THE LOCAL ACTION OF VITAMIN A ON BONE

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INTRODUCTION

There is now considerable evidence that vitamin A plays an important part in the metabolism of bone tissue. Deficiency of this vitamin results in abnormalities in the shape of the bones in several species and, as Mellanby (1938) first demonstrated in experiments on puppies, it is the failure of certain bony foramina to enlarge during growth which leads to pressure-degeneration of various nerves. This result was confirmed by Wohlbach & Bessey (1941) in young, but not in adult, rats. In a series of papers (1941, 1944, 1946), Mellanby extended his analysis of the skeletal changes in dogs. He showed that on certain skull bones the position of osteoblasts and osteoclasts became reversed, while in other regions there was a decline of osteoclastic activity. The administration of vitamin A led to the reappearance of osteoclasts in abnormally large numbers in situations where they were normally present (1946). Similar disturbances were detected throughout the skeleton, but the skull and vertebrae were most thoroughly examined. Wohlbach (1947) was able to confirm these results on dogs but gave them a somewhat different interpretation, and Irving (1949), who confined his observations to the incisor alveolar bone of rats, concluded that the basic disorder was excessive osteoblastic activity.

Since these skeletal lesions in vitamin A deficiency can be prevented by very small amounts of the vitamin, this work provides convincing evidence of its importance in normal bone growth, but skeletal disturbances also result if the vitamin is given in amounts greatly exceeding the normal requirement. Following the early demonstration by Takahashi, Nakamiya, Kawakami & Kitasato (1925) of severe toxic symptoms in rats given very large doses of fish liver-oil concentrates (Biosterin), Collazo & Rodriguez (1933) showed that, within 10 days, animals under similar treatment suffered fractures of the limb bones. This was confirmed by Bomskov & Seeman (1933) and Davies & Moore (1934), the latter using a liver-oil distillate containing less impurity than the previously used concentrates. Strauss (1934) made a histological study of bones from hypervitaminotic rats and found that the cortex of the long bones, and also the skull bones were thinned, and the occurrence of fractures was again reported by Hoff & Jeddeloh (1934–5), Weslaw, Wronski, Wroblewski & Wroblewski (1938) and Vedder & Rosenberg (1938). In 1945, Moore & Wang established that the fractures were actually due to vitamin A itself by producing them in young rats which were given 25,000–50,000 i.u. daily as a solution of crystalline vitamin A acetate. Irving (1949) reported that in rats given similar doses, the incisor alveolar bone showed a decrease of osteoblastic activity while osteoclasts remained in normal numbers. There is little published information about the detailed bone histology in hypervitaminosis A, but a summary by Wohlbach (1947) of his experiments on several species, shows that with sufficiently high doses osteoclastic resorption of the cortex of the long bones occurs, particularly in regions of active remodelling, and that with a somewhat lower and more prolonged dosage the shaft

diameters become reduced in relation to their length. The most recent contribution is that of Rodahl (1949), who noted fractures and the same curious narrowing of the long-bone shafts in young rats given either polar bear liver-oil or whale liver-oil concentrates, both of which contain high concentrations of vitamin A. It appears from the literature that in vitamin A deficiency there is a disturbance of the balance of bone deposition and resorption leading to excessive thickness of bone in certain regions, while in overdosage the situation is reversed and the skeleton is weakened. There is at present no evidence to show whether the vitamin exercises its influence on bone by a direct action on osteogenic cells, or whether a more complicated chain of metabolic disturbances is involved. The grafting technique used by the writer (1948*a*) to investigate the local action of the parathyroid on bone seemed well suited to a further analysis of the effects of vitamin A when present in excess. Some preliminary results have already been published (1948*b*), but since then further experiments have been performed, and the full data are reported in detail in the present paper.

MATERIAL AND METHODS

The grafting technique has already been described by the writer (1948*a*), and only a few details need be added. It may, however, be useful to reiterate the logic of the method. The tissue or compound under examination is attached to a small piece of parietal bone cut from a 10-day-old mouse and the two are then inserted into the cerebral hemisphere of a litter-mate and left for 2 weeks. It is argued that if the substance is capable of exerting a purely local action on bone this will be shown by changes in the immediate vicinity of its attachment. If such a change occurs, it is not likely to depend on the stimulation of some tissue remote from the graft, to which the implanted substance would have to be carried in the blood-stream, since in that case effects throughout the skeleton of the host would be expected. It is conceivable, however, that the liberation of a highly active compound from the graft site might result in a systemic effect which would in turn modify the response of the graft bone to the local action of the compound, including, for example, a non-specific action such as the mechanical pressure of the implanted material. If this were so one might expect to observe some effect on the host animal also, but it could still be argued that the systemic change might be sufficient to modify the response of the graft but not to produce any obvious change in the host. While such possibilities have not been rigorously excluded, they do not seem to be very likely.

The material used for what is conveniently referred to as the chemical implant was crystalline vitamin A_1 acetate, which was kindly provided by Sir Edward Mellanby. The ester was kept sealed in carbon dioxide in the refrigerator, and should be fairly stable under these conditions. For many of the experiments a single small chip was broken off and attached to the centre of the endocranial aspect of the piece of parietal, using solutions of human fibrinogen and thrombin with penicillin added. The graft was then thrust vertically into the cerebral hemisphere of the host through a small slit made in the skull roof. In some experiments, which were designed for a more exact comparison of the local actions of vitamin A and calciferol, the vitamin A was finely powdered, and the powder was then cemented into small pellets, of suitable size for grafting, by means of fibrin clot. In some cases either oestradiol or cholesterol was mixed with the powdered vitamin A to see whether the former sub-

stance modified the bone response; since no difference from the effects of vitamin A alone could be detected, these specimens are included in the present description.

