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An experimental study of the effects of growth on the relationship of tendons and ligaments to bone at the site of diaphyseal insertion

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Helsinki, April 1970

Tapio Videman

This article is based on earlier investigations, which have been reported in the following papers:[1)]

I: *Videman, T.* (1970) An experimental study of the effects of growth on the relationship of tendons and ligaments to bone at the site of diaphyseal insertion. Part I: Experiments with ^{35}S-sulphate and oxytetracycline. *Ann. Chir. Gynaec. Fenn. 59*, 9—21.

II: *Videman, T.* (1970 An experimental study of the effects of growth on the relationship of tendons and ligaments to bone at the site of diaphyseal insertion. Part II: Determination of growth patterns and inhibition of displacement using metal markers. *Ann. Chir. Gynaec. Fenn. 59*, 22—34.

III: *Videman, T.* (1970) An experimental study of the effects of growth on the relationship of tendons and ligaments to bone at the site of diaphyseal insertion. Part III: Autoradiographic study with ^{3}H-thymidine of the insertion area of the tendon of the pectineus muscle. *Ann. Chir. Gynaec. Fenn. 59*, 35—41.

1) A preliminary report of the results of the study was presented on October 5, 1967 (A. Langenskiöld & T. Videman).

CONTENTS

INTRODUCTION

During normal longitudinal growth in the metaphysis of a tubular bone, tendinous and ligamentous insertions maintain their positions relative to the increasing size of the bone. This means that the displacement of the site of insertion in regard to the central part of the diaphysis does not depend directly on the longitudinal growth of the metaphysis between the insertion site and the epiphyseal plate (Figs. 1 A and B).

The mechanism of the displacement of the sites of these insertions is the subject of the present study. Displacement is seen most distinctly in such diaphyseal insertions where a clearly demarcated bundles of collagenous fibres penetrate the bone. When a tendon or ligament only inserts itself into the periosteum, this displacement is associated with the relation between the growth mechanism of the periosteum and diaphysis (*Warwick & Wiles* 1934), but this problem is outside the scope of the present study.

The name Sharpey's fibres is often used in studies of the structure of the insertion areas. According to the current definition, Sharpey's fibres anchor tendons and ligaments to bone (*Ham* 1965). *Schneider* (1956) defines the term differently: Sharpey's fibres do not attach tendons to bones, but fix only the periosteum to the bone, while »tendon fibres» (Sehnenfaser) anchor the tendons and ligaments to the bone. The formation of bone at the site of insertion was termed »tendinous osteogenesis» (tendinöse Osteogenese) by *Biermann* (1957) to emphasize that tendinous tissue does not simply calcify, but that tendinous bone is being formed by gradual differentiation.

The displacement of epiphyseal insertion in relation to the diaphysis is a direct consequence of the longitudinal growth in the epiphyseal plate and is therefore not dealt with in the present work. Slight displacement of these insertions in relation to the epiphyseal nucleus takes place and the collagenous fibres remain in the bone during growth *(Schneider* 1956).

