

SOME EXPERIMENTS ON THE POSSIBLE RELATIONSHIP BETWEEN VITAMIN C AND CALCIFICATION

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Although it seems well established that adequate amounts of vitamin C are essential for the laying down of the organic matrix of bone, opinions differ as to whether it is also necessary for the production of bone salt.

Salter & Aub [1931] showed that a scorbutic diet prevented deposition of calcium in bones but did not show that vitamin C allowed calcification to take place (because vitamin C was not yet available for experimental purposes). Boyle [1938] and Boyle, Bessey & Howe [1940] state that the process of calcification continues in the teeth of guinea-pigs even when they have been for some time on a scorbutic diet. Wolbach & Bessey [1942] state that it is a generally accepted fact that vitamin C plays no part in the process of calcification. A more detailed review of the literature concerning the effect of scurvy on calcium absorption and metabolism has already been given by Bourne [1942c]. The only reference in the literature to the inhibiting effect of a scorbutic diet on the deposition of calcium in bone appears to be that of Salter & Aub. It was necessary therefore to investigate this problem further.

METHODS AND RESULTS

Forty-seven guinea-pigs were used for this work and the problem was approached in the following five ways:

- (1) Confirmation of the fact that a scorbutic diet inhibits calcification in bones and investigation of whether pure vitamin C added to a scorbutic diet permits calcification to take place normally.
- (2) Investigation of whether the calcification of bony trabeculae which are formed by regenerating bone is affected by scurvy.
- (3) Investigation of the effect of a scorbutic diet on the calcification of the bony trabeculae of costo-chondral junctions.

(4) Investigation of whether substances normally present with vitamin C in citrus fruits, i.e. citrate and vitamin P (citrin), have any effect on bone matrix formation or calcification.

(5) Further confirmation of the fact that alkaline phosphatase activity of bones is reduced in scurvy.

(1) Calcification of bones was demonstrated by Salter & Aub [1931] by the injection of sodium alizarin sulphonate into animals. The rationale of the technique has been explained by Cameron [1930]. When calcification is going on, the newly deposited bone salt is, by this method, stained pink. The less bone salt deposited the less pink colour is produced on the bone.

In the first experiment a dye kindly provided by Prof. J. C. Brash of Edinburgh was used. It was 1:2:5:8-tetrahydroxyanthraquinone (Alizarin Bordeaux). Alizarin itself is dihydroxyanthraquinone. Twelve guinea-pigs were placed on a scorbutic diet (a mash of bran, wholemeal flour, and cod-liver oil with rat cake *ad lib.*; see Bourne [1942*b*]), and were given daily doses of vitamin C by subcutaneous injection, the vitamin being injected to ensure that each animal got its exact dose. Each set of two animals received daily 5, 2.5, 1.25, 0.5 and 0.25 mg. of the vitamin. The remaining two animals received no injections of vitamin C.

A quantity of the above-mentioned dye was added to the mash each day, but the guinea-pigs as a result refused to eat the mash. It was evident also that, even if they were persuaded to eat it, those guinea-pigs which ate more than the others would get more of the dye and would therefore have their bones more intensely stained than those which ate less. Therefore after another 2 days, that is, at the end of the first week, each guinea-pig was given daily, with a pipette, 1 g. of dye suspended in 10 c.c. distilled water. This was continued for a week. The animals were then killed, the femora dissected out, carefully cleaned of muscle and fibre and examined while fresh.

The bones of animals receiving 2.5 and 5 mg. vitamin C stained the best, but the latter were no better than the former. The bones of the animals receiving 1.25, 0.5 and 0.25 mg. stained with equal intensity, but worse than those which received 2.5 and 5.0 mg.: at the same time they were better than the bones of the completely scorbutic animals. This experiment suggested then that vitamin C does play a part in the process of laying down bone salt, but it does not show how far the deposition of bone salt is simply dependent upon the availability of the necessary matrix.

(2*a*) For this experiment three guinea-pigs were placed on a scorbutic diet. In this and subsequent experiments the scorbutic diet was the same as that described by Bourne [1942*b*]. After 13 days a 1 mm. hole was bored aseptically in each femur by a method described by Bourne [1942*a*]. Feeding with dye was begun the day after the operation, i.e. after the animals had been on a scorbutic diet for 2 weeks. Of these animals, one was given 2 mg. vitamin C

daily by injection for the whole of the experiment (3 weeks), another was given 0.5 mg. vitamin C, and the third was given no supplement of the vitamin. One week after the operation the animals were killed, the femora dissected out and examined while fresh. The staining of the tissue filling the hole was best in the animal which received 2 mg. vitamin C. There was slight staining in the animal which had received 0.5 mg. of the vitamin, and there was no staining at all in the hole in the completely scorbutic animal.