The grafts were finally examined either as dry specimens after maceration in 2 % KOH, or by histological sectioning. In many cases they were stained supravitally with neutral-red in the manner previously described by the writer (1947) and the distribution of osteoblasts and osteoclasts was recorded by drawing the preparation on millimetre graph paper, using an ocular graticule and a 2 in. objective. The position of osteoclasts could be fairly accurately recorded in this way, but the osteoblasts were too numerous to be individually marked, and the drawings merely recorded the size and shape of the scattered areas occupied by these cells. In this connexion it should be noted that it was necessary to remove a layer of connective tissue from the graft surface either before or after staining, in order that the cells might be clearly visible, and some loss may have occurred in this process; as far as one could tell, however, from dissection of stained specimens under the microscope such a loss was slight, and the curiously patchy distribution of osteoblasts is unlikely to be an artefact. A staining period of 30–45 min. in a $\frac{1}{10,000}$ solution of neutral-red in 0·9 % saline was generally sufficient to give strong coloration. If supravitally stained specimens were fixed in 2 % mercuric chloride to preserve the stain, subsequent maceration was often not successful and resulted in some disintegration and distortion of the bone.

Histological material was prepared by removing the graft in a large block of brain tissue together with the overlying skull and fixing and decalcifying simultaneously in Zenker + 3 % of glacial acetic-acid. The material was double-embedded in celloidin and paraffin and after cutting serially at 8 μ was stained with Ehrlich's haematoxylin and orange G-erythrosin. The sections were in a plane parallel to the sagittal plane of the skull and therefore cut the graft bone at right angles to its surface and showed the full length from the skull wound to the deepest edge.

Animals of two inbred strains were used, C 57 black and Glaxo Champagne, and no major difference in the response of the two stocks was noticed.

Macerated and supravitally stained material

The macerated material consists of thirty-four grafts, and their distribution between different types of experiment, together with a classification of the effects on the bone is set out below (Table 1). The table gives the type and duration of the

Table 1

Type and duration of graft	Perforated	Thin area; not perforated	Resorption slight or absent	Total
Vit. A single fragment (14 days)	C 4 A 2	C 2 A 2	A 1	11
Vit. A single fragment (7 days)	A 1	A 2	A 1	4
Vit. A powdered (14 days)	C 2 A 2	A 2	A 1	7
Vit. A + cholesterol (14 days)	C 2	C 3	C 1	6
Vit. A + oestradiol (14 days)	C 5 (3♀–2♂)	C 1 (♀)	— —	6 —
Total	18	12	4	34

graft, and the letters A and C indicate Champagne and C 57 animals respectively. It will be realized that this division into categories is to some extent arbitrary, for the dividing line was not always sharp, and it is probable that a more extensive material could be arranged in a continuous series. The results are on the whole striking and consistent. More than half the bone grafts showed a definite perforation situated in the central region, and in many cases dissection revealed the vitamin residue somewhere within this hole. The perforations were generally more or less circular in outline with a smooth sharp margin, which was often formed by a band of thin bone demarcated by a step from the thicker bone nearer the graft periphery. Neutral-red revealed osteoclasts on some part of this margin (Pl. 1, fig. 8; Text-fig. 5). The

Text-figs. 1–5. Scale drawings of supravitally stained specimens showing the distribution of osteoclasts on the surface nearest to the vitamin implant. × 29. Photographs of the bones from which these figures were drawn are included in Pl. 1, so that the correspondence of bone structure and cell distribution can be studied.

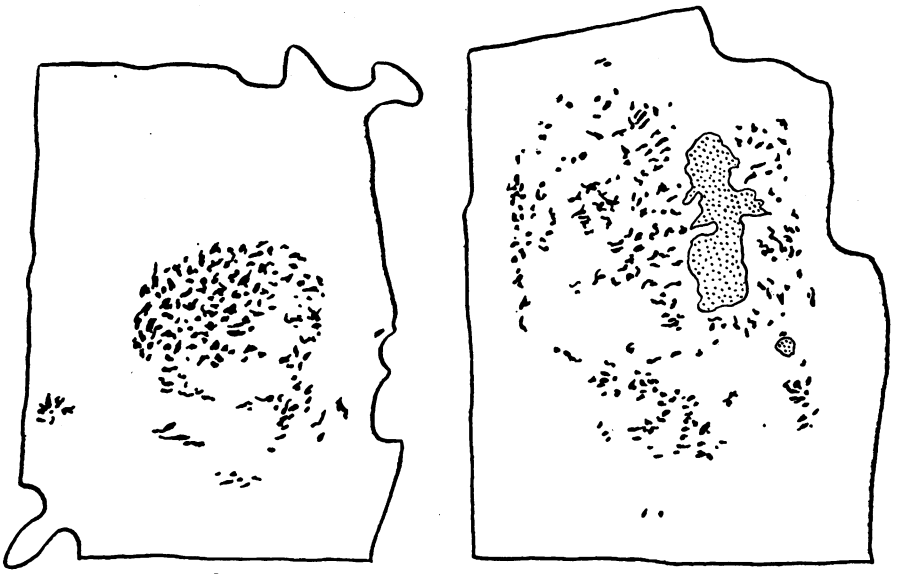
Text-fig. 1. Bone implanted with single fragment of vitamin A, and removed after 7 days. The diagram also shows areas of osteoblasts, indicated by a fine stipple, and canals in the bone. There are several scattered groups of osteoclasts in the central region. Compare Pl. 1, fig. 9, which shows the macerated specimen.

