

## **Bone Biopsy as an aid to the Study and Diagnosis of Deficiency Diseases.**

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IN the study of a disease and especially of its effects on the animal body, it is of prime importance that direct observations be made on the changes which occur in organs and tissues throughout the various stages of the malady. To complete the picture it may also be desirable to follow the course of recovery when this does take place.

Pathological examinations, as a rule, are made on the animal after it has died or has been killed for the purpose. The whole carcass is then available for dissection and it is possible to subject each and every organ to close scrutiny and to collect a choice of tissues for microscopic examination.

The picture so obtained no matter how complete, however, represents only the stage of the disease reached at the time of death. In order to overcome this difficulty it is of course possible to destroy a series of animals at various stages of the disease and even of recovery. Such a procedure naturally involves serious inconveniences and costs, and besides, the results obtained are not strictly comparable on account of the varying individual susceptibilities of animals.

In order to observe changes at successive stages of a disease recourse can be had to clinical data especially when supplemented, e.g., by chemical analysis of body fluids and excretions and for morphological changes to actual examination of small pieces of suitable tissue removed surgically (biopsy) from the living animal. The value of biopsies in pathological studies is generally admitted and there is little doubt that this method of collecting material would be used to a greater extent were the attending difficulties (surgical and after treatment) more readily overcome. The method naturally does not lend itself very well to the study of all diseases. The course of many infectious diseases being far too rapid and too short. For those having a slow and extended course, however, the method certainly has much to commend it. This was found to be the case with nutritional deficiency diseases, where its advantages can be summarized as follows:—

- (1) The state of tissues in a given animal can be studied at suitable intervals—
  - (e) during health, i.e., prior to experiment;
  - (b) during development and course of disease;
  - (c) during and after recovery.
- (2) The changes seen, being in the same animal are more strictly comparable, than when different animals are used.
- (3) There is a great saving in animals, food, accommodation, time and labour, when compared to the series of animals it would be necessary to prepare and sacrifice to yield approximately the same results. This is particularly true where the larger animals like cattle, horses, sheep, goats and pigs are concerned as it takes anything from 6 to 18 months of painstaking and strict dieting to produce appreciable pathological changes in them.
- (4) Further economy obtained from the fact that animals giving negative results, controls, or even healthy recovered animals, having yielded required data by this means, may now be utilized for other purposes.
- (5) From an experimental point of view, to be able to stop, prolong or modify the diet on a basis of the changes seen, without having to wait for the lengthy preparatory period is a distinct advantage. Observations were made in this way on advanced cases of rickets to note the reaction to addition of phosphorus in the diet. The response was immediate and striking. Within the short space of a week separating two biopsies, extensive calcification had taken place.
- (6) Lastly for diagnostic purposes in natural cases of bone diseases in order to institute correct treatment. For instance it is almost impossible to distinguish clinically between rickets and osteofibrosis in the dog.

It stands to reason of course that where biopsies become a matter of routine, the tissue required should be of fairly easy surgical access on repeated occasions without unduly endangering the life of the animal or even upsetting its health. In the case of the skeleton which was the organ interesting us mainly in these deficiency diseases, pieces of bone which fitted these conditions most satisfactorily were removed from the ribs. Among the reasons for choosing this bone, one may mention:—

- (a) Ribs are fairly superficial and with care excision of the lower extremity at the costochondral junction does not involve a major surgical operation.
- (b) They have an epiphyseal line of growth which ossifies or closes either very late in life or only partly. The resected piece should include this line and therefore comprises 2 or 3 cms. of the rib with some of the cartilage attached.

- (c) By reason of their numbers, the ribs afford the most numerous opportunities for repeated biopsy of separate yet closely similar anatomical entities. Using each side alternately—cattle have 13 pairs of ribs, horses 18 pigs 14. In practice the asternal ribs are the easiest of access and usually prove ample for ordinary purposes. The longest ribs should be used for preference since being faster growing they probably show changes in a more accentuated form.
- (d) The rib at this point is softer than most other bones so that non decalcified sections by the freezing method can be cut fairly easily, a fact which makes for rapidity and ease of diagnosis.
- (e) When due care is taken to strip and leave the periosteum in situ, the resected portion regenerates sufficiently rapidly and completely to prevent any serious impairment of the thoracic function.