(2b) One would expect lack of calcification of the repair tissue in vitamin C deficiency, because calcium salts are not deposited until osteoid trabeculae are formed, and it has been shown by Bourne [1942b] that these trabeculae do not form or are only formed in small amounts in scorbutic animals.

In the experiment now to be described, therefore, the animals were operated on (in this case a hole about 5 mm. in diameter was bored as usual in each femur) while they were still enjoying an ample diet, and immediately after the operation they were placed on a scorbutic diet. It is usually accepted that guinea-pigs take about 7 days to use up their body reserves of vitamin C [Giroud, 1938], and therefore even in a guinea-pig on a completely scorbutic diet there was probably enough vitamin C in the body reserves during the first 7 days to permit the development of osteoid trabeculae, but it was thought that there might be insufficient amounts of the vitamin (if it does play a part in calcification) to permit the deposition of normal amounts of bone salt. Three animals were used for this experiment. All were placed on a scorbutic diet on the day of the operation and were given the following doses of vitamin C each day for the succeeding week: one animal received 8 mg. vitamin C by mouth; one animal received 2 mg. vitamin C by mouth, one animal received no vitamin C.

At the conclusion of the experiment one femur of each animal was macerated in 0.5 % potassium hydroxide for a week to remove the soft tissues. It was then washed, dehydrated, cleared and stored in oil of wintergreen (methyl salicylate). The second femur in each animal was fixed, decalcified, sectioned and stained.

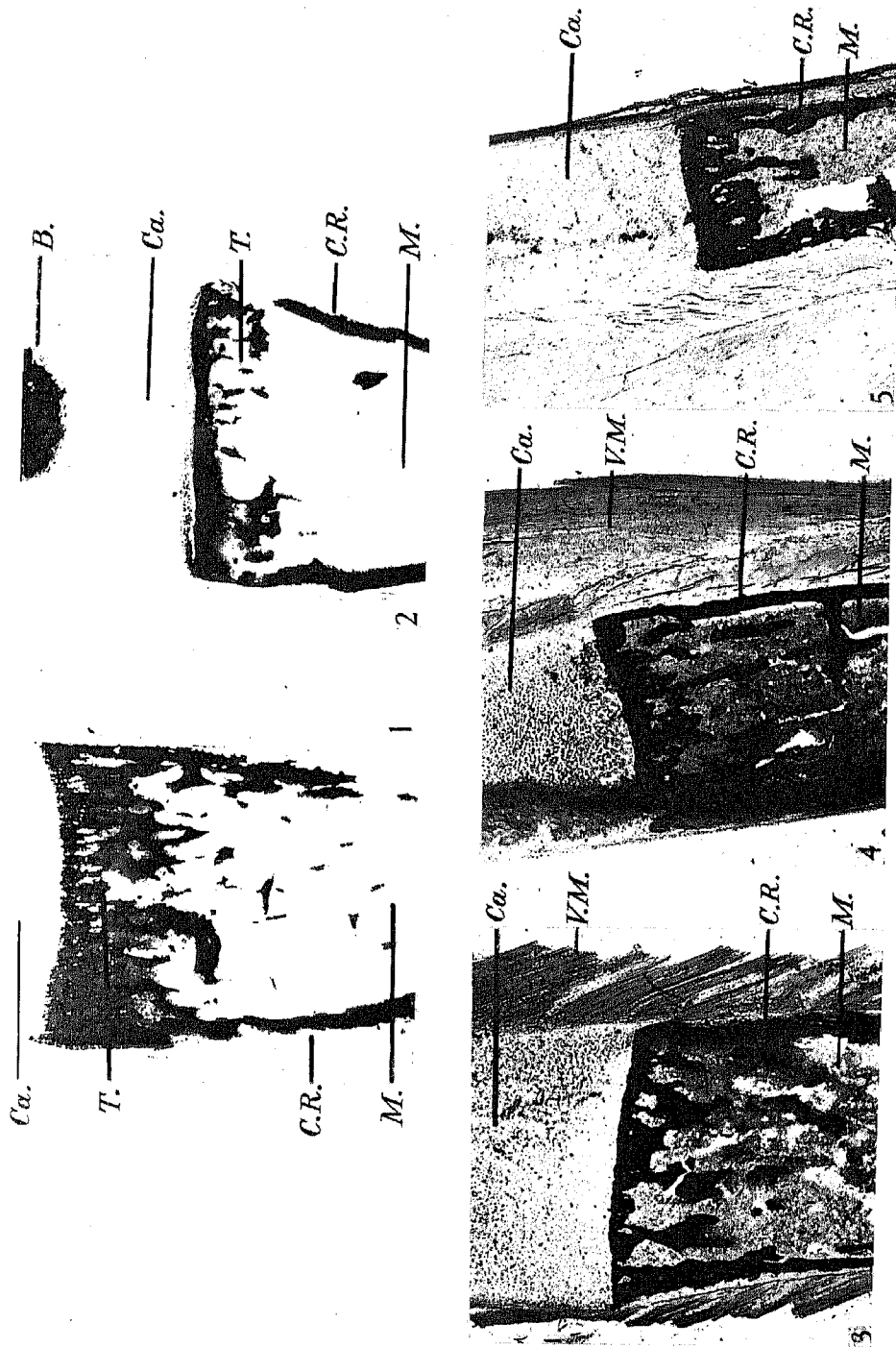
An examination of the sections showed that some van Gieson staining trabeculae were present in the holes in the femora of all three animals, but there were obviously more in the animals receiving 8 and 2 mg. respectively of vitamin C. In the macerated bones of the 8 and 2 mg. animals there was no difference in intensity of staining or in the amount of bony material in the hole. They both had more bone in the hole than the animal which had been on the scorbutic diet without any supplement of vitamin C, and they showed a more intense staining of the bone around the hole than did the scorbutic animal. This latter observation was an indication that more bone salt was in fact being laid down as a result of the vitamin C given to the animals.

