The Reversibility of the Effect of Hypervitaminosis A on Embryonic Limb Bones Cultivated in vitro

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WITH TWO PLATES

INTRODUCTION

The skeleton of young animals is profoundly affected by an abnormal amount of vitamin A in the body. In vitamin A deficiency changes in the functional activity of the osteoblasts and osteoclasts allow bone to be deposited in places where it would normally be removed, so producing excessive thickening of certain parts of the skeleton (Mellanby, 1938, 1939). Conversely, excessive vitamin A causes osteoporosis and spontaneous fractures, although the formation of new bone is not inhibited (Strauss, 1934; Wolbach & Bessey, 1942; Irving, 1949). Recent experiments have shown that the vitamin has a direct effect on skeletal tissues grown in vitro. Fell & Mellanby (1952) cultivated the long-bone rudiments of embryonic chicks and mice in medium containing vitamin A in concentrations similar to those found in the blood of animals suffering from hypervitaminosis A; in such explants the cartilage matrix lost its metachromasia and gradually disappeared (chicks, mice) while bone (mice) was rapidly resorbed.

In dogs fed on an A-deficient diet, the resulting skeletal changes could be at least partially reversed by restoring the vitamin to the food (Mellanby, 1947). The following investigation was undertaken to see whether the abnormalities produced in explanted bone-rudiments by the presence of excess vitamin A in the nutrient could be reversed in a similar way by transferring the bones to normal medium. It will be seen that under the conditions of the experiments most of the changes were not reversible, though they could be arrested.

MATERIAL AND METHODS

**Explants**

Chick embryos were removed from the egg after 6–7 days’ incubation and the humerus, ulna, radius, femur, and tibia were explanted separately.

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The culture medium

Embryo extract was made from chicks of 13–16 days' incubation. The embryo was finely minced to form a pulp which was mixed with an equal volume of Tyrode’s solution containing 1 per cent. glucose and centrifuged for 3 minutes. The extract was freshly prepared for each subculture. Another extract consisting of 1 part embryo : 2 parts of ordinary Tyrode’s solution was also made and was used for washing the tissues.

An alcoholic solution of vitamin A was added to normal fowl plasma to give a concentration of about 2,000 i.u. or 4,000 i.u. of the vitamin per 100 ml. of plasma (+A plasma). The alcoholic solution of vitamin A gave a concentration of ethanol of 0.3 per cent. or less in the final plasma. Plasma from the same bird was used for the control cultures and ethanol was added to give the same concentration as in the +A plasma. The control plasma contained between 200 i.u. and 300 i.u. of vitamin A per 100 ml. The method of obtaining the plasma and of estimating its content of vitamin A has been described by Fell & Mellanby (1952).

The final culture medium consisted of a mixture of 3 parts plasma and 1 part embryo extract. Thus in the final medium the concentration of glucose in the embryo extract was lowered to 25 per cent. of its original value and that of vitamin A in the plasma to 75 per cent.; in the following account the values for vitamin A are those estimated in the original plasma.

The watch-glass method

The watch-glass method as described by Fell & Mellanby (1952) was used. Owing to the rapid rate of growth and consequent depletion of the medium it was necessary to subcultivate the chick explants four times each week. The tissues were explanted on 12 drops of medium, were transferred to 16 drops at the first subcultivation and continued at this level throughout the experiment; the ratio of 3 parts of plasma to 1 part of embryo extract was maintained throughout the culture period.

Measurements

Camera lucida drawings of the explants were made at the beginning of the experiments and at each subcultivation. Rough estimates of the size of the epiphyses of some bones were calculated from these drawings by using a planimeter to measure the surface area.

Histological technique

The explants were fixed in acetic Zenker’s fluid for 1 hour, embedded in paraffin wax, and serially sectioned. Sections were stained with either Delafield’s haematoxylin and chromotrop 2R, 0.5 per cent. toluidine blue in 5 per cent. alcohol, or by the azan method.
RESULTS

The object of these experiments (table) was to see if limb-bone rudiments cultivated for a time in hypervitaminotic medium could recover from the effects of the excess vitamin if transplanted to normal medium.

Although all the embryos used were of approximately the same age, their stage of development varied considerably. The structure of the rudiments when explanted is summarized in the table.