majority of grafts showed a varying degree of thickening along the edge which lay in the insertion wound, but this is a feature which usually occurs in all types of graft, and is independent of the chemical implant. Although a single perforation was more usual, some specimens had one or more smaller holes lying close to the main one, and these may have been due to small particles of vitamin A adherent to the main implant, which became displaced. In some specimens (Pl. 1, fig. 6) there was a tongue of thin bone projecting into the perforation from some part of its margin, and the whole endocranial surface of such a projection might be covered with osteoclasts. The second group of specimens consisted of bones which were not actually perforated but showed a roughly circular area of thinner bone near the central region (Pl. 1,

figs. 4, 5). Such areas were slightly sunk below the surface of the surrounding bone, and often quite sharply demarcated from it by a ledge. The bone itself was sometimes homogeneous and translucent (Pl. 1, fig. 4) and sometimes more opaque and perforated by numerous evenly dispersed foramina giving it a sieve-like appearance (Pl. 1, figs. 5, 7). The thin area did not generally project much on the convex surface. These thin areas were clearly sites of resorption and incipient perforation, since supravital staining revealed numerous osteoclasts covering the whole or part of their surface nearest the vitamin (Text-figs. 2–4). It will be noticed in Text-fig. 3 and Pl. 1, fig. 5, which are of the same bone, that there is a second rather thicker area of resorption within which the thinnest area lies, and that osteoclasts are arranged along its borders. Pl. 1, fig. 7, and Text-fig. 4 show a specimen in which a large area of sieve-like bone is perforated in the centre. It is clear that osteoclastic resorption in these regularly shaped thin areas would ultimately lead to the kind of large perforations already described, and that if perforation occurred unevenly or at more than one position an intermediate stage with a projecting tongue of bone would result. The peripheral regions of the endocranial surface of the grafts showed the same kind of irregular thickening which one finds in grafts implanted with inactive substances or with no chemical implant, and this phenomenon will be mentioned again later. Osteoblasts were observed in the peripheral regions as occasional clusters of irregular shape. The origin of the thin bone will also be discussed again when dealing with the histological material, but it may be remarked here that its structure is distinguishable from the thin bone seen in ungrafted parietals (Pl. 1, fig. 1) and it is not likely to be simply the unchanged bone of the original implant. This is particularly the case with the sieve-like bone, which is, however, not absolutely distinct from the homogeneous type, since transitional forms occur. The regular shape and arrangement of the foramina and the occasional occurrence of fine surface grooves connected with them (Pl. 1, fig. 5) suggest that they carried small vessels. There is a further piece of evidence bearing on the origin of this thin bone, provided by certain grafts which were macerated after mercuric chloride fixation. In these a certain separation of bone layers occurred which enabled one to see that incompletely resorbed thin bone at the margin of a perforation was often continuous with a layer of bone covering the convex surface of the graft. It seems very probable that the layers of bone which are caused to separate by this maceration treatment are in fact newly formed layers which are comparatively loosely attached to the older bone, on the surface of which they were laid down following a pause in osteogenic activity occasioned by the grafting procedure. This suggests, then, that the perforation may involve not only the bone of the original graft but also a newly formed layer.

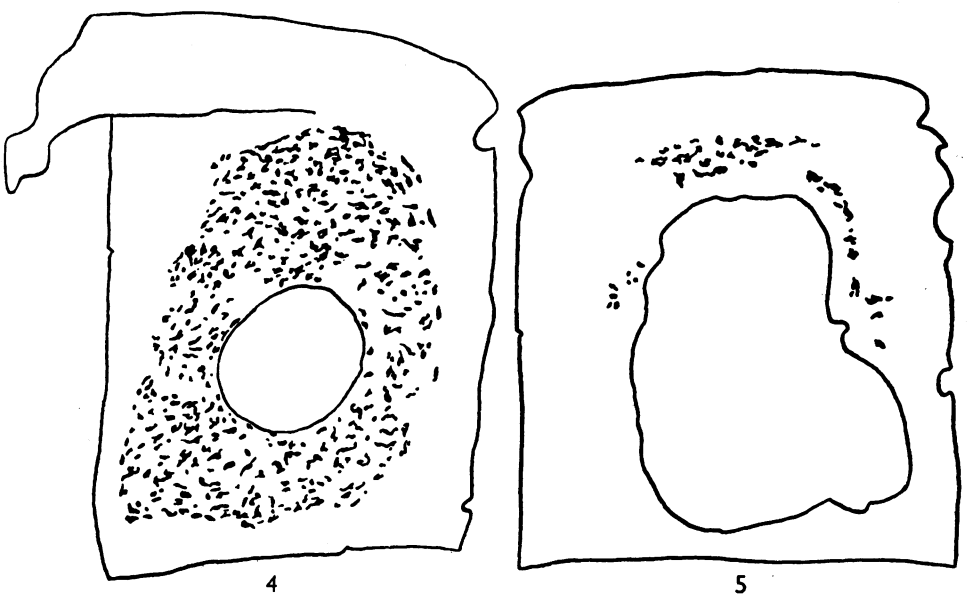
The osteoclasts which are found in resorption areas of vitamin A grafts are in no way peculiar and show a variation of form and staining intensity which could easily be matched in the growing parietals of normal mice as described by the writer (1947).

Six grafts were removed 7 days after the operation and four of them were examined by maceration. They showed relatively slight evidence of resorption; the most advanced stage was one with a small irregular area of thin bone interrupted by several ragged perforations, while in another there was a small, circular area of thinning with ill-defined margins, and bearing a number of osteoclasts. At the other extreme was a specimen in which resorption was only apparent as a minute irregular pit with



Text-fig. 2. Bone implanted with powdered vitamin A + cholesterol; removed after 14 days. Compare the bone structure shown in Pl. 1, fig. 4. Osteoclasts are clustered on a circumscribed area of thin bone. There are also some osteoclasts on the thicker bone below this area.

Text-fig. 3. Bone implanted with powdered vitamin A + cholesterol; removed after 14 days. Compare macerated specimen shown in Pl. 1, fig. 5; osteoclasts scattered over a circumscribed area of sieve-like bone, where the partially dissected remains of the vitamin are also situated (stippled area). Osteoclasts also occur near the margins of a somewhat thicker area of bone which surrounds the first area.



Text-fig. 4. Bone implanted with powdered vitamin A + oestradiol; removed after 14 days. Compare the macerated bone shown in Pl. 1, fig. 7. Osteoclasts are numerous and restricted to a large area of sieve-like bone; the centre of this area is perforated.