(3) A comparison of the hole in the macerated bone with the microscopical sections through the hole of the other femur of the same animal has therefore suggested that all trabeculae which formed were calcified. But the comparison *was* made by using two different femora albeit they came from the same animal. A method was required therefore which would permit one to see in microscopical sections whether a particular trabecula was calcified or not. It was found that pieces of the rib of a guinea-pig, bearing the costo-chondral junction could, after blocking in very hard celloidin, be sectioned without decalcification [Bloom, personal communication]. The sections could then be stained by von Kossa's silver nitrate method for demonstrating bone salt. By this means it is possible to see whether trabeculae are calcified or not.

As a preliminary test the costo-chondral junctions of a guinea-pig which had been on a scorbutic diet for 2 weeks and one which had received as much fresh green grass as it could eat during this period were treated as described above. As can be seen by inspecting Plate 1, figs. 1 and 2, there are many more bony trabeculae formed at the costo-chondral junction of the grass-fed animal than in that of the scorbutic animal. It may be noted, however, that there is an amorphous deposition of bone salt in the scorbutic animal in the region of the junction which is normally occupied by aligned cartilage cells. The cells are still there but they are not aligned and the bone salt appears to be deposited around their peripheries.

Three guinea-pigs were now placed on a scorbutic diet. Two were given supplements of 2 and 8 mg. respectively of vitamin C by mouth; the third animal received no supplement. At the end of 2 weeks costo-chondral junctions from all three animals were treated as above. On examination of the sections it could be seen that the zone of calcified trabeculae at the junction of bone and cartilage was widest in the animal receiving 8 mg. of vitamin C and smallest in the scorbutic animal: the calcified zone in the 2 mg. animal was intermediate between the scorbutic and the 8 mg. animals (see Plate 1, figs. 3-5). Some sections from these ribs were also treated with cobalt chloride and ammonium sulphide after the method of Gomori [1941] for demonstrating freshly deposited calcium phosphate. It was observed by this method that normal bone salt did not stain but that the freshly deposited bone salt at the junction of cartilage and bone did stain. The bone salt itself gradually darkened after the sections had been stained and mounted, but when they were first examined it could be seen that the area of freshly deposited bone salt was greatest in the animal receiving 8 mg. vitamin C and least in the scorbutic animal.

