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EXPERIMENTAL INVESTIGATIONS INTO POST-FOETAL OSTEOGENESIS

BY

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A. EARLIER INVESTIGATIONS[2]

1. *Classification and Histiogenesis.*

Post-foetal osteogenesis is to be seen in its most frequent and most characteristic form in the formation of a normal callus, the healing of traumatic bone lesions.

Whereas this form, *homoiotopic* osteogenesis, is associated with the osseous system itself, there is scarcely an organ or a tissue which in rarer cases may not be the seat of bone formation, of *heterotopic ossification.* True bone formation has in fact been demonstrated in renal tissue, lung tissue, lymph glands, operation scars and, above all, in muscle an tendon tissue in Myositis ossificans.

A third form of post-foetal osteogenesis is *induced* osteogenesis as a result of the transplantation of bone, marrow and periosteum.

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[2] The following summary does not aim at a complete bibliography, but *a survey of our present knowledge* with an examination of the conflicting hypotheses, the methods of research employed, and the most important results.

In the histiogenesis of newly-formed osseous tissue the main features are the following: In any fracture there are lesions —apart from those of the bone itself—of the soft parts around fragmina, the periosteum to some extent is loosened from corticalis, the marrow is dilacerated, blood-vessels and capillaries burst. This induces an aseptic, serofibrinous, inflammatory condition with the transsudation of blood and tissue fluid, followed by precipitation of fibrin between fragmina and the surrounding tissue.

The peripheral ends of the bone and any small splinters become necrotized and resorbed, and within the fracture haematome there grows a granulation tissue, in which in the course of some days it is possible to find osteoid tissue, spheroid or cuboid cells (the so-called osteoblasts), bone laminae, islets of cartilage, and proliferating capillary buds.

In the initial stage the granulation tissue consists of a loose-meshed mesenchymal network of cells, in which as early as in the second day there is an occurrence of collagenous fibrils; according to *Hansen* and *Häggkvist* these fibrils are formed in the cell ectoplasm, whereas according to *Leriche, Policard, Lauche* and others they form intercellularly. From the fifth day the collagenous bundles solidify and become "masked" by a mucoproteid, "osseomucin", into preosseous tissue or "osteoid". The cells (or the endoplasm regions) corresponding to these osteoid trabeculae are at first indistinguishable from the other mesenchymal cells, and from them emanate fibrils in between the lamellae. Only gradually do they become more spheroid or cuboid, typical "osteoblasts", and they are deposited almost epithelially along the osteoid trabeculae. Here and there, especially in the intermediate and marrow zones, islets of cartilage develop, whereafter osteogenesis proceeds along the same lines as in foetal life, except that it is more irregular, with a mixture of direct, enchondral and perichondral ossification (*Bruns, Hams, Lauche, Bennet & Bauer, Boyd,* etc.).

In heterotopic and induced osteogenesis too the actual formation of bone is preceded by degenerative and proliferative inflammatory reactions accompanied by the forming of yong

vascular connective tissue between calcified areas of matrix and osteoid lamellae; in these cases, however, the formation of cartilage is rarer than in fracture callus, and consequently the type of ossification is chiefly direct (*Meyenburg, Gruber, Dittrich,* etc.).

There is unanimity on these most elementary histological facts of post-foetal osteogenesis, but the question of the *causal genesis* of the newly-formed osseous tissue has never actually been settled. For example, *Hams* (1932) asserted that the granulation tissue arises exclusively from proliferations of specific cells already present in the periosteum, Haversian canals and bone marrow, whereas *Leriche* (1939) considers the changes in the intercellular substance to be primary, the "osteoblasts" secondary and really having little to do with bone formation: "Ces fibroblast ne deviennent ostéoblasts que quand la substance préosseuse a commencé d'aparaître".

Thus we *know* that these tissue elements make their appearance under such conditions; but *whence* they come, and particularly *what* it is that causes their development, are problems that have never yet been properly solved.

That this is so is manifested in the many conflicting hypotheses on the causality and mechanism of osteogenesis.

2. *Causal genesis; the principal hypotheses.*

Among the innumerable theories which, in conjunction with experimental and histological investigations, have been advanced on the subject of the causal genesis of post-foetal ossification, four main lines of thought: the osteoblast and the osteocyte theories, the doctrine of metaplasy and the dualistic conception, are the chief working hypotheses.

I. *The classical osteoblast theory.*

According to this hypothesis, which is traceable right back to *Duhamel* (1741), *Ollier* (1859) and *Gegenbauer* (1864), the osteogenetic power is associated solely with specific cells, osteoblasts, which already are present in periosteum, Haversian ca-

nals and endosteum and which represent the initial stages of the periostal, the intermediate and the endostal callus respectively. It is supposed that the osteoblasts are are awakened into activity by the traumatic lesion iself (*Lexer, Axhausen*).

