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THE EFFECT OF GRADED DOSES OF VITAMIN C UPON THE REGENERATION OF BONE IN GUINEA-PIGS ON A SCORBUTIC DIET

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Both experimental and clinical observations [Höjer, 1923; Aschoff & Koch, 1919] have indicated that in scurvy it was the bony tissue which showed the most constant and characteristic lesions. Typical changes were the loosening of the attachments of the periosteum to the bone, the appearance of subperiosteal haemorrhages which often extended considerable distances along the shaft, and a thinning of the cortices of the long bones and of the bony trabeculae. In addition, the marrow was found to lose its red colour and to become pale and gelatinous in appearance.

The earliest worker to investigate experimentally the effect of scurvy on the regeneration of bone was Shinya [1922], who showed that a graft of bone from a scorbutic to a normal guinea-pig would not 'take' and that conversely the transplantation of a sound bone into a scorbutic animal was ineffective; although the early death of the animals from scurvy made prolonged investigation of the latter experiment impossible. Subsequent investigators found in scorbutic guinea-pigs delay in the formation of new bone in fracture calluses [Ferraris & Lewi, 1923; Israel & Frankel, 1926; Hanke, 1935; Hertz, 1936; Lexer, 1939] and in saw cuts in the cranial bones [Watanabe, 1924; Schilozew, 1928].

Halàsz & Marx [1932] have demonstrated that if vitamin C is given to guinea-pigs in amounts exceeding the normal requirement there is no acceleration of callus formation. On the other hand, Hanke [1935] and Giangrasso [1939] claimed that in rabbits, extra vitamin C did accelerate the healing of fractures. Bourne [1942], using what was probably a more accurate technique, was unable to show that extra vitamin C had any effect in accelerating the regeneration of bone in rats (which like rabbits synthesize their own vitamin C and can be presumed therefore to be saturated with it).

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Although some of the workers mentioned have used mild or severe scurvy in their experiments none appear to have made any attempt to find the critical value for the amount of vitamin C required to promote optimum regeneration of bone. This is the object of the present work.

Methods

Twenty-eight young male guinea-pigs of approximately the same age and weight were placed on a scorbutic diet. The diet was composed of a daily mash of crushed oats, wholemeal flour, cod-liver oil and water, and of a hard cake which was provided in excess. The cake contained 'weatings', ground wheat, ground oats, ground barley, ground maize, meat and bone meal, dried skim milk, salt, dried yeast, fish meal and cod-liver oil. The animals were given varied doses of vitamin C by subcutaneous injection each day of the amounts shown below:

5 received 0·25 mg.
4 received 0·50 mg.
5 received 1·00 mg.
5 received 2·00 mg.
4 received 4·00 mg.

At the end of 1 week on this diet a hole, 1 mm. in diameter, was bored aseptically in both femora of each guinea-pig (for details see Bourne, 1942). Standard holes of this size provided exactly comparable areas for regeneration in each animal. After 7 days the animals were killed and the femora fixed, decalcified, sectioned and stained with haematoxylin and van Gieson.

Results

Animals receiving no vitamin C

There is considerable variation in the histological picture between the femora of various animals in this group and in some cases between the right and left femora of the one animal. This latter finding indicates that local factors may play as important a part, under certain circumstances, as systemic factors. One of the first normal responses to bone injury is usually a multiplication of cells in what is called the 'cambial layer' of the periosteum, that is, the inner layer of the periosteum near the bone. Even this reaction was absent in some of the femora of this group. Occurring more or less simultaneously with the cambial reaction is the aggregation of fibroblasts in the clot and the beginnings of organization, i.e. the production of fibres, presumably by the activity of the fibroblasts, which, according to Danielli [1942], may spin such fibres from a layer of protein adsorbed on to their surfaces. Fibroblasts are present in the clot in most of the femora but in some they appear to have produced no fibres. The clot was brittle and in some sections had crumbled, presumably because of the treatment it had received in sectioning process. In most of the femora, however, the clot was filled with colourless pre-collagen fibres. In some, a few of these fibres stained a faint pink with van Gieson, indicating that they had changed to mature collagen. In others there were aggregations of structureless material which stained faintly or not at all and probably represent swollen, oedematous masses of pre-collagen. In other femora there was developed in the fibrous clot a number of small islands of van Gieson staining material. In about half the femora examined there was no periosteal reaction and in none was there any reaction on the part of the endosteum. This is of