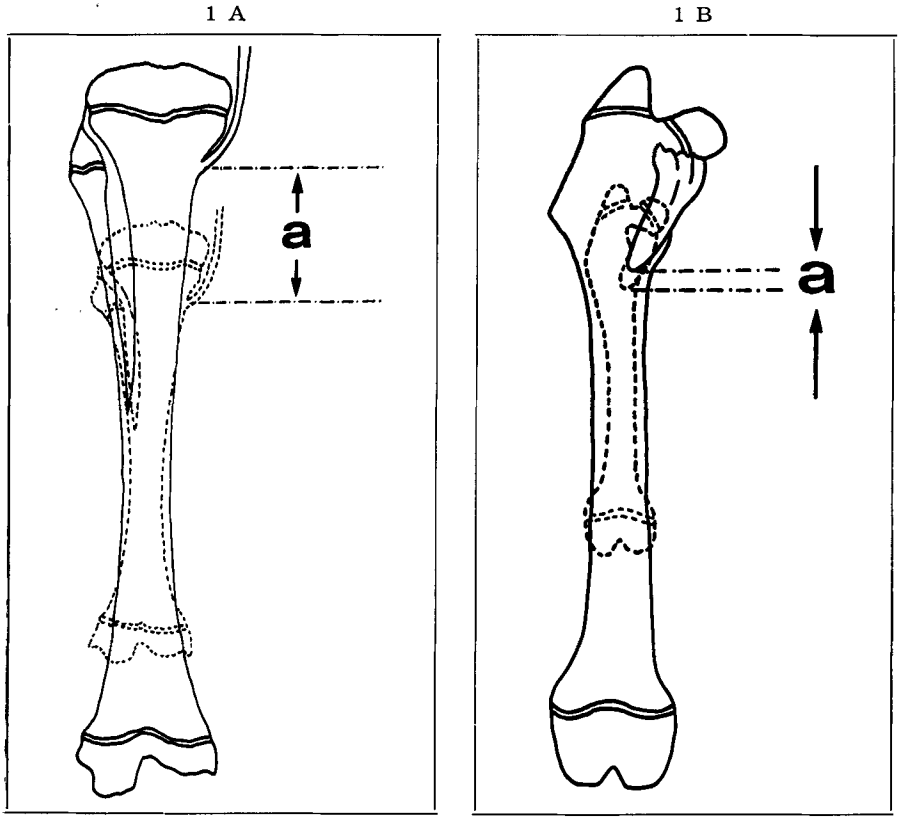

Fig. 1 A: Contour drawings of the rabbit's tibia during two different stages of growth. The tibia grows 57 per cent from the proximal epiphyseal plate and 43 per cent from the distal plate. The shape of the tibia at the younger stage is denoted by the broken line. The contour drawn in full shows a later stage when the length of the tibia had increased by 70 per cent compared to the younger stage. The displacement of the site of insertion of the medial collateral ligament during the period of growth from the first stage to the second is denoted by *a*.

Fig. 1 B: Contour drawings of the femur of a rabbit at two stages of growth. The shape of the femur at the initial observation is denoted by the broken line. *a* denotes the distance which the insertion site of the tendon of the pectineus muscle is displaced in relation to the central part of the diaphysis during growth of the bone from the size at initial observation until the size shown by the fully drawn contour. Note that 30 per cent of the longitudinal growth of the femoral diaphysis takes place at the proximal metaphysis.

The results mentioned above were obtained chiefly by histological studies, and there thus seemed reason to check them by autoradiographic studies. These studies with ^{35}S-sulphate have established that substances of the periosteum are retained in the bone during the growth process (*Amprino* 1955). However, the actual mechanism of the

displacement of the tendon insertions in relation to the central part of the diaphysis during growth (Figs. 1 A and B) has not previously been studied. The growth of the insertion areas can be studied in relation to cells, bone matrix, connective tissue surrounding the tendon and growth disturbances.

^{35}S-sulphate is a suitable medium for autoradiographic studies of the formation of the ground substance of tendons, ligaments, bone matrix and cartilage tissue i.e., all possible tissues at the site of insertion. The exact chemical composition of the radioactive compound in the insertion areas is not known in detail, but the stages of incorporation of ^{35}S-sulphate in the organism are relatively well-known (sulphated glycosamino glycans) *Dziewiatkowski* 1951, *Jackson* 1953, *Engfeldt & Westerborn* 1960, *Mancini et al.* 1961, *Brondolo & Randelli* 1962, *Crane* 1962). In practice, ^{35}S-sulphate can be used to label only the organic matrix of bone, whereas oxytetracycline can be used to label the mineral component of bone *(Ibsen & Urist* 1962). When this antibiotic is administered to growing animals, it is deposited in bone in connection with the ossification process (*Milch et al.* 1957). Because tetracyclines give a yellow fluorescence in ultraviolet light *(Saltzman* 1950), they can be used as bone label.

Another technique for studying the mechanism of growth of tendon and ligament insertions is by disruption of normal growth. Fixation of a metal wire in the metaphysis between the insertion area of a tendon (or ligament) and the epiphyseal plate, inhibits the normal displacement of the site of insertion.

Still another technique of study is based on the fact that cells in the premitotic S-phase incorporate ^{3}H-thymidine and that the labelled daughter cells can then be identified autoradiographically even after several subsequent mitoses.

MATERIAL AND METHODS

Four different methods (see below 1—4) were used for the studies of the growth mechanism at the site of insertion of tendons and ligaments.

1) *^{35}S-sulphate:* A total of 47 rabbits aged from 9—210 days at the time of administration of the labelled substance were used for the autoradiographic studies. ^{35}S-sulphate was given in a single intraperitoneal injection, 1—5 μCi per gram of body weight to each animal. The rabbits were killed 1.2 hours—60 days after the injection. The

symbol »T» in the text and in the figures refers to this time. The insertion of three tendons (the pectineus and the pectoralis major muscles and the patellar ligament) and two ligaments (the crural ligament, which corresponds to the extensor retinaculum in man, and the medial collateral ligament of the knee) were studied. Immediately after the animals were killed, the bones and tendons to be examined were dissected as a unit. These specimens were studied using routine histological and autoradiographic techniques similar to those adopted by *Langenskiöld et al.* (1967). A total of 755 autoradiographs were made from the histological sections.