Incidentally also this method has proved very useful in obtaining bone marrow for haemocytological studies, whether it be on the same, i.e., deficiency experiment animals, or in others in which the blood forming organs only had to be investigated. In our hands this method has proved simpler and more satisfactory than the usual trephining or drilling process for bone marrow. The piece of rib removed is sliced longitudinally and each piece is squeezed in a vice with cut surface uppermost. From the mass of cells thus extruded excellent smear preparations of marrow can be made.

For phosphatase determination at the chondral line of growth in ribs, this method of resection is of value; sufficient material is available in one resected portion for chemical as well as for histological examination.

#### INSTRUMENTS AND EQUIPMENT REQUIRED.

An operating table was used for small animals but the large animals were thrown on to a thick mattress made of grain bags stuffed with straw and sewn together at the edges. Two or three lengths of 1-inch cotton rope were used in throwing and securing the animals.

Instruments used included several scalpels, scissors, artery forceps, dressing forceps, wound retractors, towel clamps, sharp hooks and one each of the following: aneurism needle, wire saw, rib shears and periosteum elevator. These were sterilised in an autoclave along with the necessary cloths, swabs and gowns.

#### OPERATION.

The operation itself is very simple and calls for no special skill provided reasonable care is exercised regarding asepsis. There are nevertheless several sources of accident which have to be guarded against, particularly so when dealing with animals in advanced deficiency condition when special precautions have to be taken as outlined below.

The operative technique for the different species of normal animals is described briefly below and is then followed by the modifications suggested for experimental or abnormal animals.

#### A. *Bovines.*

Food and water should be withheld for 24 hours prior to the operation. The animal is cast by Reuff's method and chloral hydrate administered as a general anaesthetic in the form of a 10 per cent. aqueous solution intravenously at the rate of 10 gms. per 100 Kg. live weight. Should this be necessary, anaesthesia may have to be supplemented by aether inhalation. The animal should lie with its head extended, nostrils free, and on a slightly lower level than neck and abdomen.

The seat of operation which is selected by feeling for the costo-chondral bulge on the required rib, is then prepared by clipping and shaving the hair off a sufficiently large area around this site. Any of the asternal ribs except the last two pairs (which are too short) may be selected for resection. It should be noted, however, that the further forward, the broader the ribs and thicker the muscles become thus making the operation somewhat more difficult. We have made it a practice, therefore, to start with the third or fourth rib counting from behind and working forward alternately therefrom.

After suitably disinfecting the shaved skin an incision is made over and dorsal to the costo-chondral junction along the centre of the rib and down to the periosteum which is divided along the same line. With a suitable elevator the periosteum is reflected back on either side and medial aspect of the rib so that the wire saw or rib shears may be passed under the rib at the point where it is desired to cut it. The cartilage is cut through with a scalpel leaving about 1 cm. attached to the rib and the rib itself is cut or sawn through so as to remove its distal three or four cm. and adherent cartilage. All loose bits of cartilage or bone should be removed from the wound as they tend to cause sequestration. The periosteum is sutured with catgut to form a hollow sheath and the wound closed by suturing the muscles and skin separately and sealing it with collodion-iodoform cream. An adhesive strip of elastoplast as an additional precaution helps to protect the wound during the first two days usually.

*After Treatment.*—When operation is successful and the wound is well protected, none is required. There is usually a fair amount of oedematous swelling but only occasionally does suppuration occur and has to be treated on general principles. Animals on special diet have been put straight back to it and healing has taken place very well without any special attention.

#### *Special precautions for diseased animals.*

1. *Withholding of food and water* for at least 24 hours is important.