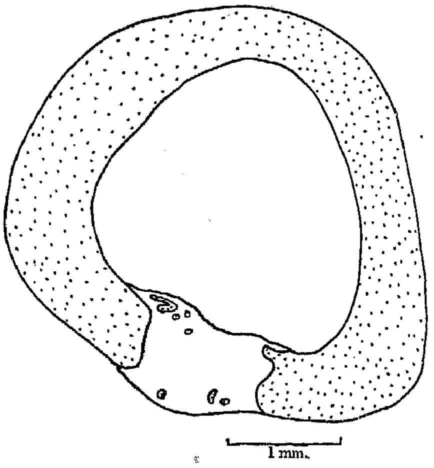

Fig. 1.

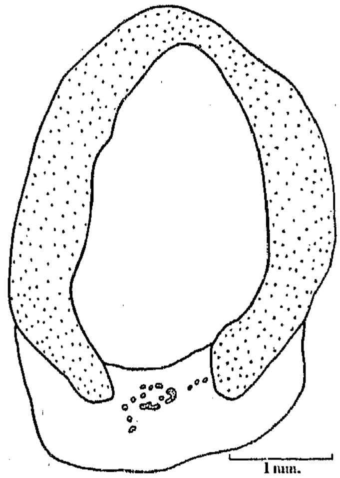

Fig. 2.

Fig. 1. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with no supplement of vitamin C.

Fig. 2. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet supplemented with 0·25 mg. vitamin C per day.

For lettering see Fig. 6.

interest because in those animals which have adequate supplements of vitamin C the endosteum produces a number of trabeculae which link up with those forming in the inner part of the hole. In some femora small chips of what appeared to be dead bone, and which had lodged in the hole as a result of the operation, showed no sign of resorption. All the cells present in the holes appeared to be fibroblasts, there was no sign of osteoblasts.

Animals receiving 0·25 *mg. vitamin C*

The most noteworthy effect of the vitamin C in this series was that in every femur except one there was a distinct reaction on the part of the cambial layer of the periosteum, but there was no formation of trabeculae between it and the uninjured bone as occurs in normal periosteal reactions. None of the femora showed any endosteal reaction. There was, as in the previous group, one femur in which there was no reaction at all to the injury. The marrow had oozed through the hole and there was no accumulation of fibroblasts and fibres in the region of the hole.

Animals receiving 0·5 mg. vitamin C

In all the femora examined in this group there was a distinct reaction on the part of the cambial layer. Although there was multiplication of the cells in this region there was no formation of trabeculae between the bone and the periosteum. Four out of seven femora examined showed a considerable reaction on the part of the periosteum, which was particularly intense near the hole.

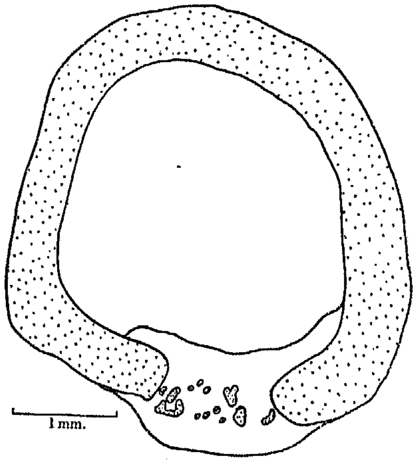

Fig. 3.

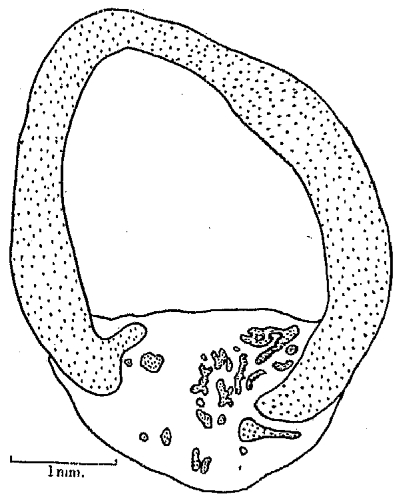

Fig. 4.

Fig. 3. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 0·5 mg. vitamin C per day.

Fig. 4. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 1·0 mg. vitamin C per day.

For lettering see Fig. 6.