Text-fig. 5. Bone implanted with powdered vitamin A + cholesterol. Compare the macerated specimen shown in Pl. 1, fig. 8. There is a large central perforation with osteoclasts located on part of the margin where thin and thick bone adjoin.

a few associated osteoclasts. Pl. 1, fig. 9, and Text-fig. 1 show the fourth specimen in which resorption can be detected as a number of small erosions near which lie clusters of osteoclasts. It is evident from this limited material that the greater part of the resorption in vitamin A grafts must occur during the second week, and this is probably due to the establishment of an adequate vascular supply by the end of the first week, as the writer also suggested in discussing parathyroid grafts (1948*a*) and intracerebral grafts of ribs (1941). It appears from these specimens that resorption may start in several isolated foci close to the vitamin implant and that these then spread to form an extensive area of thinning; during this process the number of osteoclasts increases considerably. It is also worth remarking with regard to the entire macerated material, that, as far as one can tell, the results are the same whether the vitamin is implanted as a single fragment or as a pellet of powder; nor does the inclusion of some 50 % of oestradiol in such a pellet appear to modify the resulting resorption.

Histological material

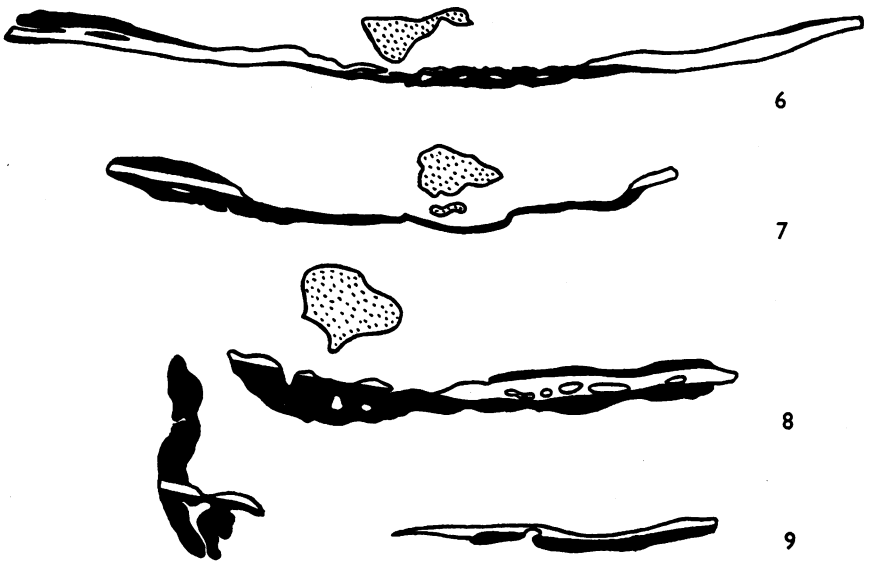
The material consists of eight specimens, in all of which a single chip of vitamin A was used for implantation. Six of the grafts were performed on C 57 animals which were killed after 14 days, and two were performed on Champagne animals, which were killed after 7 days.

Fourteen-day grafts

The vitamin residue was identified in all six specimens, but in two it had become displaced towards the skull wound. Resorption in the vicinity of the chemical implant is illustrated in the two specimens reproduced in Pl. 2, figs. 11, 12. In both of these the vitamin residue, represented by a space, lies close to the bone surface near the centre of the graft, and the bone for a considerable distance on one or both sides is very thin and contrasts with the thicker bone at the two extreme ends. In the specimen shown in Pl. 2, fig. 12, osteoclasts can be found at intervals over the whole extent of this region of thin bone but only on the surface nearest the vitamin. The thin bone itself with its rather homogeneous pale-staining matrix and living osteocytes, is distinctly reminiscent of the new bone found in the resorption areas of parathyroid grafts, but it is more continuous and has a more regular contour. Its structure is shown under higher power in Pl. 2, fig. 14. On the convex, or ectocranial aspect, there are quadrangular osteoblasts of medium size. Immediately opposite the vitamin the bone layer was actually perforated in a few sections. The thick bone has a more complex, laminated structure and contains some empty lacunae in its deeper parts; while it is very probable that part of the thickness is due to newly deposited bone it is not possible to distinguish clearly new and old bone in this specimen.

In the other specimen (Pl. 2, fig. 11), the general structure of the graft is similar; the area of thin bone has become slightly detached from the brain tissue and there is some extravasation of blood, probably due to injury during removal. Osteoclasts appear to be restricted mainly to the region where the thin central bone meets the thicker bone at the edges; the endocranial aspect of the thin bone contrasts, however, with the opposite surface in showing flattened spindle cells and very few large osteoblasts. In the thicker peripheral regions a layer of bone of fairly uniform thick-

ness can be seen lying embedded between two layers which have apparently been deposited on its surface. The condition appears to be essentially similar to that previously described in parathyroid and other tissue grafts, in which the original graft bone, which is often darker staining and may have empty lacunae, is demarcated by cement lines from newer bone laid down on both its surfaces. These



Text-figs. 6–9. Projection drawings of histological sections of grafts. Bone of original graft white, new bone black. Vitamin residue stippled. × 57.

Text-fig. 6. Graft of a single fragment of vitamin A removed after 7 days. Adjacent to the vitamin the original bone is perforated and replaced by spongy new bone. There is also some thickening by new bone deposition at the extreme edges. Compare photomicrographs of this specimen (Pl. 1, fig. 10 and Pl. 2, fig. 13).

Text-fig. 7. Graft of a single fragment of vitamin A removed after 14 days; the central area is composed of new bone and the original bone is found only at the edges. Compare photomicrograph of this specimen (Pl. 2, fig. 11).

Text-fig. 8. Graft of a single fragment of vitamin A removed after 14 days. The vitamin is somewhat displaced towards the skull wound. Opposite it the original bone graft has been perforated but the holes are sealed by a heavy deposit of new bone. The exact distribution of new and old bone on the right-hand side of the figure is difficult to make out in the specimen and is somewhat conjectural in the diagram.