2) *Oxytetracycline* (Terramycin®, Pfizer) studies were also performed, giving a single injection, 50 mg/kg of body weight, to 15 rabbits aged from 14—28 days. The rabbits were killed within 4—26 days of administration of the antibiotic. The techniques for examination of the bones and for photography were those used by *Puranen* (1966) and *Karaharju* (1967).

3) In the experiments with *implantation of metal markers,* 60 rabbits were used whose ages ranged from 11—40 days at the time of operation. Twenty-nine rabbits into which no metal markers were fixed served as the control group. Indian ink marks were used in the ligaments of some animals instead of or in addition to the metal markers. For both the metal markers and the Indian ink marks, the method employed was essentially that used by *Crawford* (1950 and 1954).

Metal markers were used in order to follow the changes by radiography. The animals were killed 11—221 days after the operation. The tendons and ligaments were carefully dissected and the location of the metal markers was verified. The overall length of the bones (from proximal epiphyseal plate to distal epiphyseal plate $= l_i$) and the distance of the metal markers from one another and from the epiphyseal plate were measured from the radiographs. The measurements of 21 rabbits were treated mathematically and statistically by a digital computer (IBM 1620) at the Computing Centre of the University of Helsinki. Growth curves and specific growth rates were determined mathematically from measurements obtained from the radiographs. The statistical means of these parameters were computed.

After fixation in 10 per cent neutral formalin and decalcification in five per cent nitric acid, the specimens were embedded in paraffin wax, sectioned at 10 μ and stained with Weigert's haematoxylin van Gieson.

4) Twenty-two rabbits whose ages ranged from 7—18 days at the time of administration of the radioisotope were used for the experiments with *^{3}H-thymidine*. Each animal received 1.3—4 μCi per gram of body weight of ^{3}H-thymidine in a single intraperitoneal injection. The animals were killed at intervals of two hours to five days after the thymidine administration. The bone and tendon were dissected as a unit immediately after killing and the tissues were fixed in 10 per cent neutral formalin and decalcified 5—15 days in a 32 per cent solution of EDTA. The specimens were embedded in paraffin wax, sectioned at five μ and cleared in xylol. Autoradiographs were prepared by the stripping film technique (Kodak AR 10). After exposure times of 1—3 months, the films were processed in Kodak D 19b developer and acid fixer and the cells were stained with methylene blue. The insertion areas were photomicrographed (magnification x 100) from these sections and the labelling of every nucleus seen in the photograph was surveyed at x 1000 magnification under a microscope. All labelled nuclei were marked with Indian ink in the photograph because from the photograph at this magnification alone it was not possible to distinguish reliably a non-labelled nucleus from a faintly labelled nucleus. The longitudinal section of the insertion region of the tendon of the pectineus muscle was divided into five areas as shown in Fig. 6. For each area, the labelling index (p) was determined from the formula:

$$p_i = \frac{s_i}{a_i \cdot n_i}, \text{ where}$$

s_i = number of autoradiographically positive cells in area i;
a_i = size of area i (measured by planimeter from the photograph);
n_i = total cells counted in area i (for the determination, the microscopic image was superimposed with 25 points arranged as in the Zeiss Intergrating Eye-Piece I).

RESULTS

1) *Incorporation of ^{35}S-sulphate:* Fig. 2 summarizes the results obtained from the ^{35}S-sulphate autoradiographs of the pectineus tendon insertion area. Two hours after administration of the labelled sulphate, the radioactivity is strongest in the insertion, periosteum, tendon and bone marrow which has not been marked at all in Fig. 2. At the site of insertion the radioactivity does not extend to the bone and there are no distinct differences in labelling intensity among the different areas