Table

Summary of Experiments 2–14

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Vit. A i.u./100 ml. added to plasma</th>
<th>Embryo</th>
<th>No. of pairs of bone rudiments</th>
<th>Days in culture</th>
<th>Stage of development of rudiments at time of explantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>+A 2,000</td>
<td>1</td>
<td>4. Humerus, radius, femur, tibia</td>
<td>16—7</td>
<td>Chondroblastic hypertrophy visible in all explants.</td>
</tr>
<tr>
<td></td>
<td>N 300</td>
<td>2</td>
<td></td>
<td>7—9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+A 4,000</td>
<td>1</td>
<td>4. Humerus, ulna, femur, tibia</td>
<td>14—5</td>
<td>Chondroblastic hypertrophy visible only in femora and humeri.</td>
</tr>
<tr>
<td></td>
<td>N 300</td>
<td>2</td>
<td>5. Humerus, ulna, radius, femur, tibia</td>
<td>5—9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+A 4,000</td>
<td>1</td>
<td>4. Humerus, tibia, radius, femur</td>
<td>——12</td>
<td>Chondroblastic hypertrophy in femora and humeri only.</td>
</tr>
<tr>
<td></td>
<td>N 200</td>
<td>2</td>
<td>4. Humerus, ulna, radius, femur</td>
<td>12—4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4—8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+A 2,000</td>
<td>1</td>
<td>4. Humerus, tibia, radius, femur</td>
<td>——15</td>
<td>Chondroblastic hypertrophy distinct and periosteal bone visible in all explants.</td>
</tr>
<tr>
<td></td>
<td>N 200</td>
<td>2</td>
<td>5. Humerus, ulna, radius, femur, tibia</td>
<td>15—4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4—11</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+A 2,000</td>
<td>1</td>
<td>5. Humerus, ulna, radius, femur, tibia</td>
<td>——16</td>
<td>Chondroblastic hypertrophy visible in all explants.</td>
</tr>
<tr>
<td></td>
<td>N 250</td>
<td>2</td>
<td></td>
<td>16—6</td>
<td>Slightly more advanced than embryo 1.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6—10</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>+A 4,000</td>
<td>1</td>
<td>5. Humerus, ulna, radius, femur, tibia</td>
<td>——18</td>
<td>Rudiments very small and soft. Chondroblastic hypertrophy visible only in femur.</td>
</tr>
<tr>
<td></td>
<td>N 300</td>
<td>2</td>
<td></td>
<td>18—9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9—9</td>
<td></td>
</tr>
</tbody>
</table>

Experimental procedure

In Experiment 2 one embryo was used and one of each pair of rudiments was cultivated in +A medium for the whole of the culture period while the other was grown in similar medium for 7 days and then in normal medium for 9 days. In all
the other experiments two embryos were used: from the first chick one of each pair of explants was cultivated in normal medium and the other in + A medium for the entire culture period; all the explants from the second embryo were cultivated in + A medium for 4–9 days and were then transferred to normal

\[\text{Normal} \quad \begin{array}{c}
24 \text{ hours} \\
3 \text{ days} \\
5 \text{ days} \\
7 \text{ days} \\
10 \text{ days} \\
14 \text{ days}
\end{array} \quad + \text{ A} \]

**TEXT-FIG. 1.** Camera lucida drawings of a pair of living humeri from a 7-day chick embryo (Exp. 3), one grown in normal and the other in + A medium (4,000 i.u./100 ml. plasma). The humerus in normal medium enlarged to more than three times its original length during 14 days' cultivation. That in + A medium grew normally at first but later shrank and became very distorted.

medium. After 2 or 3 days in normal medium one of each pair was fixed as a control and the other was maintained for the rest of the experiment. It was found that the vitamin A continued to exert an effect for 2–3 days after the bones were removed from the + A medium, probably owing to an accumulation of vitamin in the tissue; if, therefore, the control bones had been fixed when the others were
transferred to normal medium a true picture of the extent of the vitamin A effect would not have been obtained.

**Behaviour of the rudiments in normal medium**

The control rudiments cultivated in normal medium grew very well and usually increased to more than three times their original length (Text-fig. 1). The zones of hypertrophic cartilage cells, flattened proliferative cells, and epiphyseal cells differentiated in culture, and the layer of periosteal bone could be seen increasing in thickness and extending towards the ends of the diaphysis; there was no excavation of cartilage to form a marrow cavity. The soft tissue adhering to the bone grew rapidly.