Text-fig. 9. Same specimen as Text-fig. 8 at a different level in the series and showing actual perforation. Note extensive plate of new bone at the extreme left side; this lay in the scar tissue of the insertion hole.

relationships are shown more clearly in Text-fig. 7 which is made from a projection drawing of the same specimen. It will be noticed that the layer which is taken to represent the original graft is sharply discontinuous and is in fact perforated, but that the hole is bridged by the thin bone continuous with the new bone layer on the convex aspect. In the two specimens in which the vitamin was displaced towards the surface edge of the graft there is a considerable perforation in the adjacent region,

and the gap is bridged by fibroblasts. In one of these specimens the perforation, which is shown in Text-fig. 9, lies to one side of the chemical implant, opposite which the bone is continuous, but lined with osteoclasts. It can be clearly seen that much of the original graft is here perforated and that a thick layer of new bone which is also being resorbed, closes the gaps (Text-fig. 8). At the deep end of the graft new and old bone are not easily distinguishable; this is probably due to the fact that the original graft bone may have had a somewhat spongy structure owing to the inclusion of part of the lateral area of the parietal bone. In the fifth specimen the bone was continuous but, somewhat to one side of the vitamin residue, there was a zone of osteoclastic resorption forming a concavity; it is probable, that the bone in this region was also largely a new formation. The sixth specimen showed a few osteoclasts near the vitamin, but otherwise no evidence of resorption, and the reasons for this negative result are not apparent. If one attempts to link up these histological findings with the observations on macerated material, it seems probable that the two specimens with extensive areas of thinning correspond to those macerated bones showing areas of homogeneous thin bone, but nothing obviously corresponding to the sieve-like bone has been observed. One of the 7-day specimens throws some light on this matter, however.

Seven-day grafts

The specimen is illustrated in Pl. 1, fig. 10 and Pl. 2, fig. 13, and the structure is illustrated diagrammatically in Text-fig. 6. Opposite the angular vitamin fragment, and extending beyond it on one side, there is a length of bone which is spongy in structure, slightly paler staining, and contains large rounded osteocytes. This layer is continuous with a new layer of bone on the convex surface of the original graft bone, which can be distinguished at either side, and in which the lacunae are mainly empty. Osteoclasts are to be seen on the surface of the bone nearest the chemical implant and extending some distance on either side of it. In this specimen perforation and closure are therefore quite clear, and the new bone has a structure which may correspond to the sieve-like bone of macerated material. The series of the other 7-day specimen was rather incomplete; the graft was uniform in thickness with a thin layer of new bone and active osteoblasts on its ectocranial surface but only a few osteoclasts near the vitamin as evidence of a local response.

Chemical changes in the vitamin implant

Although the acetate is described as a comparatively stable ester of vitamin A when kept in air at 0° C. (Baxter & Robeson, 1942), it might well undergo oxidative destruction when implanted in the tissues at much higher temperatures. In some of the histological sections the vitamin residue had an angular form, but in others it was more rounded, and this, together with the proximity of numerous macrophages with vacuolated cytoplasm and occasional polymorphs suggested that the vitamin might be undergoing some decomposition or change of physical state. It was also noticed that when the grafts were removed the vitamin residue was opaque and that if it was mounted under a cover-slip it easily disintegrated into a fine granular material. Sometimes a core of translucent substance, like the originally implanted fragment, remained at the centre of such a granular mass. Dr S. P. Datta and Mr B. G. Overell

kindly examined a number of these residues by the method of paper chromatography for vitamin A and its derivatives which Datta, Overell & Stack-Dunne (1949) have described. The implants in these cases were of powdered vitamin made into pellets with fibrin so that the fine state of division may have favoured decomposition. While chromatographs of the material from which the implanted pellets had been made gave a strong spot for A-ester together with a trace of A-alcohol and of some unidentified derivative, no trace of vitamin A or its derivatives could be observed by this procedure in residues from 14-day grafts. Direct colorimetric estimation in solution using the Carr-Price method was therefore undertaken on pellets after 14 days in graft and also on some of comparable size which had not been grafted. The results are shown below:

Fresh pellets (i.u.)	Grafted pellets (i.u.)
(1) 19·0	(1), (2), (3) Pooled 2·0
(2) 26·0	
(3) 16·5	
(4) 22·5	(4) 1·0
	(5) 0·5

It is clear that there is very little Carr-Price chromagen left in the grafted pellets, the amount present being close to the limits of sensitivity of the method.

Grafts of β-carotene

Before discussing the results as a whole, seven macerated specimens of 14-day grafts of β-carotene must be described briefly. Since β-carotene is a substance chemically allied to vitamin A, and, indeed, is known to act as a precursor or provitamin from which vitamin A can be formed in the body, it was of some interest to examine its local action on bone. The small crystals were cemented together with fibrin for implantation and the grafts were performed on C 57 animals. On removal, the clumps of deep red crystals were easily detected lying near the centre of the bones and their sharp-edged form suggested that they had not dissolved appreciably. A few osteoclasts and scattered irregular patches of osteoblasts were detected with neutral-red, but their distribution had no obvious relation to the site of the carotene implants. The macerated bones (Pl. 1, fig. 3) showed a variable amount of irregular thickening. Similar bone structure and cell distributions are found in grafts carrying no chemical implant but simply treated by clotting fibrin on the endocranial surface (Pl. 1, fig. 2). Five grafts of this kind were performed and left for 14 days, and three for 26 days. In the latter the contrast between thick and thin areas was more pronounced. The sparsity of osteoclasts on β-carotene grafts and on those without chemical implant suggests that the relatively thin areas in such grafts are not a result of resorption but are, in the main, areas of the original graft unthickened by secondary deposition. By comparing plots of the supravitally stained cells with the macerated material it can also be seen that osteoblasts generally occur at the junctions between thick and thin areas, suggesting that in the thickest parts deposition has slowed down or ceased. One can safely conclude that β-carotene produced no local resorption or other significant change in the bone. It may be that too little dissolved to give any reaction, or it may be that β-carotene itself is in fact inactive in this respect and that insufficient conversion to vitamin A occurs in the tissues, under these conditions, to produce local resorption.