2. *Handling.*—Very great care has to be exercised in handling animals suffering from bone diseases on account of the fragility of the bones. The following examples will illustrate how easily fractures can occur. On two occasions animals bolted while being driven

and as a result of a stumble and a fall, fractured the humerus and femur respectively. Even with the animal standing and during casting we have had unpleasant experiences. A not too violent push on the hip by an assistant or the animal pivoting on one leg slightly, have sufficed on other occasions to snap the bones of a limb. Needless to say such animals had to be destroyed as healing could not be expected under the conditions of the experiment.

In order to avoid this danger as much as possible animals should be driven and handled quietly and deliberately. In taking an animal to the operating room it is preferable to let it walk there of its own free will, accompanied if need be, by some of its stable companions rather than to drag or push an unwilling and struggling animal along or even to place it on a float.

On account of struggling it is preferable to cast bovines before injecting the anaesthetic. For heavy animals (300 Kg. and over) Reuff's method can be used and every care is taken to minimise struggling, twisting and bumping. For bovines weighing less it is safer to tie the animal's legs and then lift it bodily off its feet and lower it gently on to its side on the mattress.

3. *Regurgitation*.—In ruminants, even after starvation, there is always a danger of ingesta being regurgitated during anaesthesia and leading to a fatal foreign body pneumonia. In experimental animals fed on a highly artificial diet with little roughage, fasting for 48 hours will not empty the rumen of all its liquid contents. For this reason it is essential that the animal's head should be extended straight and at a lower level than the thorax and nostrils unimpeded so that any material so brought up can drain off freely.

4. *Chloroform and Chloral Hydrate Poisoning*.—During the earlier operations it was found that bovines on a calcium deficient diet were apparently more susceptible than usual to chloroform and chloral poisoning.

Accordingly, chloroform was discarded entirely and only a dulling dose ( $\frac{1}{2}$  to  $\frac{2}{3}$  normal dose) of chloral administered. This was supplemented by local anaesthesia (novocain-adrenalin deeply on either side of the selected rib to block the costal nerve and also intracutaneously if necessary).

5. *Bleeding*.—Rather profuse bleeding and slow blood coagulation was encountered in some calcium deficient animals but not all of them. This may be slightly troublesome while stripping the periosteum and may lead to rather large haematoma formation in the wound. Beyond temporary acupressure no special haemostatic measures were adopted.

6. *Soft Bones*.—In advanced cases of disease the bones may be so soft and the costo-chondral union so loose that difficulty may be experienced in removing the portion of rib with its cartilage attached. In such cases it may be necessary to leave a ring of unstripped periosteum to bind the fragile union while manipulating and removing the rib portion.

It was also found that the use of metal rings or eyes on the ropes used to tie the legs were apt to cause appreciable indentation into the subcutaneous metatarsal and metacarpal bone.

### B. Equines.

Starving and thirsting for 24 hours prior to the operation. Chloral hydrate at the rate of 6 grams per 100 Kg. of live weight was used successfully as a general anaesthetic, supplemented by aether and novocain-adrenalin mixture for local anaesthesia. The chloral, in a 10 per cent. aqueous solution, is administered intravenously in a standing position and the animal allowed to go down of its own accord. The head is held well down, the legs secured as in the bovine so as to allow access to the costo-chondral junction of the 5th or 6th rib from behind. In equines the thoracic wall extends further back than in bovines with the result that the muscles of the flank form folds over the 3rd and 4th last ribs when the legs are secured. The operation has therefore to be done further forward with no inconvenience owing to the thin muscular layer in this region in equines.

*The operative technique and after treatment* is the same as for bovines. Rather extensive post-operative oedematous swelling of the wound and surrounding area occurs, sometimes even extending to the ventral abdominal wall. The animal becomes slightly dyspnoeic and goes partly off its feed but soon overcomes the ill effects; after 8 to 14 days the swelling completely disappears and healing is uninterrupted.

*Diseased Animals.*—Careful handling as in the case of bovines with soft and brittle bones otherwise no special precautions need be taken.