In four femora there were the beginnings of an endosteal reaction, i.e. a few small trabeculae which appeared to be formed from the endosteum near the margin of the hole. Most femora contained a number of fibres in the hole, but while in one femur of one animal there was a mass of dense fibres, in the other femur the hole contained a number of trabeculae. A number of osteoblast-like cells were present in the hole in most femora and some capillaries were present.

Animals receiving 1 mg. vitamin C

In all the eight femora examined in this group there was a considerable reaction on the part of the periosteum and the endosteum. The former showed not only cellular multiplication of the cambial layer but in most cases the formation of bony trabeculae in this layer between the periosteum and the surface of the bone, in other words the osteogenetic power of the periosteum had been retarded up to this point. The endosteum had produced bony trabeculae in most of the femora in the immediate precincts of the hole. The hole itself was in all cases densely packed with fibres which did not stain with van Gieson. In a few of the femora some of these fibres, although not arranged in trabeculae, stained with varying intensity with van Gieson. There were many capillaries in the hole and numerous osteoblasts were present, particularly in regions where trabeculae were forming.

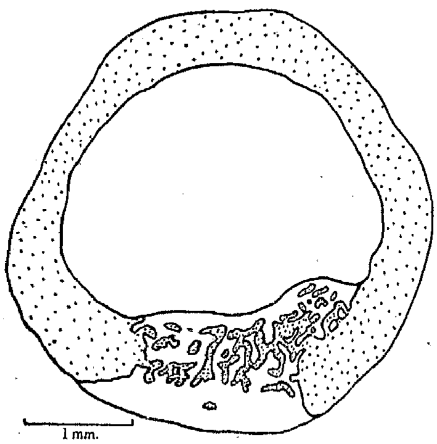

Fig 5.

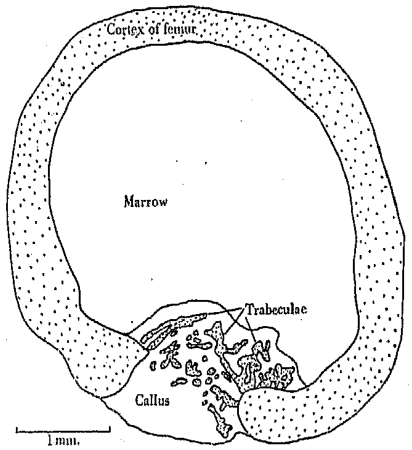

Fig. 6.

Fig. 5. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 2·0 mg. vitamin C per day.

Fig. 6. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 4·0 mg. vitamin C per day.

Animals receiving 2 mg. vitamin C.

In most of the femora of this group the inner part of the hole (near the endosteum) was filled with bony trabeculae which linked with those formed by the endosteum. The outer part of the hole was packed with fibres many of which stained with van Gieson. The periosteum in all members of this group appeared to have attained its full osteogenetic power and to have produced large numbers of thick trabeculae in association with the uninjured bone, but it had not formed many trabeculae in the outer part of the hole. Possibly this stage does not appear in the guinea-pig until later in the healing process. In a rat in most cases such a hole is completely filled with trabeculae at the end of a week. There were many osteoblasts and capillary blood vessels present in the holes of all animals.

Animals receiving 4 mg. vitamin C

In the femora of this group too there was considerable periosteal and endosteal reaction with the formation of bony trabeculae by both. Most of the holes contained numerous fibres, but generally speaking there appeared rather less van Gieson staining material than in the preceding group. This fact is illustrated in figures given later. There were numerous osteoblasts and capillary blood vessels present in the tissue filling the holes in all animals.

Summary of changes

Dose of vitamin C mg.	Periosteal reaction	Endosteal reaction	Fibres in hole	Trabeculae
None	Negative	Negative	A few	None
0·25	Slight multiplication of cells in cambial layer	Negative	Fairly numerous	None
0·50	Considerable multiplication of cells in the cambial layer	Slight reaction. Beginnings of formation of trabeculae	Numerous	None
1·00	Cambial layer forming trabeculae	Small number of trabeculae forming	Very large numbers	A few
2·00	Very large reaction. Enormous multiplication of cambial layer cells. Many trabeculae formed	Extensive formation of trabeculae	Very large numbers	Numerous, particularly in inner part of hole
4·00	Ditto	Ditto	Ditto	Ditto

An attempt was also made to obtain figures which would indicate the degree to which the formation of new bone has occurred in various groups of animals. A technique for this purpose has been described by Bourne [1942]. Briefly it is as follows.

