarf matrix and equal over the cytoplasm of the two tissues. Grain counts of sulfate and of thymidine did not differ in the two types of cartilage.

INTRODUCTION

Although achondroplasia, or dyschondroplasia, is the most common form of genetic dwarfism in man (Morch, 1941; Neel, Schull, & Takeshima, 1959; Stevenson, 1957; Shepard & Graham, 1967) and in animals (Grüneberg, 1963; Landauer, 1969a), no direct biochemical studies have been performed on the cartilage from this condition. In the absence of such biochemical and physiologic data the abnormality can be defined only in morphologic terms. In man, achondroplasia is nearly always inherited as an autosomal dominant and is characterized chiefly by disproportionate shortening of the endochondral bones with some increase in growth of the membranous skull. The histologic alterations are confined to cartilage; bone formation and fracture healing appear to be normal. The cartilaginous growth plates are decreased in thickness and the columns, if present, are irregular (Harris, 1933; Fairbanks, 1951; Weinmann

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Mucoid degeneration and cystic areas within cartilage have been described by Harris (1933) and Rubin (1964).

Experimental morphologists have concerned themselves with the forms of achondroplasia (Grüneberg, 1963; Landauer, 1969a, b). Transplantation and organ culture in chick and mouse models have shown that intrinsic factors in chondrocytes as well as humoral factors contribute to the production of the achondroplastic phenotype (Hamburger, 1941; Rudnick, 1945; Landauer, 1969b; Wolff & Kieny, 1957; Konyukhov & Paschin, 1967; Elmer, 1968). Cell dissociation studies indicate that cell adhesiveness may play a role in production of the talpid³ form of chick dwarfism (Ede & Agerbak, 1968). Endocrine dysfunctions are currently unpopular explanations for the etiology. Pituitary growth hormone failed to correct the disproportionate growth in the dog (Evans, Simpson, Meyer & Reichert, 1933) and man (Gershberg, Mari, Hulse & St Paul, 1964). In the achondroplastic mouse (cn) the growth-hormone content of the pituitary was the same as for the controls (Konyukhov & Paschin, 1967).

This report is based on organ-culture studies in newborn rabbit achondroplastics (ac/ac), chosen because achondroplasia in rabbits closely resembles achondroplasia in man (Brown & Pearce, 1945; Pearce & Brown, 1945a; Shepard, Fry & Moffett, 1969) except that the condition is inherited as an autosomal recessive (Pearce & Brown, 1945b). Their phenotypically normal littermates (Ac/ac, Ac/Ac) were the controls. We found that incorporation of ¹⁴C from glucose by the dwarf explants was increased significantly whereas incorporation of isotopes from sulfate and thymidine was not. A deviation in glucose metabolism in the mutant may be significant in view of the special reliance of cartilage on glucose for mucopolysaccharide synthesis and for energy, particularly since it is localized primarily in cartilage, the main organ involved in this pathologic condition.

**MATERIALS AND METHODS**

*Experimental animals.* This strain of achondroplastic rabbits has been described in detail (Brown & Pearce, 1945; Pearce & Brown, 1945a, b). Histological analyses have been made also on the growth of bone and cartilage in this strain (Crary & Sawin, 1963; Shepard et al. 1969). We began our colony from stock animals obtained from the Jackson Memorial Laboratory by courtesy of Dr Richard Fox. On day 32 of the timed pregnancies the does were brought into parturition by the administration of 10 i.u. of pitocin intramuscularly. Usually within 10 or 15 min the litter was delivered. These studies are based on tissue from 121 rabbit fetuses of which 31 (25-6 %) were achondroplastic. About 5 % of the fetuses had a hydranencephalic condition, usually associated with enlargement of the cranium; in some cases this caused dystocia and delay in delivery. Hydranencephaly was diagnosed by transillumination of the skull [morphological studies of the brain are still in progress (R. J. Lemire, personal communication)].
Only two fetuses had achondroplasia associated with hydranencephaly. Differentiation of the homozygous mutant (ac/ac) was made by measuring the length from the olecranon process to the tip of the upper extremity; normals were $28 \pm 0.4 \text{ mm (s.e.)}$; dwarfs were $19 \pm 0.3 \text{ mm}$. The newborn body-weight for the phenotypically normal averaged $39.9 \pm 5 \text{ g}$ and the dwarfs $28.4 \pm 2 \text{ g}$. The dwarfs were unable to reach the mother's breast; when aided, they experienced partial nasal obstruction during suckling, which resulted in inadequate nutrition and death during the first week of life.

