Growth of the cartilages of the mid-line cranial base of the Wistar rat: an autoradiographic study using $^{35}$S-sulphate

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SUMMARY Matrix synthesis in the mid-line cranial base of the rat was studied using a single injection of $^{35}$S-sulphate in animals aged 4–80 days. High uptake of $^{35}$S-sulphate in the morphologically distinct zone of early hypertrophy led to it being renamed the Matrixogenic Zone. Relocation of $^{35}$S-sulphate label into the primary spongiosa was used to estimate the growth rate of each site of endochondral ossification. This showed a marked caudorostral and temporal gradient. In addition, there was a caudorostral and temporal gradient in hypertrophic cell size, but only a temporal gradient in the rate of cell loss.

Introduction
The use of $^{35}$S-labelled sulphate as a radioactive tracer in cartilage was first used to test the hypothesis that it would be incorporated into the chondroitin sulphate of cartilage matrix (Dziewiatkowski et al., 1949). The major components of the matrix are assembled in the Golgi apparatus of the chondrocytes and are quickly passed out of the cell into the cartilage matrix. The sulphate is incorporated into glycosaminoglycan and proteoglycan components of the cartilage matrix (Serafini-Fracassini and Smith, 1974). The precise location of radioactive sulphate incorporation can be detected in histological sections using autoradiography (Rogers, 1979). The relatively high energy of the $\beta$-particles from $^{35}$S results in silver grains distributed throughout a considerable thickness of the emulsion. The poor resolution makes it impossible to carry out reliable grain counts visually. The development of photomultipliers has led to the introduction of photometric grain counting (Rogers, 1979).

In the present study a single intraperitoneal injection of $^{35}$S-labelled sulphate was used:

1. to identify areas of sulphate uptake in the five endochondral growth sites in the cranial base (Roberts and Blackwood, 1983, 1984);
2. to quantify the sulphate uptake in the five cell zones of the endochondral growth sites (Fig. 1);
3. to follow the fate of sulphate incorporated into the cartilaginous matrix as it is relocated during growth;
4. to estimate the growth rate of each of the five sites of endochondral growth.

Materials and methods
One-day-old litters of the Wistar strain of Rattus norvegicus were culled to six males and reared in a controlled environment (Roberts and Blackwood, 1983). Two litters each at 4, 8, 16, 32, 48, and 80 days were injected intraperitoneally with 4 $\mu$Ci/g body weight of $^{35}$S-sulphate in a divided dose at 09.00 hours. Two animals, one from each litter, were killed at 15 mins, 1, 2, and 24 hours, 3 and 5 days after injection. The cranial base was dissected free and fixed in 10 per cent formol saline. The tissues were processed and embedded in paraffin wax. Serial sections were cut at 5–8 $\mu$m in the sagittal plane and stained with haematoxylin and eosin. For the autoradiography two slides were coated with fluid emulsion (Ilford K5). The slides were exposed for 4 weeks at 4°C, developed in Ilford Phenisol for 8 minutes at 18°C, rinsed in distilled water, fixed in 30 per cent sodium thiosulphate for 10 minutes, washed, and dried slowly at room temperature. The slides were lightly stained with haematoxylin and mounted in Xam or Canada balsam. Spare slides were used as control to test for length of exposure and negative and positive chemo-
Delineation of the Matrixogenic zone

A preliminary assessment of labelling was made by grading labelling as light, medium, or heavy in each cell zone. Following this specimens were examined using the Leitz incident light photometer. This machine measures the amount of light reflected by silver grains in the specimen (Piller, 1977) the results are expressed as photometric units which provide a numerical value of relative intensity of light. To assess the reproducibility of the photometer a single autoradiograph was measured on 10 separate occasions over a period of several weeks.

Photometric measurements of each cell zone in each of the endochondral growth sites of the 48-day specimens killed 2 hours after injection of $^{35}$S-sulphate were made. Each measurement was made twice on each of the five cell zones from the five cartilaginous growth sites. Two sections from each of the two animals were used.

Growth rate

This was estimated by measuring the distance from the metaphyseal edge of the matrixogenic zone to the edge of the labelled matrix relocated in the metaphysis. The daily growth rate was calculated by dividing this length by the time interval over which relocation occurred, usually 5 days.

Cell size

The contribution made by any one cartilage cell to the linear growth of an endochondral growth site is expressed by its height in the direction of growth (Sissons and Kember, 1977). In the present study this dimension was measured by orientating the specimen with a rotating stage so that the stage micrometer was aligned with the axis of growth. The distance measured was the diameter of the cell including the proximal metaphyseal bar of matrix in the axis of growth. Cells were selected using random number tables.

Rate of cell loss

This was calculated for each endochondral growth site for all of the ages studied by dividing the growth rate estimated from the autoradiographs (data from Table 2) by the size of the last complete cell in the hypertrophic zone (data from Table 3). In this way the number of cells lost per day from the receding edge of the growth plate was calculated.