**Text-fig. 2.** Camera lucida drawings of a pair of living humeri from a 7-day chick embryo (Exp. 3). Both rudiments were grown in +A medium (4,000 i.u./100 ml. plasma) for 5 days and were then transferred to normal medium; one was fixed after 2 days and the other after 9 days in normal medium. Both the rudiments were affected by the excess vitamin and on transfer to normal medium there was no further growth of the shaft although the epiphyses continued to enlarge.
Sections of these bones fixed after 12–14 days in vitro showed that the shaft consisted of a middle region of large hypertrophied chondroblasts and the two zones of flattened cells beyond which the small-celled epiphyses could be distinguished (Plate 1, figs. A, E, H). Even after 15 days in vitro the cartilage cells were surprisingly healthy and there was little degeneration in either the shaft or epiphyses, but after 18 days some cells were necrotic. Mitoses were common in the epiphyses. The chondroblasts of the shaft were separated by thick partitions of matrix which was highly basophilic when stained with Delafield's haematoxylin and which stained metachromatically with toluidine blue. The layer of periosteal bone, which extended to the ends of the shaft, was usually very thick and occasionally some cartilage cells were enclosed in it. Most of the osteoblasts were healthy and they were usually arranged in fairly regular rows along the longitudinal axis of the rudiment.

Effect of hypervitaminosis

The explants grown in a concentration of vitamin A of about 2,000 i.u. per 100 ml. of plasma did not appear different from the controls until they had been in culture for 4 days, but those grown in a concentration of about 4,000 i.u. per 100 ml. were much more severely affected (Text-fig. 1). The cartilage of the hypervitaminotic explants differentiated into the usual three zones of cells and periosteal bone was formed quite as rapidly as in the controls but after about 4 days the growth rate declined and, as compared with the controls, the shafts became shorter and thinner; the epiphyses, although they continued to enlarge until about the seventh day, were also smaller than in the controls. Constrictions appeared at the ends of the shaft in the humerus and femur at about the sixth day in the lower concentration and at about the fourth day with the higher dose; by the end of the experiment usually all the bones were constricted and very distorted. After 7–8 days in culture the explants began to shrink although the surrounding soft tissue proliferated more extensively than in the normal medium.

Microscopical examination showed that after 4 or 5 days in + A medium the basophilia was usually lost from the periphery and ends of the shaft but remained in the centre of the diaphysis and in the epiphyses. After 7 days' cultivation, even in the lower concentration of the vitamin, the matrix of the shaft was usually completely colourless and the periphery of the epiphyses was also pale when stained with Delafield's haematoxylin. In the non-basophilic parts the matrix of the shaft was reduced, the cells were usually smaller than normal and there appeared to be more degenerate cells than in the areas which still stained with the haematoxylin. There were usually few degenerate cells in the epiphyses and mitotic figures were frequently observed.

After 12–15 days in culture the histological differences between the treated and control explants were very great (Plate 1, cf. figs. A and E with B and F). In the + A medium most of the bones had become so distorted that it was difficult to distinguish the three zones of cells in the sections. There was usually as much
periosteal bone as in the controls, but the osteoblasts, some of which were necrotic, were arranged in a less regular manner. The cartilage matrix showed most pronounced abnormalities. The amount of matrix between the chondroblasts in the shaft was reduced and had completely lost its basophilia and metachromasia and was colourless when stained with Delafield's haematoxylin or toluidine blue. The cells of the shaft were considerably smaller and less vacuolated than those of the controls and a higher proportion was necrotic. In places the matrix had disappeared completely and two or three chondroblasts were enclosed in one capsule; mitoses were sometimes observed in these cells (cf. Fell & Mellanby, 1952). After about 12 days in +A medium the cells of the shafts of some rudiments were completely necrotic and the matrix appeared to consist of a network of very loosely arranged fibres (Plate 1, figs. B and I). In other explants, however, the cells appeared quite healthy, mitotic figures were very frequently seen, and the intercellular substance was more compact (Plate 1, figs. F and G). The matrix of the epiphyses was less severely affected than that of the shaft, and basophilic and metachromatic areas were usually visible in the centre of the epiphyses although the periphery was pale. There were fewer degenerate cells than in the shaft but mitoses were seldom seen.

The extent of the changes produced by the excess vitamin A was directly related not only to the length of time spent in the +A medium but also to the stage of development of the rudiments when explanted, the younger primordia being more rapidly and more drastically changed than the older ones (Plate 1, cf. figs. B and I with figs. F and G). There were also differences in the effect on individual bones; the femur was always affected more than the other rudiments while the ulna and radius were the least changed.

The effect of transfer from +A to normal medium

The degree of recovery which the hypervitaminotic bones underwent when they were transferred to normal medium depended upon the severity of the damage they had sustained. In Experiment 5, for example, the rudiments were scarcely changed by their 4 days' contact with excess vitamin A and, when put into normal medium, they continued to grow and develop as well as the controls grown in normal medium throughout. This apparent lack of effect was partly due to the short time spent in +A medium but also to the facts that the lower concentration of the vitamin was used and that the rudiments were at an advanced stage of development when explanted (see table). In Experiment 4, however, which may be taken as a typical example, younger rudiments were explanted and after 4 days in the higher concentration of the vitamin they were severely affected (Plate 1, fig. C; Plate 2, fig. M).