Dwarf and control litter-mates of similar weight, whenever possible, were decapitated during the first day of life and the small bones of the extremities (usually metacarpals, metatarsals, and first phalanges) were cleaned of tendons, muscles, and bone cuffs before explantation. Because the achondroplastic explants were shorter and sometimes narrower, control experiments with slices of cartilage were performed. Pieces of kidney and heart from dwarf and control newborns were cut into approximately $1.0 \text{ mm}$ cubes with a razor and explanted. To evaluate the effect of immaturity, we studied and compared cartilage from 22-day with 32-day New Zealand rabbit fetuses.

### Table 1. Media used

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waymouth's*</td>
<td>3.7</td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>Waymouth's* without glucose</td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid 1.0 mg/ml</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Penicillin, kanomycin and mycostatin in NaCl (9.0 gm/l)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Isotope diluted in Waymouth's used above</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Rabbit serum</td>
<td></td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Obtained from Grand Island Biological Co., New York.

**Organ-culture methods.** The cartilage explants were placed on stainless-steel grids in plastic Petri plates 6 cm in diameter (Falcon Plastics, Los Angeles). For measurement of CO₂ production, plastic Petri plates with a well diameter of 3.0 cm and an outer trap diameter of 0.6 cm were used (Falcon Plastics, microdiffusion dish). Filter papers impregnated with 10% KOH in the surrounding well trapped CO₂. In these latter experiments 15–45 mg of cartilage tissue was added to the grid in each Petri plate. The three types of medium used are detailed in Table 1. Medium A was used for most isotope incorporation experiments but medium B in 2 ml volumes was used when CO₂ was to be trapped and lactate measured. In experiments with [¹⁴C]glucose and [¹⁴C]galactose in medium B ¹⁴C uptake was increased approximately tenfold over similar experiments in medium A. Before using medium B the explants were washed for 4–5 min in glucose-free Waymouth's medium. Explants of kidney and heart were studied in medium B using the same technique as for cartilage. For experiments lasting over 2 days in length medium C was used. Serum was obtained...
from blood drawn from sexually mature rabbits; no anticoagulant was used. Incubation was in 95 % air and 5 % CO₂ for 24 h except when the chambers were gassed before incubation with nitrogen. Gassing with nitrogen reduced the oxygen content to less than 1 %. A 2·5 l. desiccator jar moisturized by lining with water-impregnated paper was used as a gas chamber. The volume of the jar exceeded that of the tissue by a factor of at least 10000. Incubation temperature was 38 °C. In comparing ¹⁴CO₂ evolution from glucose labeled on carbon 1 with that labeled on the 6 position, incubation was done in medium B. The same explants were re-used in paired studies of the two types of labeled glucose. A 1- or 2-day recovery period in medium C separated the two tests.

Isotope measurement. Specific activities and commercial sources of the isotopes are shown in Table 2. The uniformly labeled [¹⁴C]glucose was analysed on thin-layer chromatography; 97 % of the radioactivity was found associated with the spot for carrier glucose. The glucose labeled on position 1 and 6 was obtained from Nuclear Chicago Company, Chicago, and had specific activities of 54 mCi/mm. At the end of incubation the explants were thoroughly washed in Waymouth's medium and were wet-weighed by the method of Biggers (1960a). After 5 min exposure to a boiling water bath each explant was separately digested for 24 h in 0·1 ml of pronase (2 mg/ml in 0·2 M Tris buffer pH 8·0) (Calbiochem, Los Angeles) at 38 °C. At the end of the digestion period in pronase hydroxide of Hyamine was added and the sample transferred to toluene liquid scintillation solution (Shepard, 1963) and counted using a Packard Tricarb, 3214. It was necessary to count the [¹⁴C]lactate and ¹⁴CO₂ in a dioxane solution of p-dioxane containing 120 g naphthalene, 4·0 g 2,5 diphenyloxazole and 0·05 g 1,4-bis-2-(5-phenyloxazolyl)-benzene/l. Counting efficiencies for ¹⁴C, ³⁵S, and ³H were 78, 30, and 41 % respectively. Corrections for sample quenching were made after addition of internal standard.