DISCUSSION

The work presented here makes it clear that vitamin A acetate is capable of producing active local bone resorption if it is brought into close contact with the surface of young bone. This result seems, in general, to be consistent with reports of experiments on the whole animal, which show that bone overgrowth is the major result of vitamin A deficiency and that resorption and weakening of the skeleton occur if the vitamin is given in excess. There is no good reason to think that in the graft experiments resorption results from other than a local action since the host animals showed none of the signs of general hypervitaminosis such as a failure to grow, unkempt hair, or inactivity; nor did there appear to be a generalized skeletal resorption in the host and the resorption of the graft was confined to the neighbourhood of the vitamin. It can in fact be calculated from the analyses of the vitamin content of the implants, that even if the whole implant were given systemically as a single dose and were completely absorbed it would still be only about one-tenth of the dose required to produce hypervitaminosis, assuming the body weight/dose relationship to be of the same order as it is in young rats. It is still not possible, however, to determine the exact locus of action of the vitamin in these experiments since the graft is a fairly complex structure which includes other tissue elements, besides the osteogenic cells. There is some evidence, derived from the writer's observations on parathyroid grafts (1948*a*) that, during the first few days after grafting, the osteoblasts on the surface of a graft become a disorganized layer of spindle-cells and that any osteoclasts which may have been present disappear. One finds very few osteoclasts on grafts of bone without chemical implants, or on those implanted with certain inactive substances, and it has been demonstrated here that in grafts removed after 7 days they are restricted to the region of the vitamin, and that after 14 days their number has increased considerably. The vitamin therefore seems to stimulate the differentiation and multiplication of these cells, and perhaps also their functional activity. Whether it does so by acting directly on them or on their precursors, which seems the most likely mechanism, or by some indirect means such as the control of the local vascular supply, one cannot say. Certainly resorption does not seem to start until a vascular supply is established, but this may simply mean that the response of the cells or the ability of the vitamin to reach them is limited by this factor. It is also important to notice that osteoblastic activity in the resorption areas is by no means suppressed, although on the actual surface where osteoclasts are numerous, active osteoblasts are seldom found. Irving's (1949) hypothesis, that the vitamin acts by suppressing osteoblastic activity, while leaving osteoclastic resorption unchanged but uncompensated, cannot apply here, since osteoclasts appear to be formed *de novo* in the vicinity of the implant, and to resorb the bone in its immediate neighbourhood, while simultaneous osteoblastic activity at the periphery and on the opposite surface of the bone may be considerable.

It would be helpful to know the actual local dosage of vitamin A in the graft experiments, but for various reasons no useful estimate can be made. There is not enough known about the solubility of the vitamin when present in the solid form, about the properties of the surrounding solvent medium, nor about the rate of the removal of the dissolved material from the site of action either by decomposition or by replacement of the solvent. In addition to all this the evidence that the ester is

progressively destroyed raises the further question whether it is in fact the ester or some derivative which stimulates resorption. According to Clausen, McCoord, Baum, Steadman, Rydeen & Breese (1940) the normal blood vitamin A in man is in the form of the alcohol, and Glover, Goodwin & Morton (1947) have presented evidence that the blood vitamin level in rats is more closely correlated with the level of A alcohol in the liver than with the level of the esterified vitamin in that organ, which suggests that the latter is a storage compound. Although the blood vitamin A may be very much increased in hypervitaminotic rats, as Walker, Eylenburg & Moore (1947) and other workers have shown, it is not known whether this rise is due to the ester or to some other form. The investigation of other vitamin A compounds by the present technique would be of some interest.

Turning to the facts disclosed by Mellanby's work, it is apparent that the situation in A deficiency is not simple. Osteoclasts are by no means absent, but they occur in unusual situations, and the precise character of the defects is not the same in different regions of the skeleton. In experiments on the whole animal either vitamin deficiency or excess is likely to lead to changes in tissues other than the skeleton, and even if the effects on the bones are to some extent direct ones, they are liable to occur in an environment which has been rendered abnormal in various other respects. Since, according to the commonly accepted view, osteoclasts and osteoblasts perform complementary functions, which in normal growth are somehow spatially co-ordinated, it would not be surprising if vitamin A and also other essential substances affected both cell types but in an opposite sense. The precise result of deprivation or excess of the substance in any particular case might then be expected to depend on such factors as its local concentration at the bone surface, and the degree of activity of the two cell types at that particular region of the skeleton and at that particular developmental period, and this would at the same time be influenced by various other factors on which the metabolic activity of bone tissue also depends. It is possible that some of Mellanby's observations might be unified by postulating that deprivation of vitamin A affects the response of the bone tissue to mechanical pressure, so that in some areas resorption no longer results from a given degree of pressure exerted by adjacent organs, while in others the response is reversed and deposition of bone occurs. The continual rise of pressure due to this defect might explain why such an abnormally great outburst of osteoclastic activity occurs when the vitamin is again supplied. It may be pointed out, in conclusion, that even if vitamin A deficiency or excess leads to changes in both osteoblastic and osteoclastic activity by a predominantly local action, various possible mechanisms might be suggested between which one cannot decide; it might act on each of the two cell types, or on only one of them, the correlated response of the other being mediated by some essentially independent mechanism, or again it might act mainly on some common precursor cell causing differentiation in one or other direction. Our knowledge of the mode of origin and fate of osteoclasts is not at present sufficient, however, to provide a secure basis for further speculations.

SUMMARY

1. Fragments of crystalline vitamin A acetate were attached to small pieces of parietal bone cut from 10-day-old mice, and the combination inserted into the cerebral hemisphere of litter-mates.

2. The grafts were removed after 7 or 14 days and were examined either by supravital staining with neutral-red followed by maceration or as histological sections.

3. Well-marked resorption, accompanied by numerous osteoclasts, and often leading to perforation of the bone was apparent after 14 days.

4. The significance of the results is discussed in relation to existing knowledge of the skeletal effects of vitamin A deficiency and of overdosage with this vitamin.