When the rudiments were transferred to normal medium the changes produced by the hypervitaminosis were arrested (Plate 1, cf. figs. B and D; Text-fig. 2), but if the shaft had ceased to grow in the +A medium there was no further elongation after transfer although the width sometimes increased. The epiphyses
were less drastically altered than the shaft and usually began to enlarge again in normal medium even though they had been shrinking previously (Text-fig. 2). Text-fig. 3 shows the relative sizes of the distal epiphyses of three comparable femora at intervals during Experiment 14 and the enlargement on transfer from +A to normal medium can be seen.

**Text-fig. 3.** The sizes of the distal epiphyses of three comparable femora at intervals during Experiment 14. The epiphysis of the rudiment which was transferred to normal medium began to increase in size again although it had been shrinking while in the +A medium (4,000 i.u. vitamin A/100 ml. plasma).

† indicates transfer to normal medium. ○ = 16 days in +A medium.
+ = 16 days in normal medium. ● = 9 days in +A, 7 days in normal medium.

The transfer seemed to favour the formation of periosteal bone, and histological examination showed that in places a particularly thick layer was deposited which probably accounted for the increase in the width of the shaft mentioned above. Cartilage matrix which had disappeared from the diaphysis was not reformed nor was basophilia recovered where it had been lost (Plate 1, cf. figs. c and d). The diaphysial chondroblasts, however, largely recovered but although most of them looked healthy they were very different in appearance from the hypertrophic cells seen in the normal controls. The shaft as a whole resembled osteoid tissue rather than hypertrophic cartilage (Plate 2, figs. j and k), particu-
larly in the region just beneath the layer of periosteal bone where the cells were very similar to the adjacent osteoblasts. In places the sheath of bone merged almost imperceptibly with the cartilage and the cells seemed to show a gradation from the young osteocytes in the osseous layer to the slightly hypertrophic cells in the interior of the shaft (Plate 2, cf. figs. 1 and L). Even the central chondroblasts, however, were smaller and less vacuolated than the hypertrophic cells of the controls (cf. Plate 2, fig. L and Plate 1, fig. H). In sections stained with Delafield's haematoxylin and chromotrop, the cartilage matrix which had lost its usual basophilia took up the pink of the chromotrop as did the periosteal bone. On the other hand, after Azan staining it acquired the pale blue coloration of normal cartilage and not the typical dark blue of osteoid tissue, but there was no sharp division of the colours where the character of the cells was changing as described above. The cartilage cells seemed to be more numerous than in the controls but this was probably due to crowding of the cells through loss of intercellular material, although mitoses were occasionally seen.

After the rudiments had been transferred to normal medium, many more mitotic cells could be seen in the epiphyses than in the explants grown either in normal or in high A medium throughout the experiment. The cells were similar in appearance to those of the normal controls and relatively few were necrotic but, as in the shaft, there was no restoration of lost matrix or of metachromasia.

**DISCUSSION**

These experiments show that excess vitamin A affects chick limb-bone rudiments cultivated *in vitro* in several ways. Differentiation of the cartilage cells continues but the growth of the rudiments is arrested and the cartilage matrix shrinks and loses its basophilia and metachromasia. Certain bones are affected more than others and the changes vary according to the stage at which the rudiments were explanted. These results confirm those of Fell & Mellanby (1952).

The changes produced by a few days' exposure to high vitamin A are only partly reversible by returning the explants to normal culture medium; the short treatment with the vitamin does not impair the viability of the chondroblasts and seems to increase their reproductive capacity (Lasnitzki, personal communication), but it inhibits their ability to form normal cartilage matrix even after the excess vitamin A has been withdrawn. Thus when the treated rudiments are returned to normal medium the epiphyses resume their growth, but their enlargement is mainly due to profuse mitosis; the matrix does not seem to increase in amount nor does it regain its former basophilia and metachromasia. In the shaft also there is no regeneration of matrix after the transfer, and although the chondroblasts recover their healthy appearance they do not regain their characteristic swollen, vacuolated structure; they resemble osteoblasts rather than chondroblasts and it is interesting that they sometimes undergo mitosis which is rarely, if ever, seen in normal, mature hypertrophic cells. Whether as a result of exposure to high vitamin A the chondroblasts have permanently lost their
ability to form normal matrix or whether on prolonged cultivation in normal medium this function would eventually return, is not known.

SUMMARY

1. Experiments were made to study the reversibility of the effects of hypervitaminosis A on embryonic long-bones in vitro.