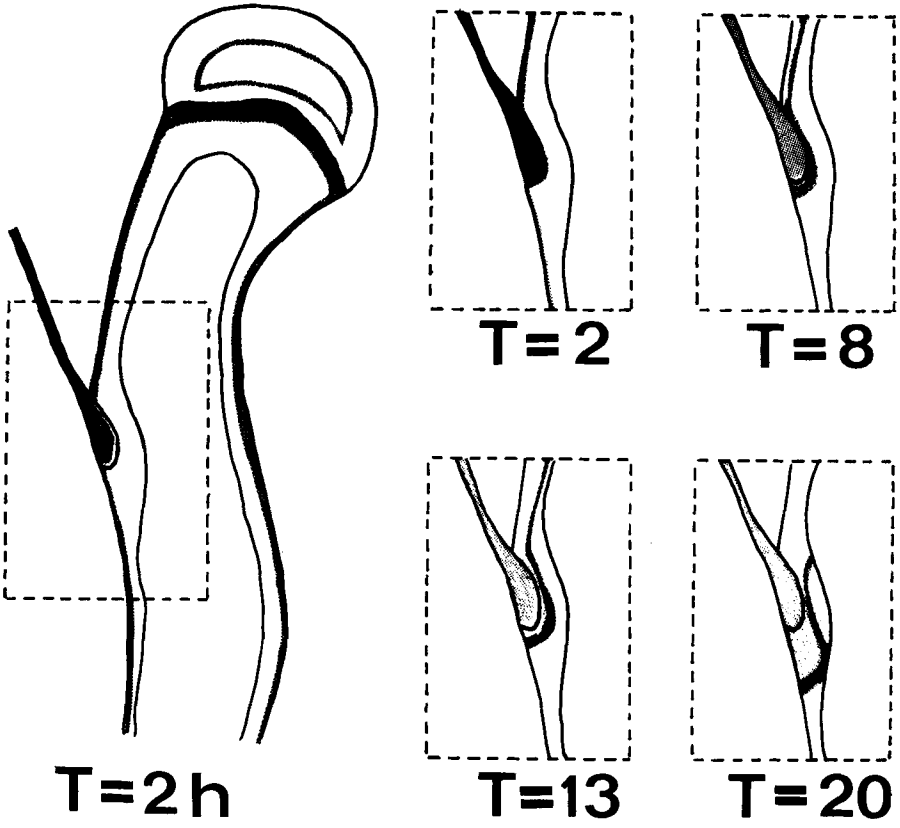

Fig. 2: Schematic summary of the results from autoradiographic studies. Outlines have been drawn for the proximal end of the femur and the tendon of the pectineus muscle with its insertion. Radioactivity is denoted in grey. Two days after the administration of the isotope the bone at the insertion region displays a very narrow radioactive area which remains in the bone. Twenty days after the injection, a radioactive zone is seen in the bone below the tendon-bone boundary of the insertion area. There is a very faintly radioactive area between this zone and the tendon-bone boundary. The exposure times were chosen to show the most radioactive areas as equally dark in all five phases (h = hours, other figures days).

of the region of insertion. However, two days after the administration of the radioisotope an essential change occurred in the localisation of the radioactivity and there was labelling in the bone adjacent to the tendon-bone boundary of the insertion area. Eight days after the administration of ^{35}S-sulphate, a weakly radioactive area could be noted in the tendon-bone boundary. It was clearly distinguished from the more strongly radioactive tendon and bone. The change is obvious when this phase is compared with the preceding phase. Intensity of

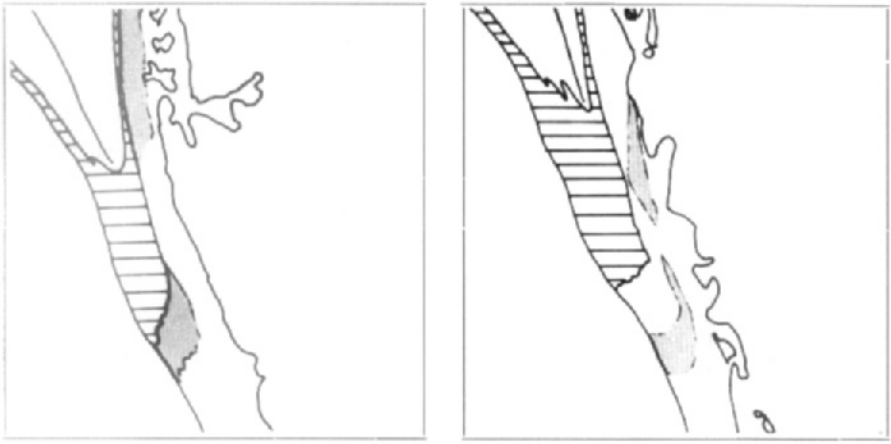

Fig. 3: Diagram of tetracycline studies: The grey shading represents the yellow fluorescence of the distal insertion area of the pectineus muscle of rabbit femur. The animal was given oxytetracycline four days before it was killed (left). In the picture on the right, the rabbit was given antibiotics 25 days before it was killed, and there is non-fluorescing bone between the tendon-bone borderline and the fluorescing bone.

labelling in the photographs of the different phases of this study cannot be directly compared as the exposure times had to be varied in relation to the time from the administration of the labelled substance (cf. *Langenskiöld et al.* 1967).