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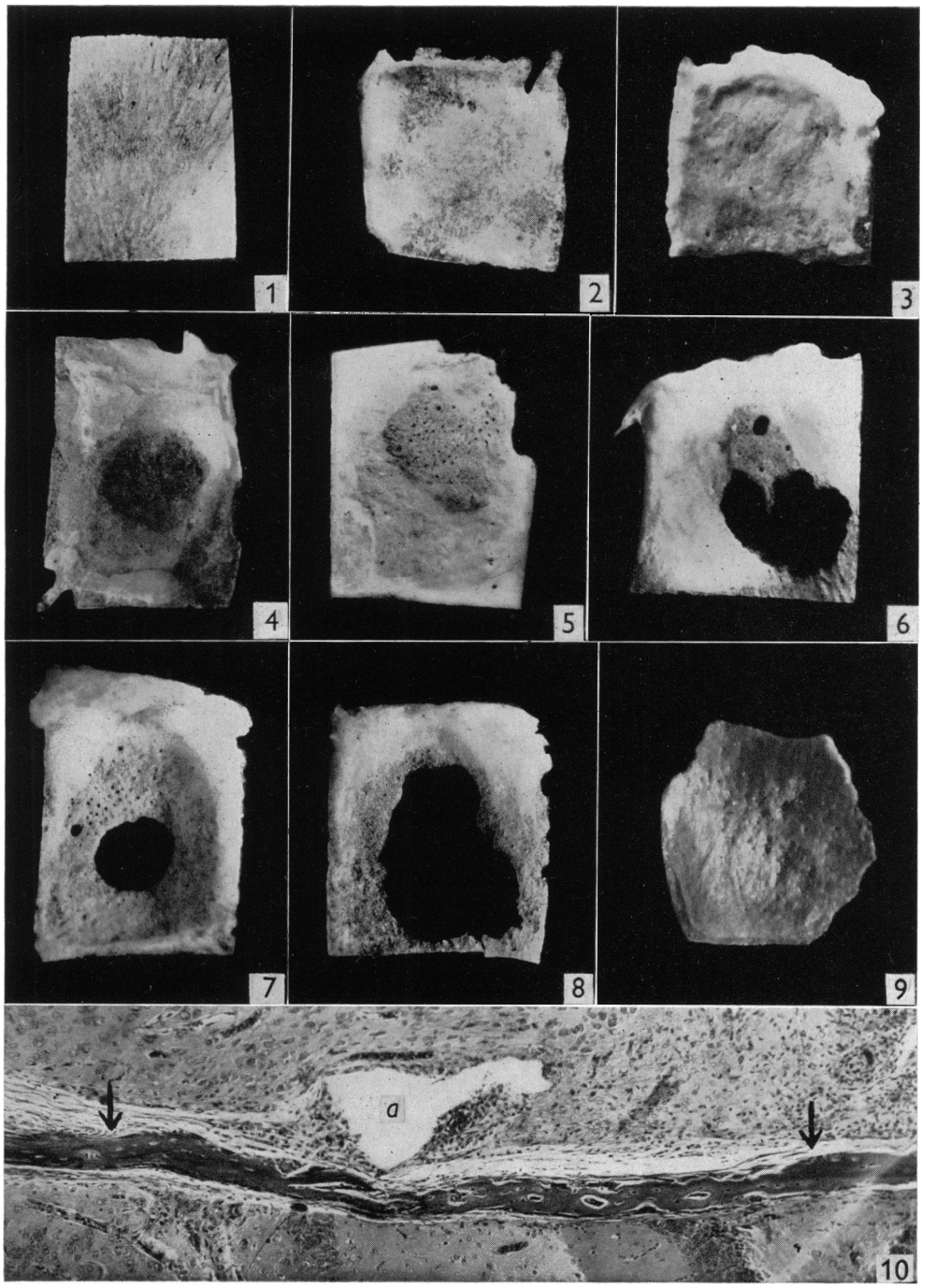