The method of Barker & Summerson (1941) was used for separating [¹⁴C]-lactate in the medium; only two radioactive spots were found after thin-layer chromatography and they corresponded exactly to carrier lactate and glucose. Glucose was measured by the glucose oxidase method (Marks, 1959).

Tissue radioautography. The histological and radioautographic methods have been described previously (Shepard, 1967; Shepard et al. 1969). The sections were stained by toluidine blue, periodic acid–Schiff, and Richardson's method. For quantitating labeled nuclei after [³H]thymidine exposure the explants were serially sectioned, and the labeled cells counted. The tissue sections were projected on paper of standardized thickness, and cut-outs of the outline of the cartilage were made. These tracings were then weighed, from which a calculation of the number of labeled nuclei per cubic millimeter of cartilage was made. The grain counts for glucose and sulfate had to be made in areas of resting cartilage because it proved impossible to define exactly the borders of other areas of the cartilaginous growth plate. The grain counts were made on tissue exposed for 24 h to the isotope. The counts of ¹⁴C and ³⁵S grains were performed by using
Table 2. Isotope incorporation into control and experimental cartilage

<table>
<thead>
<tr>
<th>Substance</th>
<th>Label</th>
<th>Medium</th>
<th>Sp. activity in media (mC/mM)</th>
<th>Control (mean ± SE)</th>
<th>Dwarf (mean ± SE)</th>
<th>D.F.</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>U-14C*</td>
<td>A</td>
<td>8.09 ± 0.60</td>
<td>1.03 ± 0.06</td>
<td>0.31 ± 0.07</td>
<td>25</td>
<td>3.13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>U-14C*</td>
<td>B</td>
<td>55.8</td>
<td>1.32 ± 0.10</td>
<td>0.75 ± 0.07</td>
<td>27</td>
<td>2.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>U-14C*</td>
<td>B</td>
<td>30.7</td>
<td>0.64 ± 0.09</td>
<td>0.31 ± 0.07</td>
<td>8</td>
<td>4.99</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Galactose</td>
<td>35S carrier free*</td>
<td>A</td>
<td>35</td>
<td>16.18 ± 2.17</td>
<td>0.70 ± 0.07</td>
<td>29</td>
<td>5.44</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Thymidine</td>
<td>3H (methyl)</td>
<td>A</td>
<td>6722</td>
<td>0.77 ± 0.08</td>
<td>32</td>
<td>35</td>
<td>9.81</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Obtained from Nuclear-Chicago Co., Chicago, Ill.
† Obtained from New England Nuclear Co., Boston, Mass.
§ N.S. not statistically significant, P > 0.05.

Table 3. Isotope incorporation into 22- and 33-day fetal rabbit cartilage

<table>
<thead>
<tr>
<th>Substance</th>
<th>Label</th>
<th>Medium</th>
<th>Sp. activity in media (mC/mM)</th>
<th>22 day (mean ± SE)</th>
<th>33 day (mean ± SE)</th>
<th>D.F.</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>U-14C</td>
<td>A</td>
<td>55.8</td>
<td>2.10 ± 0.43</td>
<td>4.60 ± 0.48</td>
<td>20</td>
<td>2.64</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Sulfate</td>
<td>35S carrier free*</td>
<td>A</td>
<td>15.4</td>
<td>17.06 ± 0.90</td>
<td>4.60 ± 0.48</td>
<td>18</td>
<td>5.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thymidine</td>
<td>3H (methyl)</td>
<td>A</td>
<td>6722</td>
<td>3.22 ± 0.10</td>
<td>0.87 ± 0.10</td>
<td>18</td>
<td>9.29</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Obtained from Nuclear-Chicago Co., Chicago, Ill.
† Obtained from New England Nuclear Co., Boston, Mass.
§ N.S. not statistically significant, P > 0.05.
phase-contrast microscopy under oil immersion on sections of 1 \( \mu \) thickness stained with toluidine.