The trabeculae in five random sections taken through the region of the middle of the hole in each femur were projected on to paper and drawn. The weight of a standard rectangle of paper which fitted into the hole was obtained and the trabeculae contained in this rectangle were drawn and subsequently cut out and weighed. The division of the second weight by the first gave a figure called the 'trabecular index'. Since some of the indices obtained in this work were so small it was found necessary for convenience to multiply all indices by 100.

The results obtained by the application of this technique were as follows:

		Trabecular index times 100
5 animals on a scorbutic diet.	No supplement of vitamin C	7·73 ± 2·25
5 ,, ,, ,,	Supplement 0·25 mg. vitamin C	6·70 ± 2·30
4 ,, ,, ,,	,, 0·50 mg. ,,	9·74 ± 2·48
5 ,, ,, ,,	,, 1·00 mg. ,,	19·41 ± 3·53
5 ,, ,, ,,	,, 2·00 mg. ,,	23·73 ± 6·75
4 ,, ,, ,,	,, 4·00 mg. ,,	18·09 ± 4·48

The apparent decrease in the amount of trabeculae formed in the group receiving 0·25 mg. vitamin C compared with that of the animals receiving no vitamin C supplement, is probably spurious. In these two groups, and also in the next group (that receiving 0·50 mg. of vitamin C per animal per day), there was no formation of true trabeculae. Small spots of van Gieson staining material were found and occasionally relatively large areas which appeared to have been formed by the clumping together and fusing of pre-collagen fibres were seen. These masses stained very faintly, and it was difficult to decide whether to include them as trabeculae or not. However, they were included and since there were rather more of them in the completely scorbutic group than in the group receiving 0·25 mg. vitamin C, their inclusion as trabeculae weighted the results towards the former group.

There was a slight increase in the amount of van Gieson staining material present in the next group (0·25 mg. vitamin C).

In the 1 mg. group there was a considerable increase in the van Gieson staining material, and in this group for the first time the isolated islands of van Gieson staining material linked up to form trabeculae. In the 2 mg. group this process was carried a stage further. The drop in the amount of van Gieson staining material in the 4 mg. group may be due to the retarding effect of the higher dose of vitamin C, but it seems more likely to be due to experimental error. In fact it is very difficult satisfactorily to apply the technique of estimation of trabeculae to this work owing to the difficulty in the scorbutic and partially scorbutic animals of deciding what material to include as trabeculae and what not to include; under the circumstances the application of Fisher's '*t*' test for significance to the figures would be of little value.

Discussion

These results show that without adequate vitamin C there is practically a complete inhibition of the reparative processes in a damaged bone. As the dose of vitamin C increases, one reparative function after another comes to life, until, with a daily supplement of 2 mg., regeneration appears to be taking a normal course. The critical dose lies somewhere between 1 and 0·5 mg. per day as can be seen from the list of trabecular indices. There is still further improvement in the histological picture when 2 mg. vitamin C are given per day but no apparent further improvement at the 4 mg. level.

The effects of complete deficiency of vitamin C obtained in this work are similar to those obtained by other workers [e.g. Wolbach & Howe, 1926; Hertz, 1936]. Hertz points out that in a completely scorbutic animal, following bone injury, there is no periosteal hyperaemia, and that in general it is difficult for a scorbutic animal to produce an inflammatory reaction in response to injury, and that, in the absence of such a reaction, the normal processes of healing cannot be introduced. The enlargement of the cambial layer of the periosteum, Hertz believes to be an essential stage of this inflammatory reaction.

Hertz [1936], like Watanabe [1924], noticed a delayed absorption of the haematoma at the site of the fracture in scorbutic animals. The same phenomenon has been observed in this work. In the animals receiving no vitamin C and in those receiving 0·25 mg. of the vitamin, at the end of the experimental period (7 days after injury) the whole of the connective tissue supporting the muscles associated with the femur was permeated with blood which had not been absorbed. The impression given was that the blood had not clotted very readily and that it had continued to ooze from the hole for some time after the operation. Finally, it extended through practically the whole of the soft tissues of the upper part of the leg. The connective tissue was (as has been found by other workers) obviously oedematous and had a soft jelly-like appearance. The animals receiving 0·5 mg. of vitamin C showed a small haematoma at the site of injury, but in the 1·00 mg. group and the 2·0 and the 4·0 mg. group it had disappeared by the time the animals were killed.