The changes in labelling pattern in the early stages became more pronounced and, by 13 days after the administration of the isotope, the labelling was most prominent in the bone bordering the tendon insertion. This radioactive zone was farther from the tendon-bone boundary than in the previous phase. The tendon-bone boundary and the bone close to this area were very weakly labelled. Radioactivity in the actual insertion and in the tendon was moderate. At later stages after the administration of ^{35}S-sulphate, the strongly radioactive zone in bone was more distal from the tendon-bone boundary.

2) *Binding of oxytetracycline to the bone:* Fig. 3 is a drawing of longitudinal section made through the insertion of the tendon of the pectineus muscle. Four days after the administration of the antibiotic, fluorescing bone (the grey areas in Fig. 3 represent bone that fluoresces yellow in ultraviolet light) is seen on the distal side of the site of insertion in a narrow zone close to the tendon-bone boundary. Fluorescing bone is also seen subperiosteally on the proximal aspect of the insertion. Three weeks later, the fluorescing zone was still visible, but non-

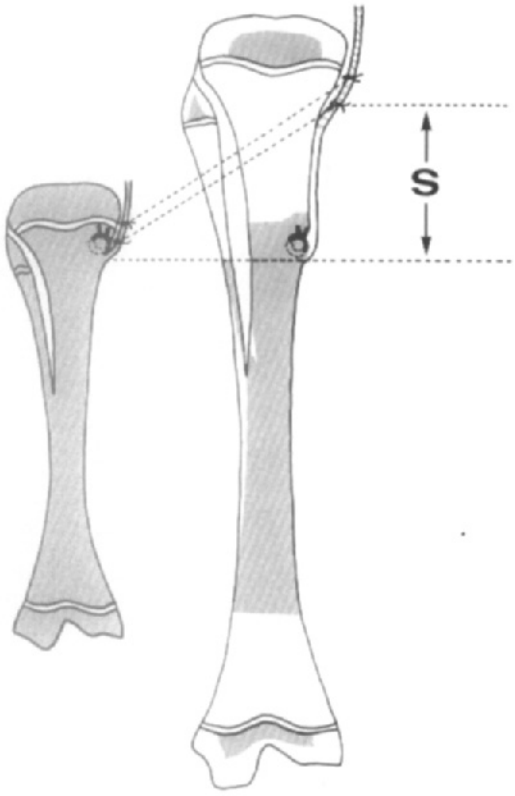

Fig. 4: Contour drawings of the rabbit's tibia during two different stages of growth. At the stage seen on the right it is about 70 per cent larger than at the stage immediately postoperatively seen on the left. The growth of two different segments of the ligament are marked with dotted lines. The insertion segment displaying the greatest relative growth is denoted by *s*. The growth of the rest of the ligament is interstitial, which accounts for the displacement of the metal markers in the ligament downwards in relation to the epiphyseal plate. New tissue formed is indicated by light areas.

fluorescing bone could be observed between it and the connective tissue of the insertion.

3) *Implantation of metal markers:* When a metal wire loop was fixed in the bone between the site of insertion of the medial collateral ligament and the proximal epiphyseal plate of the tibia, the ligament insertion remained at its original site (Fig. 4) and the ligament grew in length corresponding to the length of the new part of the diaphysis formed between the marker and the epiphysis. The histological sections of the medial collateral ligament of the knee (Figs. 5 A and B) showed that the ligament lay on the surface of the periosteum to which it was partially attached. The diameter of the lengthened ligament was uniform from the level of the epihysis to the metal marker and the ligament was attached to the diaphysis just distal to the marker. In six of 51 rabbits, the tendon or the ligament was inserted partially and in three entirely on the epiphyseal side of the metal marker in