The distribution of \( ^{14} \text{C} \) grains in thick-sectioned tissue radioautographs appeared to differ in the control and experimental explants. To quantitate this difference radioautographs of 1-0 \( \mu \) thickness prepared from several growth plates were divided into four columns starting at the periphery; these columns were further subdivided during grain counting by use of an oblong-shaped aperture in one ocular of a binocular microscope (Fig. 5). The means and standard errors of grid areas from five columns were used for determining the statistical significance of differences.

Statistical analyses were performed by standard methods as described by Edwards (1955). For determinations of probabilities the table of distribution of \( t \) of Fisher & Yates (1963) was used.

RESULTS

Incorporation. During organ culture a significantly increased amount of \( ^{14} \text{C} \) from glucose and from galactose was retained by the dwarf cartilage; no such increase for \( ^{35} \text{S} \) from sulfate or for \( ^{3} \text{H} \) from thymidine was demonstrated (Table 2). Slices of the dwarf and control cartilages were exposed to \([^{14}\text{C}]\)glucose and a significant increase of uptake by dwarf cartilage was present. The chemical analysis made of the media (medium C) after 2 days of culture showed utilization of glucose by dwarf cartilage to be 0-122 mg/mg of weight, a substantial amount over the 0-069 of the control, thus confirming our earlier observation.

Organ explants of heart and kidney from the dwarf and control fetuses were subjected to the same process. The dwarf hearts incorporated 3769 ± 292 dpm/mg; the controls 4971 ± 996 dpm/mg of wet weight (D.F. 8; \( t \), 1-2; \( P > 0-05 \)). Dwarf kidney incorporated 279 ± 32; the control 289 ± 34 dpm/mg. The relatively equal uptake of \( ^{14} \text{C} \) from glucose by both these organs in the dwarf and control groups as opposed to the larger uptake of dwarf cartilage over control seemed to suggest a possible specificity of dwarf cartilage for excessive incorporation of \( ^{14} \text{C} \) from glucose.

The results of isotope incorporation by 22-day fetal rabbit cartilage are compared with 33-day fetal cartilage in Table 3. Non-carrier New Zealand rabbit fetuses were used in this single instance because the ac carrier mothers were felt to be too precious for sacrifice or operation. The more immature tissue incorporated all three isotopes to a greater extent, suggesting that the achondroplastic's increase in glucose alone is not simply a function of immaturity of the dwarf cartilage (Crary & Sawin, 1963).

End-products of \([^{14}\text{C}]\)glucose. An overall increase of \( ^{14} \text{CO}_2 \) and \([^{14}\text{C}]\)lactate production from the dwarf explants was found (Table 4).

\([^{14}\text{C}]\)1-Glucose versus \([^{14}\text{C}]\)6-Glucose. The ratios of \( ^{14} \text{CO}_2 \) from glucose labeled on the 1 and on the 6 position were compared at 1, 3, and 24 h. Evolution of \( ^{14} \text{CO}_2 \) from the glucose labeled on the one position was more rapid than
Culture studies of achondroplastic cartilage

353

that on the six position in both the dwarf and control cartilage organ culture. The dwarf cartilage produced $^{14}\text{CO}_2$ at a more rapid rate at all intervals. The ratio of $^{14}\text{CO}_2$ from glucose labeled on the 1 position and on the 6 position at 3 h was $9.6 \pm 1.6$ in the control and $7.8 \pm 5.6$ in the dwarf. The differences were not significant (D.F. 8; $t = 1.07$; $P > 0.05$). Generally, the ratios increased with prolonged organ culture when the experiment was repeated using the same tissue. This may suggest that the Krebs' cycle became inactivated in comparison with the pentose phosphate pathway. For instance, within the first 4 days of explantation a pair of Petri plates with normal cartilage gave ratios of 13.6 and 15.3 h after the isotopes were added; the same pair gave ratios of 28 and 58 at the end of 2 weeks.