The weakest point of the osteoblast theory is the demonstration of the supposedly pre-existing osteoblasts on which the entire hypothesis rests. It is a well-known fact that young growing bones in the deepest layer of the periosteum contain a cell-rich layer (the Cambium) with coarse spheroid or cuboid cells, the supposed starting point of periostal ossification; but in adults these "osteoblasts" are quite hypothetic. "La couche osteogene n'existe pas", says *Leriche*. The only cells to be found in the periosteum of the fully grown bones are elongated with flat nuclei, and they differ in no way morphologically from typical fibroblasts (*Lauche, Bennet & Bauer,* etc.). They occur sporadically, here and there, and at many places in the skeleton they are lacking entirely. The same applies to the Haversian canals and the endosteum (*Weidenreich, Petersen*).

Nevertheless, the modern osteoblast doctrine reckons with the presence of morphologically undifferentiated, but osteoblastically determined specific cells which, as a result of the hyperaemia in the region of the fracture, or through the influence of certian histamine- or acetylcholine-like substances released by the lesion, become differentiated into typical osteoblasts.

It is only with much difficulty that heterotopic ossification can be explained on the basis of the osteoblast theory, which either assumes various adjuvant hypotheses such as detached periostal elements (*Sudeck*), the occurrence of dislocated germs, i.e. dislocated osteoblasts (*Lexer*), and resting undifferentiated mesenchymal cells (*Rhode*) as the initial factor in bone formation, or simply rejects heterotopic ossification as an isolated phenomen with no genetic connection with bone formation on the whole.

II. *The bone cell theory.*

Authors as early as *Goodsir* (1845) and *Sedillot* (1867) argued against the doctrine of the overwhelming importance of

the periosteum in osteogenesis; indeed, *Goodsir* even called it a "limiting membrane" passively enveloping the "hive" of living bone cells which both build up and break down osseous tissue. *Goodsir*'s bone cell theory was not defined more precisely until the appearance of *Mac Ewen*'s monograph "*The Growth of Bone*" in 1912. The periosteum is discredited with having any influence at all on osteogenesis, it being held that all osseous growth emanates from the bone cells themselves—the osteocytes, or as *Mac Ewen* calls them, "the mature osteoblasts".

Mac Ewen says that the bone cells have possibilities of proliferation at least as great as epithelial cells, and that they are more easily freed by small pieces of bone than by large, coherent flakes covered with periosteum, for which reason multiple small pieces are preferable as transplantation material.

Häggkvist too believes that after the resorption of the osseous tissue is complete, the "endoplasm regions" of the osseus tissue may again differentiate into "osteoblasts" and thereafter again produce bone.

In a critical discussion on this theory one may refer—as in the case of the osteoblast theory—to the difficulty of explaining heterotopic ossification (*Mac Ewen* mentions the possibility of haematogenous metastasis), and also to the fact that mitotic cell divisions are very rarely observed in the bone cells themselves, so that the supposedly great capacity for proliferation must be described as rather improbable and at any rate as quite unproven.

III. *The metaplasia theory.*

In contrast to the two hypotheses already described, osteogenesis according to the doctrine of metaplasia is not associated with pre-existing specific cells, but arises out of the unspecific, undifferentiated cells in the young connective tissue which always occurs in osteogenetic processes. The irritant that causes the differentiation of connective tissue into osteogenetic tissue is supposed to emanate from the fractured or transplanted bone.

However, here again the theory, which was advanced by

Baschkirzew & Petrow 1912, does not directly explain heterotopic ossification, although it is easier to imagine the presence of an osteogenetic irritant in the form of a hormone, an inductor or an enzyme, tranported in the blood stream to non-osseous regions than a haematogenous metastasis of detached bone cells.

The chief objection at the moment to the metaplasia theory is that no one has convincingly described or demonstrated the factor or factors that cause the supposed metaplasia of the connective tissue.

IV. *Osteogenetic dualism.*

This theory may be described as a compromise between the osteoblast theory and the metaplasia doctrine, as it assumes that post-foetal bone formation may emanate from pre-existing osteoblastic cells in the periosteum and bone marrow and it may also be caused by the metaplasia of undifferentiated mesenchymal cells (*Bier* 1918). Periostal and intermediate callus, for example, are assumed to be formed of osteoblasts, whereas parossal callus and heterotopic ossification are considered to be metaplastic (*Bancroft* 1918, *Lauche* 1937).

As one of the causes of metaplastic osteogenesis *Bier* assumes the existence of "örtlich entstehenden Hormonen, die von der Markhöhle ausgeht".

It will be seen that the theory covers both observations which seem to support the osteoblast theory and observations which have their most natural explanation in the metaplasia doctrine; but it has this conspicuous weakness, that it endeavours to explain similar or at any rate closely related histogenetic processes in two quite different and contradictory ways, i.e. by specific cell activity and by humeral determination of unspecific tissue. Thus the objections to both theories apply to the dualistic view, that is to say the difficulty of demonstrating pre-existing osteoblasts and that of producing "marrow hormones" or other active osteogenetic preparations.