These results appear to suggest that vitamin C is associated with the process of calcification. Yet we should note that even in the scorbutic animals such trabeculae as are formed at the costo-chondral junctions appear to be as heavily calcified as those present in the animals receiving adequate vitamin C, although they are much fewer in number.



Figs. 1-5.



Figs. 6-10.

(4) Of recent years it has been suggested (originally by Armentano, Bentsáth, Béres, Rusznyák & Szent-Györgyi [1936]) that some of the effects of vitamin C deficiency, in particular those resulting in haemorrhage, have been due to a substance closely associated with vitamin C, chemically a flavone derivative. It is called vitamin P. For this investigation a preparation, free of vitamin C but made from a mixture of lemon rind and juice, which contained a mixture of flavones, was used. Many of the earlier workers have used citrus juices to supply vitamin C in their experiments on the effects of scorbutic diets on tissue regeneration. These juices contain an appreciable proportion of citric acid. Bone also contains quantities of the same substance (nearly 2% [Dickens, 1941]). The shells of birds' eggs may contain up to 0.3% citric acid [Thunberg, 1941]. The acid is believed to be present in both bone and shell as calcium citrate. Hathaway & Meyer [1939] have shown that when citric acid is added to a rachitic diet it has a calcifying action. These facts suggest, therefore, that some of the results of earlier workers on bone formation and vitamin C deficiency may be due partly to the citric acid in the juice they used.

A combined experiment was therefore carried out to test both the effect of citrin (vitamin P) and of sodium citrate on calcification and bone formation. At the same time it was decided to give a natural supplement, such as grass, to some animals for comparison.

Twenty guinea-pigs of similar weight were placed on a scorbutic diet for 2 weeks and were given dietary supplements as shown below. At the end of 1 week a hole was bored in both femora of each animal and they were all killed at the end of the next week, after having been injected for the last 3 days with 1 c.c. of a 2% solution of sodium alizarin sulphonate. One femur from each animal was fixed in formalin, decalcified, sectioned and stained. The other femur was macerated in potassium hydroxide, washed and cleared in oil of wintergreen.

The following supplements were given:

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|----------|---------------|---|
| Group 1. | 4 guinea-pigs | received no supplement of vitamin C. |
| 2. | 4 | 50 mg. vitamin C daily by mouth. |
| 3. | 4 | 50 mg. vitamin C daily by mouth + 25 mg. citrin. |
| 4. | 4 | 50 mg. of vitamin C daily by mouth + 0.5 g. sodium citrate. |
| 5. | 4 guinea-pigs | consumed daily a sufficient amount of fresh grass to provide them with 20-30 mg. vitamin C a day. |

Large doses of the various substances were given in order to ensure production of the maximum (if any) effect.

Examination of the sections of the femora showed that there were practically no osteoid trabeculae formed in the hole in the scorbutic animals. Groups 2, 3, 4 and 5 had all formed more trabeculae than the scorbutic group, but there was no detectable difference between the amount of bone formed in them. Examination of the macerated femora showed the same relationships

between the intensity of staining of the femora of the different animals. These results suggest that neither citrin (vitamin P) nor sodium citrate (substances of widely differing chemical nature) has any effect in the formation of the fibrous matrix of bone (no difference in the amount of osteoid formed) or in the deposition of calcium salts.

These conclusions have been subsequently confirmed by a similar experiment in which undecalcified sections of the costo-chondral junctions of six guinea-pigs were stained with von Kossa's silver nitrate method for demonstrating bone salt. Histological examination of holes bored in skulls of the same animals which had been healing for 1 week were also made. No difference in the calcification of the costo-chondral junctions or in the amount of bony trabeculae formed in the holes of the skull could be detected between those animals receiving only vitamin C and those receiving only citrin or sodium citrate.

It is of interest that Hartzell & Stone [1942] have found that vitamin P has no effect on wound healing.

(5) If vitamin C is associated with calcification, then one would expect the alkaline phosphatase activity of bones to decrease with scurvy. It has been shown by Bourne [1943] that in holes bored in the femora of scorbutic guinea-pigs little phosphatase can be demonstrated by Gomori's technique after 1 week's healing. But in holes bored in the femora of normal guinea-pigs, a concentration of phosphatase can be seen in the developing trabeculae by this time. One would not expect much phosphatase to be present in the tissue filling a hole in a bone until the trabeculae begin to develop and vitamin C deficiency, in any case, retards the formation of trabeculae, so the reduced amount of phosphatase in the scorbutic holes may simply be due to their absence. Nevertheless, in most animals the normal periosteum appears to react more strongly to Gomori's technique than does the periosteum of the scorbutic animal [Bourne, 1943]. But it appears that further information on the relationship between vitamin C deficiency and phosphatase activity is needed.