Table 4. Distribution of $^{14}\text{C}$ from uniformly labeled glucose* in cartilage explants

<table>
<thead>
<tr>
<th>End products</th>
<th>Control</th>
<th>Dwarf</th>
<th>D.F.</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporated into tissue</td>
<td>$5.31 \pm 0.59$</td>
<td>$9.74 \pm 0.67$</td>
<td>8</td>
<td>3.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>$13.7 \pm 0.43$</td>
<td>$27.2 \pm 1.50$</td>
<td>8</td>
<td>7.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactate</td>
<td>$31.5 \pm 3.1$</td>
<td>$49.1 \pm 3.8$</td>
<td>8</td>
<td>3.55</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* $^{14}\text{C}$ glucose $9.78 \times 10^6$ dpm added to 2.0 ml of the glucose-free medium; 20–40 mg of cartilage per Petri plate.

Table 5. Grain counts – number and distribution

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dwarf</th>
<th>D.F.</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}\text{C}$ from glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total*</td>
<td>$5 \pm 1$</td>
<td>$19 \pm 2$</td>
<td>38</td>
<td>7.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Matrix†</td>
<td>$210 \pm 4$</td>
<td>$181 \pm 5$</td>
<td>8</td>
<td>4.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cytoplasm†</td>
<td>$68 \pm 4$</td>
<td>$76 \pm 3$</td>
<td>8</td>
<td>1.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nucleus†</td>
<td>$22 \pm 3$</td>
<td>$43 \pm 3$</td>
<td>8</td>
<td>5.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$^{35}\text{S}$ from sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total*</td>
<td>$57 \pm 4$</td>
<td>$52 \pm 4$</td>
<td>38</td>
<td>0.9</td>
<td>&gt;0.05†</td>
</tr>
<tr>
<td>Matrix†</td>
<td>$147 \pm 7$</td>
<td>$158 \pm 2$</td>
<td>8</td>
<td>0.63</td>
<td>&gt;0.05†</td>
</tr>
<tr>
<td>Cytoplasm†</td>
<td>$120 \pm 6$</td>
<td>$106 \pm 12$</td>
<td>8</td>
<td>0.98</td>
<td>&gt;0.05†</td>
</tr>
<tr>
<td>Nucleus†</td>
<td>$33 \pm 2$</td>
<td>$36 \pm 5$</td>
<td>8</td>
<td>0.19</td>
<td>&gt;0.05†</td>
</tr>
</tbody>
</table>

* Counted via square aperture in ocular of microscope.
† Average of five separate analyses each consisting of 300 grains.
‡ Not significant.

Radioautographic localization of isotopes – $^{14}\text{C}$ from glucose and galactose. Radioautographs of dwarf resting cartilage showed a significant increase in the number of grains from uniformly labeled glucose (Table 5) and galactose. In both types of cartilage the edges of the resting cartilage and of the cartilage growth plates both showed a higher concentration than the centers. This gradual decrease in grains toward the center was more marked in the control than in
the dwarf. Although the $^{14}\text{C}$ from galactose was not counted it appeared to be localized in a pattern similar to that of the glucose. The distribution and cellular localization are illustrated in Figs. 1–6.

In a previous paper Shepard et al. (1969) have described the occurrence of cells with pyknotic nuclei and absence of surrounding moats. These presumably

Fig. 3. Radioautograph of cartilaginous growth plate of control explant exposed 24 h to uniformly labeled [$^{14}\text{C}$]glucose in medium B. Many grains are located at the edge of the growth plate at the upper right and fewer over the central part. Calcifying cartilage toward the bottom. Fixed in neutral formalin, embedded in paraffin wax and sectioned at 8 $\mu$ thickness. After 24 h exposure to nuclear emulsion the slide was developed and stained with toluidine blue.

Fig. 4. Radioautograph of cartilaginous growth plate of dwarf $ac/ac$ explant exposed 24 h to uniformly labeled [$^{14}\text{C}$]glucose in medium B. The orientation is similar to Fig. 3, with the edge of the plate to the right. Note the persistence of grain density toward the center of the plate. Conditions identical to those of Fig. 3.

Fig. 1. Radioautograph of resting cartilage from control cartilage explant exposed to uniformly labeled [$^{14}\text{C}$]glucose for 24 h in medium B. Fixed in 2% osmium tetroxide, embedded in epon epoxy resin and sectioned at 1 $\mu$ thickness. Developed after 4 days’ exposure to nuclear emulsion and stained with toluidine blue.