2. Limb-bone rudiments from 6- to 7-day chick embryos were cultivated by the watch-glass method, first in medium to which excess vitamin A had been added and then in normal medium. Suitable controls were also grown.

3. The excess vitamin arrested the growth of the explants and caused the cartilage matrix to shrink and lose its basophilia and metachromasia. The extent of the damage depended upon the length of time spent in +A medium, the concentration of the vitamin, the stage of development of the rudiment when explanted, and the particular rudiment treated.

4. The degree of recovery was related to the severity of the changes produced by the vitamin. In the shaft these changes were arrested but there was no restoration of lost matrix; the cells appeared healthy but resembled osteoblasts rather than normal hypertrophic chondroblasts. The epiphyses also partially recovered and began to enlarge again when transferred to normal medium; they had a high mitotic rate in comparison with the normal controls.

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REFERENCES

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Plate 1
Plate 2
EXPLANATION OF PLATES

PLATE 1

Fig. A. Femur from Experiment 4 after 12 days' cultivation in normal medium. The differentiation of the chondroblasts can be seen and the cartilage matrix is strongly basophilic. \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. B. Femur from the opposite side of the same chick as shown in Fig. A after 12 days' cultivation in medium containing 4,000 i.u. vitamin A/100 ml. of plasma. The rudiment is very small, and the cartilage has almost disappeared (see Fig. I). \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. C. Femur from Experiment 4 after 4 days' cultivation in +A medium (4,000 i.u. vitamin A/100 ml. of plasma) and 3 days in normal medium. The bone is rather distorted and one epiphysis has almost disappeared. The basophilia has been lost from part of the shaft and from the outer region of the remaining epiphysis (ep) (see Fig. M). \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. D. Femur from the opposite side of the same chick as the explant shown in Fig. C after 4 days' cultivation in +A medium and 8 days in normal medium. The remaining epiphysis (ep) is much larger than the one seen in Fig. C, but there has been no recovery of basophilia in either the shaft or the epiphysis (see Figs. J, K). \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. E. Femur from Experiment 3 after 14 days' cultivation in normal medium. This shows the differentiation of the cartilage cells and the highly basophilic matrix (see Fig. H). \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. F. Femur from the opposite side of the same chick as shown in Fig. E after 14 days' cultivation in +A medium (4,000 i.u. vitamin A/100 ml. plasma). When explanted this rudiment was at a more advanced stage of development than that shown in Fig. B and was less changed by the vitamin A. The basophilia has been lost but the matrix is compact and most of the cells are healthy. \( \times 17. \) (Delafield's haematoxylin and chromotrop.)

Fig. G. Part of the shaft of the femur shown in Fig. F. Groups of chondroblasts are enclosed in single capsules and two mitotic figures (m) can be seen. \( \times 1,200. \) (Delafield's haematoxylin and chromotrop.)

Fig. H. Part of the diaphyseal region of the femur shown in Fig. E. The periosteum is at the top and beneath this is the sheath of periosteal bone with the osteocytes arranged in a fairly regular manner. The large hypertrophied chondroblasts are separated by thick bars of basophilic matrix. \( \times 600. \) (Delafield's haematoxylin and chromotrop.)

Fig. I. Part of the femur shown in Fig. B. Many of the cells are necrotic and the matrix has disappeared almost entirely although a few pale fibres remain. The periosteal bone is less dense than in the normal control and many of the osteocytes are necrotic. \( \times 170. \) (Delafield's haematoxylin and chromotrop.)

PLATE 2

Fig. J. Part of the shaft of the section shown in Fig. D. This shows the layer of periosteal bone which seems to merge with the cells of the shaft as the cells directly beneath the bone layer are very similar in appearance to osteoblasts. \( \times 600. \) (Delafield's haematoxylin and chromotrop.)

Fig. K. Another region of the shaft of the femur shown in Fig. D. The chondroblasts are healthy but they are smaller and more compact than those of the normal controls. \( \times 600. \) (Delafield's haematoxylin and chromotrop.)

Fig. L. The interior of the shaft of the femur shown in Fig. D. These cells also appear healthy and some are slightly hypertrophic but they are still smaller and less vacuolated than those seen in Fig. H. \( \times 600. \) (Delafield's haematoxylin and chromotrop.)

Fig. M. Part of the shaft of the femur shown in Fig. C. The chondroblasts show a greater degree of hypertrophy than those seen in Fig. L. Some capsules contain more than one chondroblast and the matrix has lost its basophilia. \( \times 600. \) (Delafield's haematoxylin and chromotrop.)

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