Four guinea-pigs were placed on a scorbutic diet for 3 weeks. Two were given daily supplements of 10 mg. vitamin C, each by mouth. At the conclusion of the experiment, pieces of rib, including the costo-chondral junction, were fixed in absolute alcohol subsequently embedded in celloidin and cut into sections 30μ thick without decalcification. Sections were treated by Gomori's phosphatase technique in which the sites of phosphatase activity were stained black. At the line of junction of bone and cartilage in the normal animals was a thick black band which was continuous with the periosteum. The endosteum of the trabeculae near the junction, the osteocytes and what appear to be the white cells of the bone marrow, all gave an intense black colour indicating that they had a high concentration of phosphatase. In the costo-chondral junctions of the scorbutic animals, the intensity of staining of the periosteum

seemed to be reduced only slightly, but the broad band of phosphatase at the junction was reduced to a narrow line.

Sections of the same bones were also incubated with Gomori's substrate mixture and sodium alizarin sulphonate (technique described by Bourne [1943]). By this method a red-coloured precipitate of calcium phosphate is laid down at the site of phosphatase activity. These preparations gave identical results with those obtained by the previous method.

These experiments suggest that vitamin C is associated in some way with phosphatase activity in the bones. It is of interest to note that while these experiments were in progress Gould & Schwachman [1942] published results obtained by a quantitative investigation which showed that bone phosphatase was reduced in scurvy. More recently, Schwachman & Gould [1942] have shown that there was a reduction of serum phosphatase in guinea-pigs on a scorbutic diet and that the phosphatase level returned to normal on the administration of vitamin C as ascorbic acid.

DISCUSSION

The results described in this paper have suggested that less bone salt is laid down by normal and regenerating bone in scorbutic animals than in those in which pure vitamin C is given. But the deposition of bone salt is an orderly and timed process in normal animals. That is to say, bone salt does not appear to be deposited (except in severe scurvy) until there is an adequate fibrous matrix to receive it. Urist & McLean [1941] have shown that as osteoid trabeculae are in the process of being formed they already have a deposit of bone salt. The production of the fibrous matrix of bone and the deposition of bone salt are therefore probably simultaneous processes. It would seem that as long as there is sufficient vitamin C to produce matrix then that matrix will be calcified. This is supported by the fact that in the costo-chondral junctions mentioned earlier, although the number of trabeculae is reduced in scurvy, those which were present still stained quite intensely with von Kossa's bone-salt method. The apparent failure of long bones to deposit bone salt in scurvy, therefore, may be due to the fact that no matrix has developed for its reception. It has been shown, however [Bourne, 1943], that bone matrix when formed appears to contain phosphatase, and since there is now evidence that vitamin C is associated in bone with the presence of phosphatase, it seems from this point of view that there may be some relation between vitamin C and calcification. The function of vitamin C in bone formation appears to be to facilitate the production, not just of bone matrix, but of bone matrix impregnated with phosphatase. There is no evidence at the moment that vitamin C can be regarded as a coenzyme of phosphatase in calcificatory processes. In fact Schwachmann & Gould [1942] find that there is no activation of serum phosphatase *in vitro* by vitamin C.

The apparent reduction of phosphatase activity in scurvy is therefore probably due to a reduction in the amount of bone matrix produced. Any matrix that is produced even in vitamin C deficiency will apparently have as much phosphatase as the matrix of a normal animal. Therefore it seems that if any matrix is formed at all in vitamin C deficiency it will be as heavily calcified as the matrix formed in normal animals. It is thus impossible to separate the functions of vitamin C as a substance facilitating bone-matrix formation and as a substance facilitating calcification, since these two processes are simultaneous.

SUMMARY

1. It has been shown that the deposition of bone salt in normal and regenerating bone is retarded in scurvy, but that pure synthetic vitamin C permits this process to take place.

2. That it is actually vitamin C and not some associated impurity that is responsible for this is suggested by the fact that the administration of vitamin P (citrin) and sodium citrate did not result in the formation of more osteoid trabeculae or the deposition of more bone salt than vitamin C alone.