Fig. 2. Radioautograph of resting cartilage from $ac/ac$ dwarf cartilage explant to show generalized increase in number of grains. Conditions identical to Fig. 1.
dead cells increased toward the center of the dwarf cartilage and were devoid of overlying grains in radioautographs (Fig. 5).

Fig. 6 shows comparisons of grain counts of columns paralleling the edge of

Fig. 5. Radioautograph of dwarf cartilage exposed to [14C]glucose for 24 h in medium B to show lack of grains over two 'dead' cells (arrows). Preparation as in Fig. 1. Phase contrast.
the growth plates, and it is clear that the larger number over the dwarf cartilage is significant.

Table 5 details the decreased grain counts overlying central parts of the resting cartilage in the control. The distribution of grains lying over matrix, cytoplasm, and nucleus are given in Table 5. Over the matrix the proportion of grains was increased in the control; however, grains over the nuclei in the dwarf were increased significantly. No difference in the proportion of cytoplasmic grains was detected.

![Graph showing grain counting results](image)

**Fig. 6.** Method and results of grain counting after exposing explants for 24 h to uniformly labeled [14C]glucose. Histological details identical to those of Figs. 1 and 2. Four columns, each 55 μ in width, were subdivided into fields approximately 31 μ in height. The average number of grains per field for each column is represented on the left. Eight columns through the growth plates of a normal and a dwarf were counted. The mean differences were analysed by Student's t test.

35S from sulfate. The general distribution of grains was even over all areas of both the experimental and control slides, except at the edges where the concentration was slightly reduced (see Fig. 7). After the organ explant was exposed for 24 h and then washed, the isotope was concentrated mainly over the matrix, with a smaller amount over the cells. No significant differences in grain counts or cell-matrix localization were detected (Table 5) between the two groups. The dead-looking cells in the dwarf cartilage did not concentrate 35S.
$^3$H from thymidine. The $^3$H was localized over or close to the nuclei. In both types of cartilage a higher concentration of labeled cells was found over the perichondrium and over the proliferative zone of the columns in the growth plates (Fig. 8). The number of labeled cells in serially sectioned control and
dwarf cartilage was $211 \times 10^3$/mm$^3$ and $290 \times 10^3$/mm$^3$ respectively. The average number of grains per labeled nucleus as observed in 1 $\mu$m sections was not significantly different in the two types of cartilage (control, $8.0 \pm 0.9$; dwarf, $8.1 \pm 1.0$).

**DISCUSSION**

The utilization of $[^{14}\text{C}]$glucose by cartilage explants from achondroplastic (ac) rabbits was greater than that by phenotypically normal litter-mates used as controls. Higher glucose disappearance from media during longer periods of organ culture in the dwarf appeared to support this finding. Evidence that this increased metabolism by the cartilage tissue was not solely due to the reported immaturity of the dwarf skeleton (Crary & Sawin, 1963) is provided by comparison of 22-day with 33-day fetal rabbit cartilage. The 22-day cartilage explants...
incorporated all three isotopes from glucose, sulfate, and thymidine to a greater amount, whereas the dwarf cartilage showed greater utilization of only the isotope from glucose.

Data to explain the increased incorporation of $^{14}$C from galactose are insufficient. In cultures of chick embryonic cartilage (Kieny, 1958; Biggers, 1965) and fetal rat cartilage (Heyner, 1960) galactose failed to support growth in the absence of glucose.

That excess glucose utilization is localized in cartilage, the affected tissue in achondroplasia, and not in kidney or heart suggests a possible relationship to the pathogenesis of achondroplasia. It is altogether possible that the increased utilization of glucose may be only a compensatory factor for some deficiency, such as faulty fatty acid or amino acid metabolism. Carbon dioxide and lactate production were not demonstrated to be qualitatively different in the dwarf and the control, nor was evidence found for a difference in the proportion of C-1 to C-6 carbon utilization.