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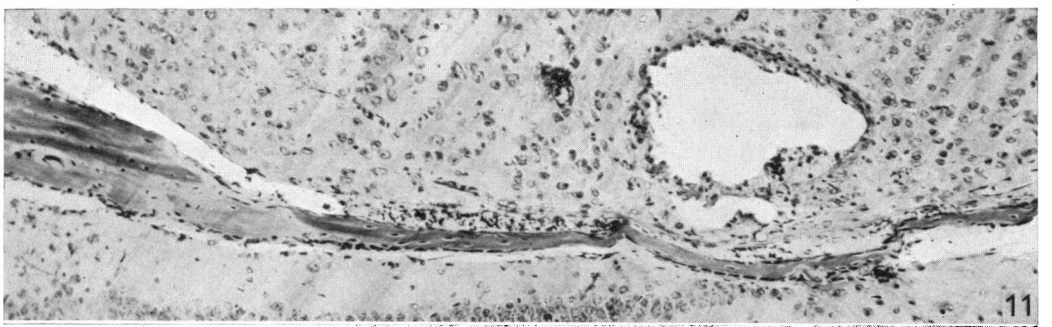
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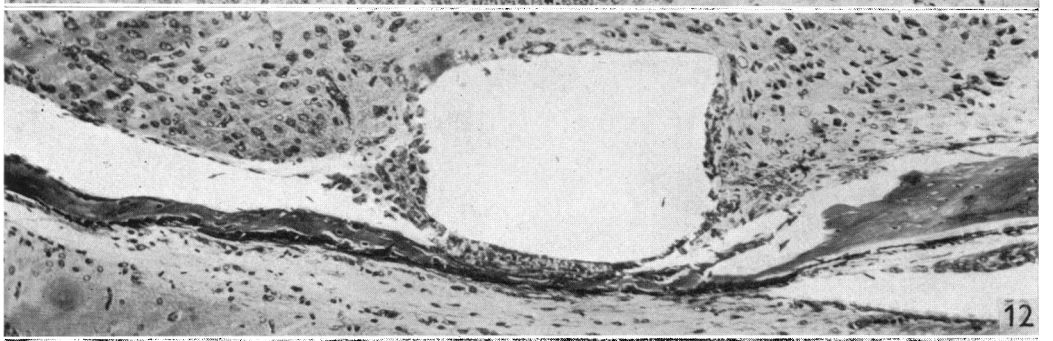
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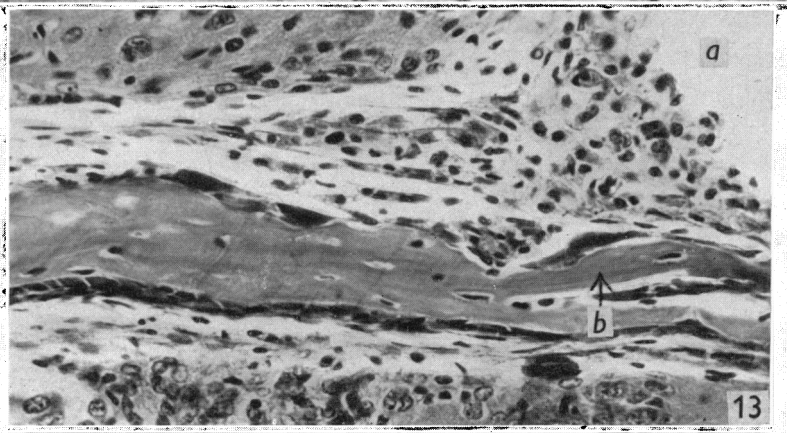
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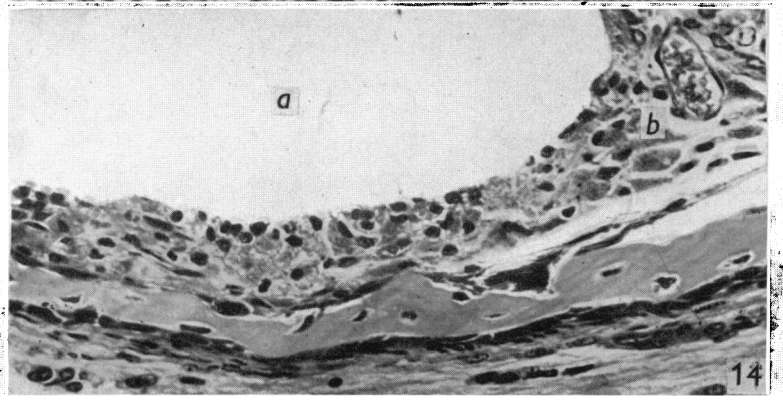
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EXPLANATION OF PLATES

PLATE 1

Figs. 1–9. Macerated grafts showing, in the majority of cases, the structure of the endocranial surface on which the chemical implant lay. The edge nearest the skull-wound is uppermost. × 16.

Fig. 1. Ungrafted piece of parietal bone cut from the antero-lateral region of a 10-day-old animal.

Fig. 2. No chemical implant; graft removed after 14 days. Irregular thickening of the surface.

Fig. 3. β-Carotene pellet; graft removed after 14 days. Irregular thickening of the surface.

Fig. 4. Powdered vitamin A + cholesterol; removed after 14 days. Slightly sunken area of thin bone with a few perforations in its central region; thickening of the peripheral region of the graft with the original thickness of the marginal bone showing in some places. Compare plot of osteoclasts, Text-fig. 2.

Fig. 5. Powdered vitamin A + cholesterol; removed after 14 days. Slightly sunken, rounded area of thin, sieve-like bone showing fine canals leading from the foramina. Compare plot of osteoclasts on this bone, Text-fig. 3.

Fig. 6. Vitamin A, single fragment; removed after 14 days. The convex ectocranial surface is seen in this specimen. A large single perforation with a tongue of thin bone remaining at one side.

Fig. 7. Powdered vitamin A + oestradiol; removed after 14 days. There is a large area of sieve-like bone perforated in the centre; the upper edge is very much thickened. Compare plot of osteoclasts on this bone, Text-fig. 4.

Fig. 8. Vitamin A + cholesterol; removed after 14 days. There is a large perforation with a margin of thin bone. Compare plot of osteoclasts on this specimen, Text-fig. 5.

Fig. 9. Vitamin A, single fragment; removed after 14 days. The edges of the specimen have been broken after maceration and the bone is considerably distorted by curling. In the central region are several small areas of commencing resorption. Compare the plot of osteoclasts, Text-fig. 1.

Fig. 10. Section of a graft of a single fragment of vitamin A, removed after 7 days. The central region opposite the vitamin (*a*) is composed of spongy bone which is distinct from the bone at the two sides. Osteoclasts are numerous on the surface nearest the vitamin between the points marked by arrows. Compare the projection drawing of this specimen, Text-fig. 6. × 125.

PLATE 2

Fig. 11. Section of a graft of a single fragment of vitamin A, removed after 14 days. On the left side the bone is thickened by new deposit. The central region on either side of the vitamin is formed of thin bone. The vitamin residue consists of two pieces, both rounded in form. Compare the projection drawing of this specimen, Text-fig. 7. × 125.

Fig. 12. Section of a graft of a single fragment of vitamin A, removed after 14 days. Opposite the vitamin residue and for some distance on the left-hand side of it the bone is thin and is lined by numerous osteoclasts on the surface nearest the vitamin. × 125.

Fig. 13. High-power field of the bone adjacent to the vitamin implant (*a*) in Pl. 1, fig. 10. Near the vitamin are macrophages. The bone of the original graft is clearly distinguishable at (*b*) and is being attacked by an osteoclast. It is faintly demarcated by a cement line from a layer of new bone on its lower surface. × 210.

Fig. 14. High-power field of the bone adjacent to the vitamin implant (*a*) in the same graft as is shown in Pl. 2, fig. 12, but from an adjacent section. The space left by the vitamin fragment is surrounded by foamy macrophages and the nearby vessels are somewhat congested (*b*). The bone layer shows numerous osteoclasts on the surface nearest the vitamin; it contains living osteocytes and has deeply staining osteoblasts on its lower surface. × 210.