This work confirms the results of other workers that there is a delay in the development of collagen fibres and delay in their maturation [e.g. Hunt, 1941] in scurvy.

In view of the present war situation it would be of interest to attempt to interpret the results with guinea-pigs in terms of human requirements. This, however, is no easy task.

Göthlin [1934] has stated that the stage of scurvy in guinea-pigs which is recognizable only by means of microscopic alterations in the teeth is equivalent to that prescorbutic stage in man which is indicated by fragility of the capillaries. He found that such teeth changes could be prevented in the guinea-pig by a minimum dose of 1·33 mg. a day of vitamin C and that 19–27 mg. a day were required to prevent capillary changes in man. Therefore the factor for converting guinea-pig doses of vitamin C to human doses should be approximately 17, and anything less than 17 mg. of vitamin C a day for a human being with a fractured bone would be likely to retard seriously the healing of the fracture and 34 mg. a day would be required to secure the maximum formation of callus.

On the other hand, Kellie & Zilva [1939] state that the daily dose necessary to maintain the condition of saturation in a human being is 30–40 mg., and that a guinea-pig requires for the same purposes about 20 mg. [Zilva, 1936]. These results suggest that a human being needs only twice as much vitamin C as a guinea-pig, and that therefore 4 mg. of the vitamin per day in a human being with an injured bone would be sufficient to induce regenerative changes equivalent to those obtained in the guinea-pig with 2 mg.

But a guinea-pig requires 0·5–1 mg. of vitamin C a day to protect it from macroscopic signs of scurvy, and Fox, Dangerfield & Gottlich [1940] found that a daily intake of 12–25 mg. of vitamin C resulted in the appearance of scurvy in twelve out of 950 mine labourers studied. Apparently then an intake of this order is approaching the scorbutic danger level. The vitamin C demands of mine labourers may be greater than those of persons lying in bed in hospital with a fracture, but even so it seems that anything less than 10 mg. a day in such a patient would be undesirable. If we accept 10 mg. of vitamin C as the possible minimum amount required to prevent macroscopic scurvy in such patients, then we see that it is necessary to multiply a guinea-pig dose by 15–20 in order to obtain the human equivalent. Therefore to secure the same degree of bone healing present in a guinea-pig with 2 mg. of vitamin C a day, human beings should receive about 40 mg., and anything less than 20 mg. would seriously retard the healing process. Fox *et al.* [1940], however, found no evidence that fractures or wounds healed more rapidly in mine labourers on 40 mg. of vitamin C than on those receiving 12–25 mg. per day. But they admit that comparison was difficult owing to the difference in situation and severity of the lesions.

There are many other complications, for example Crandon [see Crandon, Lund & Dill, 1940] found that it took him 169 days to develop macroscopic scurvy on a vitamin C-free diet, Wolbach & Howe [1926] found that when the bones of scorbutic guinea-pigs were injured there was a temporary healing process initiated in the incisor teeth, and Lauber, Nafziger & Bersin [1937] found that a complete fracture of the humerus in rabbits caused an increase in the excretion of vitamin C.

Nevertheless, if we assume that 40 mg. vitamin C a day is needed to secure maximum regeneration of injured bone then the results are in keeping with recent observations on the vitamin C requirement of man [see Smith, 1938; Harris, 1941]. In any case it is desirable that patients with fractured bones should be given at least 40 mg. vitamin C a day as long as there is the slightest doubt about the efficacy of smaller doses.

Summary

1. The effect of the injection of graded doses of vitamin C on the regeneration of bone in guinea-pigs has been investigated by measuring the amount of trabeculae formed in a hole bored in the femur at the end of one week.

2. It has been found that guinea-pigs require 2 mg. of vitamin C a day by injection to secure adequate regeneration of bone and that less than 1 mg. seriously retards regeneration.

3. It is suggested that the corresponding doses to produce the same results in human beings may be 40 mg. and 20 mg. vitamin C.

4. Pure synthetic vitamin C is alone able to promote regeneration of bone in scorbutic guinea-pigs.

I am indebted to Roche Products for a supply of vitamin C for these experiments.

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