### C. Sheep and Goats.

Starving and thirsting as for bovines. The animal is placed on the operating table and the legs secured. The head is held lower than the level of the thorax to allow of free drainage of regurgitated ingesta as in the case of bovines. Intrajugular infusion of chloral hydrate (10 per cent. aqueous solution) at the rate of 2 grams per 10 Kg. and, where necessary, supplementary aether inhalation proved satisfactory.

The operation is proceeded with as described in A. After stripping the periosteum from the lateral surface of the rib, an aneurism needle is passed between the periosteum and its lower surface just above the point where it is intended to sever the rib; the needle is worked downwards to the floating end of the rib, thus stripping the periosteum completely from its lower surface. The rib is cut with a curved rib shears or bone forceps, holding the free end of the isolated portion with a tissue forceps and dissecting it away from adherent tissues, finally cutting through the cartilage below its junction to the end of the rib. The periosteum is sutured with fine catgut and the operation completed as in the case of bovines.

*After Treatment.*—No special care is necessary. Practically no swelling occurs and healing takes place without interruption.

*Diseased Animals.*—The technique applied in experimental animals differs in no way from that in the normal, excepting that due care must be exercised to minimise struggling and prevent inhalation of regurgitated ingesta.

#### D. *Porcines.*

Starving and thirsting for 24 hours. Pernoxton was used for general anaesthesia with great success at the rate of 1 cc. per 5 Kg. of body weight injected into the ear vein. Local anaesthesia was unnecessary.

The control measures, operative technique and after treatment were the same as for sheep and goats. Pigs stood the operation better than any of the other species.

*Diseased Animals.*—There is no difference in technique between normal and diseased animals excepting that infinite care must be exercised to prevent fractures and the cartilage breaking off from the rib during operation when ribs are soft and brittle.

#### E. *Rats.*

Starving and thirsting are unnecessary. Exposure for a few minutes in an aetherised chamber and subsequent aether inhalation from a swab of cotton wool proved successful as a method of general anaesthesia. The site is prepared for operation and palpated carefully for the costal-arch in the region of the 5th or 6th rib (from behind). Incise the skin and underlying tissues just above the arch over a rib and locate the costo-chondral union by careful examination.

The periosteum is too delicate to allow of stripping and suturing so that the required portion of the rib is dissected out *in toto* and cut off with a fine pair of scissors. Care should be taken not to penetrate the pleural cavity which is separated from the inner surface of the rib by a relatively thick layer of muscle.

The operation is completed as in the other species.

*After Treatment.*—Recovery is uneventful and rapid. It is noteworthy that this operation in the rat is practically bloodless.

#### F. *Birds.*

In chickens the operation is very simple. The feathers are plucked over the site of the operation which is determined by palpation as in all the other species, the 2nd last rib being the most suitable one. The area is disinfected with tincture of iodine, infiltrated with 2 cc. novocain-adrenalin mixture. The costo-chondral joint is exposed by a cut through skin and muscles. The periosteum here too, is too delicate to be stripped and the same procedure is followed as in the rat. The number of ribs easily accessible for this operation is very limited in the fowl. There are only seven pairs of ribs all told.

*After Treatment.*—Ordinary care is all that is necessary.

## SIMPLIFIED DIAGNOSIS.

The procedure and suggestions outlined above for the resection of portions of ribs in bone studies represent the result and experience gained in some 77 biopsies performed by the writers (21 in bovines, 4 in horses, 7 in goats, 9 in sheep, 28 in pigs, 4 rats and 4 fowls).

At the outset the object of the biopsy was more for the purpose of rapidly determining the degree of progress of a particular deficiency disease, i.e., to know whether it was sufficiently advanced to warrant destruction of that animal for a complete histological study, rather than for making a diagnosis. Later it became evident that a diagnosis reflecting fairly reliably the general condition of the skeleton as a whole could be made from such biopsy material as well as from material collected after death. It was with a view to the eventual applicability and utility of the method in the smaller laboratory animals that the operation was tried on the rat and fowl. The former was generally regarded as suitable and time saving in certain nutrition experiments. It is perhaps advisable at this stage to describe how the process of diagnoses was further simplified.