3. The amount of phosphatase present in costo-chondral junctions was reduced in scorbutic animals.

4. It appears likely that one of the functions of vitamin C is to allow the production of a phosphatase-impregnated bone matrix upon which bone salt is immediately deposited.

5. Vitamin C may play some part in the formation or stabilization of alkaline phosphatase.

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EXPLANATION OF PLATES 1 AND 2

All figures $\times 20$. Except where indicated all sections are taken through the centre of the costo-chondral junction and parallel with the long axis of the rib.

PLATE 1

- Fig. 1. Undecalcified section of costo-chondral junction of rib of guinea-pig receiving ample diet of greenstuff. Stained von Kossa's method for demonstrating bone salt. Numerous calcified trabeculae may be seen extending from the junction.
- Fig. 2. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet for 2 weeks. Stained von Kossa's method. Number of trabeculae greatly reduced, but the trabeculae which are present stain just as intensely as those in fig. 1.
- Fig. 3. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet for 2 weeks (see Exp. 4). Small number of trabeculae present.
- Fig. 4. Undecalcified section of costo-chondral junction of rib of guinea-pig on a scorbutic diet with a daily supplement of 2 mg. vitamin C. A greater number of trabeculae than in fig. 3 can be seen.
- Fig. 5. Undecalcified section of costo-chondral junction of rib of guinea-pig receiving a scorbutic diet with a supplement of 8 mg. vitamin C. The calcified area at the junction may be seen to be wider than in either figs. 3 or 4. The apparent thinness of this section compared with the two preceding figures is due to the fact that it was cut parallel with the narrow axis of the rib, whereas the latter were cut parallel with the broad axis.

PLATE 2

- Fig. 6. Undecalcified section of costo-chondral junction of guinea-pig on a scorbutic diet for 3 weeks but receiving a daily supplement of 10 mg. vitamin C. The section was treated by Gomori's alkaline phosphatase technique. A broad black band (*A.*) continuous on the right with the periosteum (*P.*) can be seen at the junction. These deep black areas indicate the site of the phosphatase activity. (The periosteum had been removed on the left of the section.) The marrow and the trabeculae also stain deep black, indicating the presence of appreciable amounts of alkaline phosphatase. The dark staining of the cartilage matrix is of no significance, since it is also present in the control (fig. 8).
- Fig. 7. Undecalcified section of costo-chondral junction of guinea-pig receiving a scorbutic diet for 3 weeks. The line of phosphatase at the junction (*A.*) is reduced to a very thin band. The marrow and periosteum and trabeculae still stain intensely. The white area just above the junction is fibrous tissue. This is present because the rib had been fixed in a bent position while out. Cartilage can be seen above it and below it at the junction.
- Fig. 8. Undecalcified section of costo-chondral junction of guinea-pig's rib. Phosphatase control. Passed through Gomori's reagents for demonstrating freshly deposited phosphate but without prior incubation with phosphatase substrate. The cartilage stains black. There is a thin light-staining band of preformed, presumably freshly deposited, phosphate at the junction. The marrow stains very slightly. The endosteal lining of the bony trabeculae and the surfaces of the osteocytes in the trabeculae stain black. It should be noted that true bone has not stained.
- Fig. 9. Undecalcified section of costo-chondral junction of guinea-pig receiving scorbutic diet for 3 weeks with daily supplement of 10 mg. vitamin C. Stained by modification of Gomori's alkaline phosphatase method, described by Bourne [1943]. In this method sodium alizarin

sulphonate is added to the substrate mixture and the calcium phosphate precipitated by the phosphatase activity is therefore red. A broad red band (*A.*) identical in position with the black band of fig. 6 may be seen. Nuclei of cartilage cells have stained and bone marrow is positive.

Fig. 10. Undecalcified section of costo-chondral junction of guinea-pig on a scorbutic diet for 3 weeks. The line of phosphatase is very reduced in size (*A.*) although it stains intensely. The marrow still stains. The staining of the osteocytes is very obvious in this and the preceding figure.

Explanation of lettering: *Ca.* cartilage, *T.* trabeculae, *C.R.* cortex of rib, *M.* marrow, *B.* deposit of bone salt in cartilage, *A.* line of phosphatase (see descriptions of figures), *V.M.* voluntary muscle, *P.* periosteum.