Results

Incorporation of $^{35}$S-sulphate

Examination of the autoradiographs by light microscopy reveals that the general pattern of autoradiographic labelling is similar for each site of growth at each of the six ages studied. After 15 minutes there are a small number of silver grains localized over the chondrocytes with only a very small number over the surrounding matrix.
Figure 2  Basioccipital basisphenoid synchondrosis 2 hours after injection $^{35}$S-sulphate. The heavy labelling over the matrixogenic zone is clearly seen (arrowed). (Magnification x 94.)

Table 1  Autoradiographic grain count measured by incident light photometry. 48-day Wistar rats 2 hours after injection of $^{35}$S-sulphate.

<table>
<thead>
<tr>
<th></th>
<th>CZ</th>
<th>PZ</th>
<th>MZ</th>
<th>HZ</th>
<th>EZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO</td>
<td>47</td>
<td>114</td>
<td>375</td>
<td>79</td>
<td>74</td>
</tr>
<tr>
<td>BS (caud)</td>
<td>78</td>
<td>126</td>
<td>470</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>BS (rost)</td>
<td>68</td>
<td>137</td>
<td>305</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>PS (caud)</td>
<td>58</td>
<td>93</td>
<td>227</td>
<td>61</td>
<td>16</td>
</tr>
<tr>
<td>PS (rost)</td>
<td>37</td>
<td>35</td>
<td>172</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The data has been corrected for the background level which was 80 units.

**Key**
- Cell zones: CZ = central zone, PZ = proliferative zone, MZ = matrixogenic zone, HZ = hypertrophic zone, EZ = erosive zone.
- Growth sites: BO = basioccipital, BS (caud) = basisphenoid caudal, BS (rost) = basisphenoid rostral, PS (caud) = presphenoid caudal, PS (rost) = presphenoid rostral (caudal edge of nasal septum).

Within 1 and 2 hours, respectively, the grain yield over the cells and matrix is similar. It is apparent that the greatest yield is over the matrixogenic zone (Fig. 2). Delineation of this functional cell zone was demonstrated by photometric measurements on the 48-day specimens (Table 1). The reproducibility of the photometer readings gave a coefficient of variation of 6-2 per cent. The photometer readings corrected for general background, reflect the significantly higher degree of labelling over the matrixogenic zone. It is apparent that at 1, 3, and 5 days after the injection the labelled matrix is being relocated from the cartilaginous growth plate into the bony medulla (Fig. 3).

**Growth rate**
The values for the growth rate (Table 2) show a marked caudorostral gradient in each age of animal and a marked decrease in growth rate at each site of endochondral growth with increasing age.

**Cell size**
The diameter of cells in the direction of growth are shown in Table 3. There is a marked caudorostral gradient with a marked reduction in cell size with increasing age. This is apparent in each endochondral growth site.

**Cell loss**
The rate of cell loss in each endochondral growth site and at each age studied is shown in Table 4. The most distinctive feature of this is the constancy of cell loss in all five endochondral growth sites. The rate of cell loss declines steadily with increasing age.

**Discussion**
It is apparent that the uptake of $^{35}$S-sulphate is an indicator of the pathway of sulphate incorpora-
Figure 3 Basiooccipital basisphenoid synchondrosis. Five days after injection of $^{35}$S-sulphate. The labelled matrix has been relocated into the primary spongiosa. The amount of growth can be estimated by measuring the distance between the arrows for each growth centre. The moderately heavy labelling over the matrixogenic zone is probably due to recirculation of $^{35}$S-sulphate from breakdown of cartilage matrix. (Magnification $\times$ 94.)

Table 2 Growth rate of sites of endochondral growth and ossification estimated from $^{35}$S-labelled material (stripping film) 5-day specimens.

<table>
<thead>
<tr>
<th>Growth rate ($\mu$m/d)</th>
<th>4 days</th>
<th>8 days</th>
<th>16 days</th>
<th>32 days</th>
<th>48 days</th>
<th>80 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO (caud)</td>
<td>107</td>
<td>92</td>
<td>73</td>
<td>48</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>BS (caud)</td>
<td>105</td>
<td>90</td>
<td>66</td>
<td>42</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>BS (rost)</td>
<td>92</td>
<td>87</td>
<td>67</td>
<td>40</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>PS (caud)</td>
<td>88</td>
<td>92</td>
<td>64</td>
<td>39</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>PS (rost)</td>
<td>—</td>
<td>73</td>
<td>52</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

Key: as for Table 1.

Table 3 Cell diameters measured in the direction of growth of the five sites of endochondral growth (units = $\mu$m).

<table>
<thead>
<tr>
<th></th>
<th>4 days</th>
<th>8 days</th>
<th>16 days</th>
<th>32 days</th>
<th>48 days</th>
<th>80 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO</td>
<td>41.7</td>
<td>43.7</td>
<td>32.5</td>
<td>26.6</td>
<td>23.5</td>
<td>21.2</td>
</tr>
<tr>
<td>BS (caud)</td>
<td>40.8</td>
<td>37.5</td>
<td>32.7</td>
<td>24.4</td>
<td>23.7</td>
<td>19.4</td>
</tr>
<tr>
<td>BS (rost)</td>
<td>39.4</td>
<td>35.8</td>
<td>28.1</td>
<td>23.4</td>
<td>18.5</td>
<td>16.2</td>
</tr>
<tr>
<td>PS (caud)</td>
<td>35.9</td>
<td>34.9</td>
<td>26.5</td>
<td>21.2</td>
<td>19.4</td>
<td>12.9</td>
</tr>
<tr>
<td>PS (rost)</td>
<td>33.4</td>
<td>27.1</td>
<td>24.0</td>
<td>21.2</td>
<td>16.6</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Key: as for Table 1.