Glucose metabolism by cartilage is uniquely different from that of other tissues. Biggers, Rinaldini & Webb (1957) and Biggers (1960b) have shown that cultured cartilage is especially dependent on glucose as a nutrient, a point confirmed by Kieny (1958) and extended to fetal rat cartilage by Heyner & Biggers (1958). Glucose acts as a source of both acid mucopolysaccharides and collagen via conversion through proline (Lucy, Webb & Biggers, 1961; Biggers, 1965). In addition, cartilage, by nature of its relatively anerobic environment, relies more on glycolysis than do other tissues (Whitehead & Weidmann, 1959; Pawelek, 1969). Landauer has recently summarized the extensive evidence that micro- melody in the chick is closely connected to a defect in carbohydrate metabolism (Landauer, 1969b; Zwilling & DeBell, 1950). Space does not allow adequate discussion of Landauer's analysis by use of insulin, 6-aminonicotinamide, 2-deoxy-D-glucose as micromelia-producing-teratogens and the protective action of riboflavin and nicotinamide. These special requirements for glucose catabolism might cause a generalized biochemical defect of hereditary origin to manifest itself primarily in cartilage tissue and produce the achondroplastic syndrome.

The low incorporation of $^{14}$C from glucose into the explants during incubation in nitrogen supports the findings of Whitehead & Weidmann (1959), who showed that limb cartilage of kittens contained active oxidative enzyme systems. These authors point out that many of the previous studies indicating lack of oxidative systems in cartilage were performed on noncalcifying articular cartilage. Our findings that the dwarf cartilage continues to metabolize more $^{14}$C than the normal under reduced oxygen may indicate that the observed increase is due to a problem in anerobic glycolysis.

The end-products of glucose metabolism from our organ-explant experiments were roughly similar to those of Guri, Plume & Bernstein (1967), who incubated rat epiphyseal cartilage for 3 h and found lactate, CO$_2$, and tissue incorporation to represent 70 %, 8 %, and 23 % of the recovered end-products. Findings in the
rabbit not reported in detail in this paper indicated that 86 %, 4 % and 10 % of the radioactivity were distributed in lactate, CO₂, and tissue fractions respectively after 24 h culture in the glucose-containing medium. The end-products found following organ culture in medium without added cold glucose were 60, 29, and 11 % in the lactate, CO₂, and tissue fractions respectively.

The ratio of ¹⁴CO₂ from metabolism of 1- and 6- carbon-labeled glucose by both types of cartilage was close to that found by Vilee & Loring (1961) in human fetal adrenal. Therefore, if this ratio technique is a valid measure of glucose metabolic pathways, a larger amount of pentose phosphate shunt activity relative to that of the Krebs' cycle pathway is present in fetal cartilage than in most organs.

The radioautographic demonstration that less ¹⁴C from glucose is localized in the centers of normal cartilage explants than in the periphery may mean that as these cells are isolated from the nutrients or gases they compensate by a change in nutritional pathways. Decrease in central localization of ¹⁴C was less evident in the dwarf explants. Previous findings have indicated that the dwarf rabbit cartilage has dead chondrocytes which increase in number toward the center and that the isogenic capsules of the growth plates contain a reduced number of cells toward the center (Shepard et al. 1969). It is, of course, possible that the lack of central ¹⁴C grains is but some artifact introduced by the explanting methods. Identification of the chemical nature of this ¹⁴C should contribute to the enlightenment on this subject.

The cell localization of ¹⁴C from glucose in the thin sections was significantly higher overlying the nuclei of the dwarf than of the control cartilage (Table 6). Glucose has been shown to be a precursor for both ribose and deoxy-ribose nucleic acids in chick cartilage (Lucy et al. 1961). The number of grains from [³H]thymidine was not increased over the dwarf nuclei, suggesting that a ribonucleic acid or histone component is increased rather than deoxynucleic acid. Chalkley counts in our previous studies showed that the dwarf nuclear area was greater than that of the control (Shepard et al. 1969). The enlarged nuclei and increased ¹⁴C incorporation in dwarf cartilage could be explained by an increase in the manufacture of messenger ribonucleic acid. Such an increase in ribonucleic acid could be secondary to cell mechanisms directed toward unsuccessful correction of an enzyme deficiency.

The uptake of ³⁵S from sulfate and its tissue localization were similar in the two types of cartilage. This suggests that acid mucopolysaccharide production is normal in the achondroplastic. Mathews (1967) has reported that chondroitin sulfate analyses of the creeper chick mutant did not differ from controls.

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REFERENCES


Culture studies of achondroplastic cartilage


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