5 A 5 B

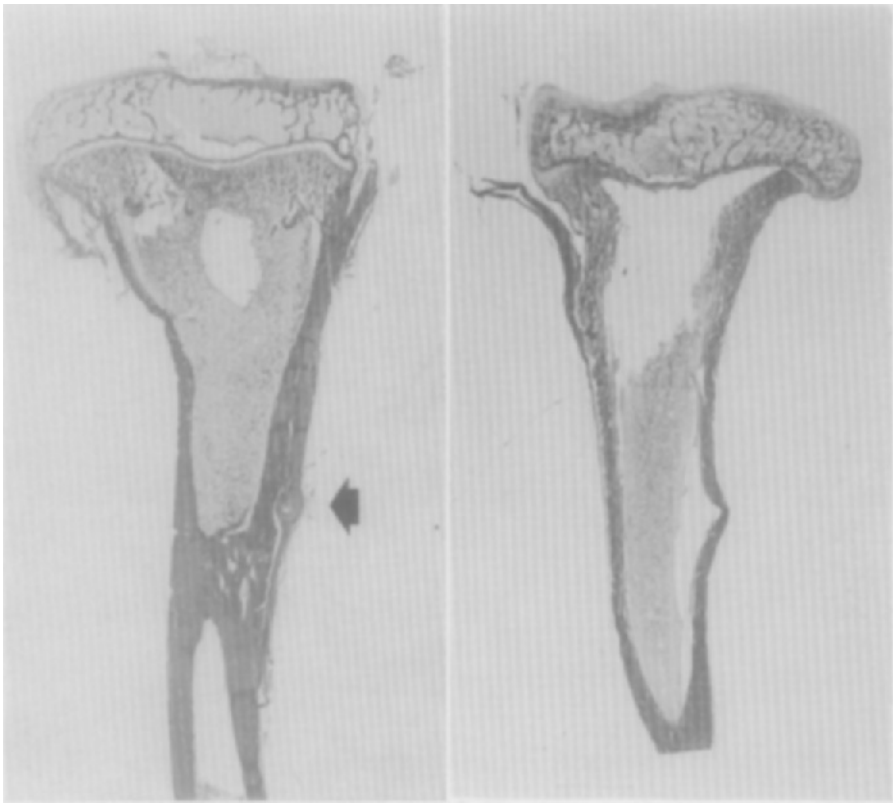

Fig. 5 A (histological sections): Section from the proximal end of the tibia;- the abnormally long medial collateral ligament runs along the right edge of the section. The location of a metal wire which had arrested displacement of the site of insertion is indicated by the arrow.

Fig. 5 B: Normal insertion of the ligament on the left edge of the control sections.

the bone. In all cases the insertion was more distal from the epiphysis than in the controls.

»Insertion segment» (*s* in Fig. 4), refers to the distance between the metal markers in the bone and in the tendon (or ligament). The increase in the length of the insertion segment was 75.2 ± 2.9 per cent of the increase in the metaphyseal length during growth.

In the control group, the displacement of the insertion site referable to the central part of the diaphysis was compared to the increase in the metaphyseal length during growth. The mean result was 74.8 ± 0.5 per cent. The rate of displacement of the insertion site and the rate of growth of the »insertion segment» were thus 74.8 and 75.2 per cent