There is a low level of $^{35}$S-sulphate incorporation in all cellular zones except the erosive zone. In the matrixogenic zone the amount of $^{35}$S-sulphate incorporated is two-and-a-half times that of the proliferative zone and more than four times the level of incorporation in the central and hypertrophic zones. This finding lends considerable support to the hypothesis that this zone, formerly called the early hypertrophic zone should be renamed the
<table>
<thead>
<tr>
<th></th>
<th>4 days</th>
<th>8 days</th>
<th>16 days</th>
<th>32 days</th>
<th>48 days</th>
<th>80 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO</td>
<td>2.6</td>
<td>2.1</td>
<td>2.3</td>
<td>1.8</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>BS (caud)</td>
<td>2.6</td>
<td>2.4</td>
<td>2.0</td>
<td>1.7</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>BS (rost)</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>1.7</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>PS (caud)</td>
<td>2.5</td>
<td>2.6</td>
<td>2.4</td>
<td>1.8</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>PS (rost)</td>
<td>—</td>
<td>2.7</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Key: as for Table 1.

Matrixogenic Zone (Roberts and Blackwood, 1983). The principal function of the cells in this zone is to produce cartilaginous matrix, important for the tissue separating force of an endochondral growth centre (Copray et al., 1986). The large capacity for matrix production in this zone gives each endochondral growth site the potential for large variations in matrix formation, presumably depending upon the intensity of physiological stimuli such as growth hormone. The scheme of functional activity appears to be that cells in the proliferative zone divide several times (Kember, 1983), then engage in considerable metabolic activity to produce cartilage matrix: the newly named Matrixogenic Zone. Subsequent to this further cytochemical changes occur which prepare the cartilage matrix for calcification (Greenspan and Blackwood, 1966) and the cells themselves for cell-death or apoptosis (Kerr et al., 1972).

One other feature of $^{35}$S-sulphate incorporation is that cartilaginous matrix production occurs, albeit at a lower level, in the cells of the proliferative zone. This indicates that cells undergoing DNA synthesis and cell division continue to manufacture glycosaminoglycans for export from the cell. This has been alluded to by Lutfi (1974).

Previous attempts at the estimation of growth rate have used the passage of $^3$H-thymidine labelled nuclei through the proliferative, matrixogenic, hypertrophic, and erosive zones (Kember, 1960, 1978). This method has the disadvantage that the timing of the loss of labelled nuclei from the endochondral growth site via the erosive zone is imprecise (Roberts and Blackwood, 1984). An alternative method of measurement direct from radiographs is reasonably accurate (Vilmann, 1969, Roberts and Blackwood, 1983), but does not allow investigators to discriminate between the growth contribution of the caudal and rostral ends of the basis-phenoid and presphenoid bones.

An interesting feature of this study, not previously reported, is the marked caudo-rostral gradient at all ages studied. This is a considerable refinement when compared to simple radiography (Roberts and Blackwood, 1983). This trend has already been revealed by $^3$H-thymidine autoradiography (Roberts and Blackwood, 1984), but as already indicated this is a less reliable method of estimating growth. The data presented here have considerably higher values than those from the $^3$H-thymidine by a factor of some 40 per cent. We consider that this data represents a more reliable estimate of the absolute growth rate because it relies on the direct measurement of the labelled and displaced matrix.

The size of the last hypertrophic cell and matrix also shows a clear caudo-rostral gradient at all ages studied. Comparison of these sizes with the tibia of rats (Thorngren and Hansson, 1973), the tibia of humans (Kember and Sissons, 1976; Sissons and Kember, 1977), shows that the ultimate size of the hypertrophic chondrocytes appears unique to each individual growth site. It is not known if this hypertrophic cell size is due to a specific controlling mechanism or whether it is closely correlated with the growth rate of each endochondral site. Strong evidence for this latter hypothesis comes from the observation of significant differences in hypertrophic cell size in normal and hypophysectomized rats (Thorngren and Hansson, 1973) and from the reduction in cell size with increasing age observed in the data presented in Table 3. As age increases growth is slowed (Table 2), and at the same time the hypertrophic cells become smaller.

One other feature of the data is the remarkable consistency in the rate of cells lost per day at each growth site. This suggests that factors outside the
endochondrial growth apparatus are responsible for the rate of vascular erosion presumably in the metaphysis. The data shows that this also varies with age, the rate of cell loss decreasing as the animals get older.

It is apparent that the three main factors influencing the growth rate of endochondral growth sites are the rate of cell division (Roberts and Blackwood, 1984), the rate of matrix production alluded to above, and the ultimate size of the final hypertrophic cell immediately before vascular erosion. Little is known about the factors that control the relative tempo of these growth processes.

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