*Choice of a Single Bone.*

When the preparation and examination of histological sections of bone from a large scale experiment has to be carried out, the time, labour and space involved in fixation, decalcification, embedding (celloidin and paraffin), cutting and staining of a representative set of several bones from each animal, becomes a serious limiting factor to the rate of progress of the work. Moreover, the results of such examinations are often not available for months after the death of the animal when a close correlation of the clinical and biochemical data with the histo-pathological is no longer possible. It became evident quite early in our attempted studies, that material to be examined would have to be curtailed considerably, if working diagnoses were to be turned out so as to keep pace with the progress of the experiments. It was accordingly decided to restrict the material to be examined to one bone, namely the rib and likewise to simplify and short cut the method of section preparation.

The procedure outlined below has now been adopted as a matter of routine in regard to animals in nutrition experiments and has also proved of value for rapid diagnosis in any animals where bone diseases are suspected. It has the merits of more or less standardising the comparative study of the skeleton, as reflected in the one bone and the results can be made available the same day if necessary.

*Preparation of Section.*

On removal the portion of rib may be dropped into 10 per cent. formalin direct or if some of it is required for other purposes, a longitudinal 2-4 m.m. slice is cut off from the centre and fixed in formol while the remaining pieces are available in the fresh state for phosphatase determination or bone marrow smears as the case may be.



After fixation, which may be accelerated of course by boiling for a few minutes in formalin, sections are cut on the freezing microtome in the ordinary way. Stains used for routine purposes are Haemalum-eosin, van Gieson and von Kossa's silver nitrate method.

In ribs of young animals or those suffering from advanced mineral deficiency, satisfactory sections are cut without any difficulty, but in harder and in normal adult ribs, this method plays havoc with microtome knives. It is nevertheless quite feasible to cut fairly satisfactory sections on the non-decalcified normal rib, a feat that would be almost impossible on other more compact bones. Microtome knives that have been used for ordinary work and discarded, are still quite suitable for this purpose. It also facilitates matters to make the piece to be cut smaller and smaller in proportion to the hardness of the bone, always provided that a portion of the epiphyseal line is included in the section.

Sections so obtained have unfortunately to be rather thick, anything up to  $40\mu$ , but are good enough as a rule to show up all the essential changes. Non-decalcification of the bone not only simplifies and saves time, but is a distinct advantage in itself in that it allows of direct observation of the degree of calcification a point of some importance in the diagnoses of early rickets for instance. Finer sections for more detailed histological studies can if required be cut from the remaining piece of rib after decalcification, embedding, etc., at a later date.

#### RESULTS OF NUTRITION EXPERIMENTS.

Finally it should be mentioned that the results of the nutrition experiments worked upon, i.e., the actual clinical, biochemical and histopathological findings as well as the significance of the various mineral constituents and their ratios in the diet will form the subject of separate papers to appear later.

#### SUMMARY.

1. The objects and advantages of bone biopsy in the histological, haemocytological and biochemical study and diagnosis of nutritional deficiency diseases are enumerated.
2. The technique for such biopsies is described for the various domesticated animals, stress being laid on the likely sources of accidents and methods employed to prevent these.
3. The necessity for simplifying and accelerating methods of diagnoses of bone conditions when dealing with large numbers of animals is stressed and the methods evolved towards this end are described.

#### REFERENCES.

- VALADE, P. M. (1932). L'Utilité des biopsies. *Recueil de Médecine Vétérinaire*, Tome CVIII, No. 11.
- QUINLAN, J., DE KOCK, G., AND MARAIS, I. P. (1935). The Operation of Splenectomy in Horses, Cattle, Sheep, Goats, Pigs, Dogs, and some South African Antelopes. A Summary of the Results of 98 Splenectomies. *Onderstepoort Journal of Veterinary Science and Animal Industry*, Vol. 5, No. 1, July, 1935.