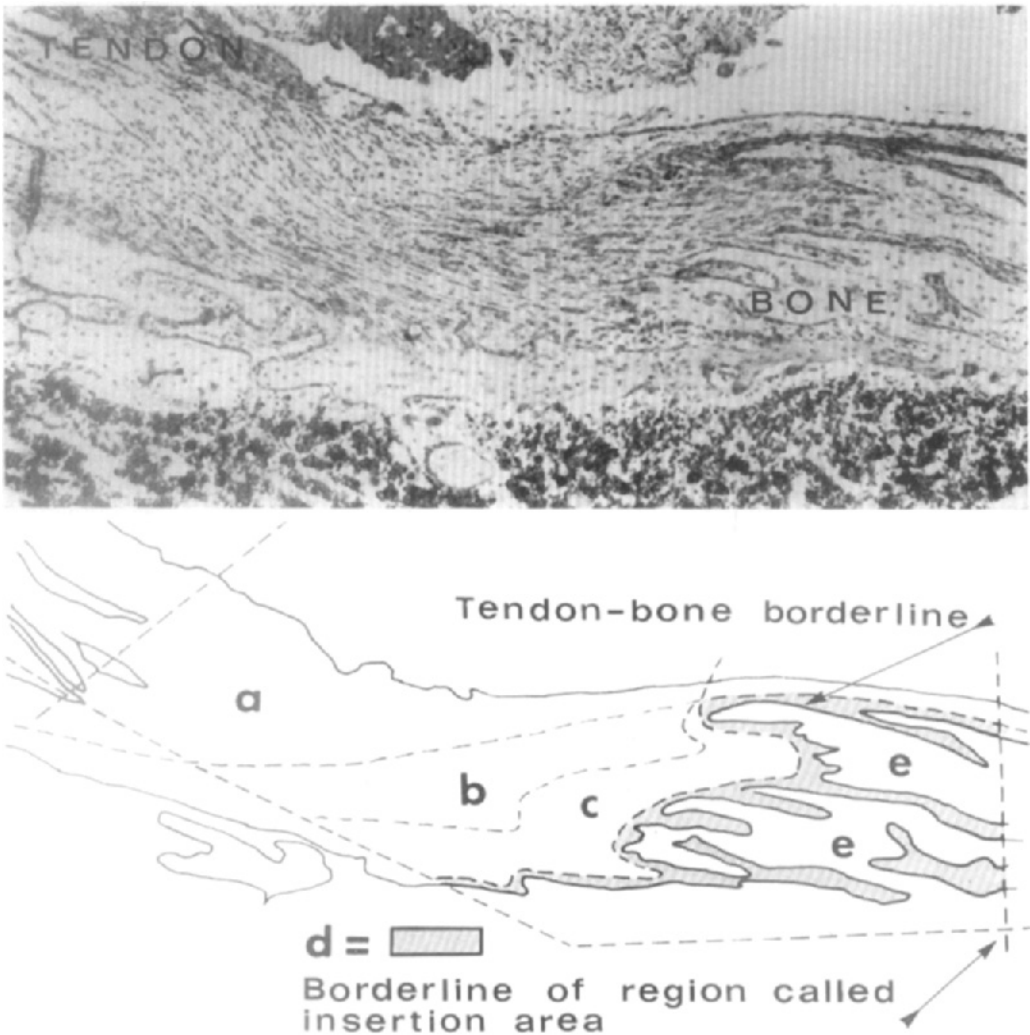

Fig. 6: Histological section (top) of the area of insertion of the pectineus muscle in the femur. The tendinous fibres run from the upper left corner of the microphotograph down to the right. There is bone on the right and bone marrow in the lower part of the picture.

The contour drawing shows how the region of the insertion area in the upper photograph was divided into five areas. The tendon-bone boundary is drawn with continuous lines. The broken line between areas *a* and *b* is a continuation to the boundary between the fibrotic and osteogenic layers of the periosteum. Area *d* (shaded in the picture) skirts the bony trabeculae. The zone between areas *a* and *d* has been divided by halving the distance of the above boundary lines at each point. Bone is denoted by *e*. The limits of the insertion area are drawn in straight broken lines. The line down to the middle (on the left) is parallel to the tendinous fibres, but the other limits are somewhat arbitrary.

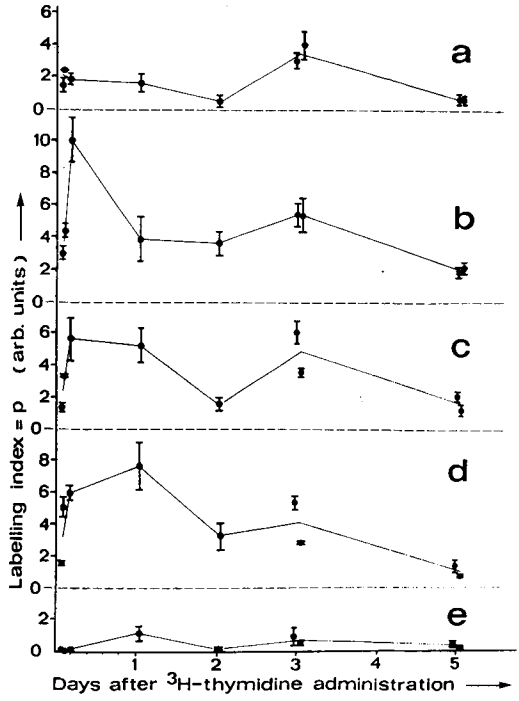

Fig. 7: Graphic representation of the time course of the labelling index in the different areas, *a*, *b*, *c*, *d* and *e*, of the tendon insertion area (cf. Fig. 6). The curves are based on results calculated from 64 autoradiographs. Each point represents the mean ± SD of different specimens from one rabbit.

of the rate of growth of the metaphysis. These percentages were not statistically different.

4) *Incorporation of ^{3}H-thymidine:* The longitudinal section of the insertion region of the pectineus muscle was divided into five areas (*a*, *b*, *c*, *d*, and *e* in Fig. 6) and the labelling index (*p*) of each area was calculated. Fig. 7 illustrates the labelling index of areas *a*, *b*, *c*, *d* and *e* as a function of time. The cells in the DNA synthesis phase were located chiefly in areas *b* and *d* judging from the preparation where the labelled cells had not yet divided (e.g. 2.3 hours after the injection). One day after the administration of the thymidine, labelled cells were numerous in area *c*, as well. After five days, the number of autoradiographically positive cells was decreased in areas *b*, *c* and *d*, but distinctly increased in area *e*, i.e., in bone.

The following is a summary of the changes seen in each area.

Area *a*. Labelling index in the distal part of the tendon of the pectineus muscle, i.e., in area *a*, remained on the whole unchanged and there was no definite peak in the curve.

Area *b*. There appeared to be a peak at about five hours in area *b*.

Area *c*. Precise determination of the peak in the labelling index of the cells in area *c* was difficult, but the peak fell mainly within the region of half a day and certainly at a later time than in area *b*.

Area *d*. This area, the insertion region adjacent to the bone trabeculae, had a peak at one day.

Area *e*. In area *e*, i.e., within bone, no distinct peak was noted; the slope of the time course curve was initially ascending, but descended slowly thereafter.

DISCUSSION

The autoradiographs obtained after labelling with ^{35}S-sulphate showed that the radioactivity originally taken up by the connective tissue cells of the insertion area is deposited in the bone and appears farther from the site of insertion as the bone grows. Therefore, the tendon-bone boundary of the region of insertion moves further from the central part of the diaphysis because of the interposition of newly-formed, unlabelled bone. It should be noted that 95 per cent of the ^{35}S-sulphate injected is excreted within five days in the urine and stools, and no appreciable radiosulphate is present in the blood 1—2 days after an intraperitoneal injection *(Dziewiatkowski* 1949). It has been demonstrated chromatographically that in slices of cartilage about 90 per cent of the total radioactivity is in chondroitin sulphate *(Dziewiatkowski* 1962). It is important to note, however, that the results obtained in the present study only reflect the behaviour of ^{35}S-containing substances which are not removed from the tissue by the preparative techniques used.

The pictures obtained using the tetracycline technique confirm the findings of new bone formation along the tendon-bone boundary. New tendinous tissue must also form to replace that which has been incorporated in bone.

The metal marker experiments indicated that the zone of most rapid tendon growth is in the insertion area, close to the tendon-bone boundary. However, it is not possible to determine the zone exactly with this technique. Fig. 2 shows that two days after the administration of the ^{35}S-sulphate the bone at the insertion area displayed a very narrow radioactive zone which remained in the bone. When 8—13 days had elapsed from the administration of the isotope, a relatively narrow, more weakly radioactive zone, clearly distinguished from the

stronger radioactivity of the environment, could be seen on the tendon-bone boundary.

These results may be explained as follows: the rate at which tendinous tissue is produced in the insertion area is not the same in all parts of the area, but there is a zone in which the connective tissue is synthesized more rapidly than elsewhere in the environment. Part of the connective tissue formed at the time of administration of ^{35}S-sulphate is incorporated in the bone and, following this, new connective tissue is laid down, especially in the zone of most rapid growth, which is not radioactive. Another possible explanation is that the radioactive connective tissue is in soluble form along the tendon-bone boundary and the pale zone is thus an artefact. However, the results using ^{3}H-thymidine show that there are zones in the insertion region which have varying population kinetics, although the boundaries between them are not sharp. Relatively few mitoses take place in zone *c* compared with the adjoining zones. It is thus possible that on moving from area *b* to area *c* cellular proliferation diminishes and synthesis of the intracellular substance increases. Hence, the insertion area can be compared functionally with the epiphyseal plate in which it is possible to distinguish between, e.g., a cell proliferation layer and a layer of mature cells which synthesize the intercellular substance.

In addition, population kinetic studies established that the boundary between the tendon itself and the insertion area runs between zones *a* and *b*. The growth in area *a* was diffuse, as in the description of tendon growth given by *Crawford* (1950) and *Hughes* (1956). Also, the lengthening of the distance between the metal markers in the medial collateral ligament corresponds with this diffuse mode of growth of tendons. The results of the metal marker experiments shown in Fig. 4 are in good agreement with the results obtained by other methods. Longitudinal tendon growth in the insertion is proportional to the bone growth originating from the epiphysis, and the most rapid tendon growth obviously occurs in zone *c* (Fig. 6) close to the tendon-bone boundary. During normal growth the insertion area of the tendon shifts in regard to the central part of the diaphysis together with the formation of tendon, new bone originating along the tendon-bone boundary. Substances of the tendon tissue were retained in the bone during the growth process.

SUMMARY

The aim of the present study was to elucidate the displacement of the sites of insertion of tendons and ligaments in regard to the central part of diaphysis during growth of tubular bones. The problem was studied using ^{35}S-sulphate and autoradiographic technique; tetracycline technique and metal markers fixed to tendons, ligaments and bone; ^{3}H-thymidine and stripping film autoradiographic technique. A total of 144 growing rabbits and additional control animals were used for the experiments.

Two different processes were noted as occurring simultaneously in the region of insertion during growth: longitudinal growth of tendon or ligament in the insertion area which is proportional to epiphyseal growth; and direct incorporation of newly-formed tendinous tissue in osseous tissue.

According to these events, the sites of insertion of tendons and ligaments in long tubular bones remain relatively unchanged topographically despite bone growth.

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