3. *Grouping of Earlier Investigations.*

For the purpose of obtaining some measure of survey over

the voluminous literature on post-foetal osteogenesis it is practical to divide the earlier investigations into five groups:

1. Morphological-descriptive investigations (especially callus microscopy),
2. Experimental-surgical (partial resections, transplantation experiments),
3. Experimental-physiological investigations (especially extraction experiments),
4. Investigations into the histochemistry of ossification, and
5. Explantation experiments.

Each of these groups will be examined separately in the following.

5. *Morphological Investigations (callus microscopy).*

Until the appearance of *Duhamel*'s publications 1739-43[1] the prevalent opinion was that of Galene, according to whom bone fractures were cemented together by a juice, *succus ossificus,* which flowed out of the fracture ends and slowly coagulated; but the callus thereby formed never became real bone, *callus non est organicus.*

On the basis of macroscopic examinations of fractured pigeon bones *Duhamel* drew attention to the connection of the young callus tissue with periosteum "externum" and "internum"; and by means of feeding the pigeons on madder red[2], a vital colour that is deposited in young, newly-formed osseous tissue, he demonstrated the growth of the callus tissue with lamellar apposition under the periosteum. He stated that the newly-formed

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[1] *Duhamel*'s two centuries old but exemplary investigations are so much the more remarkable as, Sieur of the du Monceau estate, his only contact with medical science was through Winsløv's lectures in Paris, which he attended when a young law student. However, he soon took up investigations in relation to agricultural science and was elected to the Academy at the age of 27.

[2] Madder red is obtained from the creeping plant *Asperula tinctoria,* and its capacity for vital staining of bone tissue was described for the first time in 1581 by *Lemnius* (de miraculi occultis); it was rediscovered later by *John Belchier* (Phil. Transaction *39.* 287. 1736).

tissue was cellular and living, though this view was not generally accepted until about a hundred years later when *Heine* made his resection experiments (1836) and after *Ollier*'s transplantations (1858).

v. Haller and *Hunter*, who considered that "succus ossificus" came from the arteries in the bone and its surroundings, sharply opposed *Duhamel*'s periosteum theory. *Detleff, Howship, Scarpa, Troja* and others also championed more or less modified Galenic views.

Nevertheless some authors, especially in France, supported *Duhamel*'s opinion, e.g. *Fougeroux* 1760, *Dupuytren* 1808 and later *Bréschet, Villermé, Cruveilhier* and *Flourens*.

Dupuytren made a sharp distinction between the provisional and the definitive callus. He held that the provisional callus, which develops in the course of the first weeks, emanates from the periosteum and the marrow, whereas the fracture ends are not united until after three or four months by the definitive callus, which originates from the fracture surfaces themselves; simultaneously the provisional callus begins to be absorbed.

According to *Cruveilhier* and *Flourens*, marrow and fracture surfaces have no part in the formation of the callus, which comes exclusively from the periosteum and the surrounding soft tissues.

With *Virchow*'s doctrine of specific cells, which dominated the latter half of last century, the tendency is to bring osteogenesis in under the concept of specific cell activity. *Goodsir* (1845) had already described osteogenic cells under the periosteum and in the Haversian canals, the latter being described as "the oldest and most active of the cellular elements of the bone"; and *Ollier* (1858) studied thin sections of periosteum covering young growing osseous tissue and described a "couche osteogène" which was well-defined in young animals, but consisted of more scattered cells in adults.

The first more detailed description of the so-called osteogenic cells, however, was given by *Gegenbauer* (1864), who gave them the name by which they are known today, "osteoblasts". He described their situation in the deepest layer of the periosteum and in the primary marrow cavities, and regarded the

lamellar systems under the periosteum and around the primary marrow cavities as being formed by a kind of secretion process from the osteoblasts.

On the basis of these observations lies the specific osteoblastic theory which still prevails in most manuals and textbooks; *Cowdry*'s *Special Cytology* (*Hams* 1932) still has it that the starting point of osteogenesis is specific periostal, Haversian and endostal osteoblasts, and it actually utters a "warning" against the acceptance of other and unnecessary hypotheses.

In Denmark *John Hertz*, for example, as recently as in 1936 writes: "My specimens distinctly show that bone regeneration chiefly comes from the periosteum, but also that the endosteum and the marrow take part in the formation of the callus, though to a smaller extent", an opinion that is identical with that of *Heine* (1836), *Asada* (1927) and several others, and on the whole with that of *Bergmann* (1924), who, however, credits the marrow endosteum with more importance. Earlier students of experimental fracture like *Lebert* (1845) consider that the periosteum is the sole source of callus formation, a view that is adhered to i.a. by *Maas* (1877). On the other hand, *Hjørdis Jørgensen*'s findings from her studies of fracture healing by osteosynthesis (1942) are given the most natural explanation on the basis of modern forms of the metaplasia theory, to which the author indeed draws attention in her conclusions.

Nevertheless, *Bonome, Lexer, Leriche* and others are emphatic in their contention that in callus studies one cannot decide morphologically the origin of the bone tissue that is formed and of the cells that make their appearance in relation to this tissue (—les fractures experimentales ne nous ont rien dit de nouveau—); and already in 1885 Bonome refers to experimental defect resection and partial transplantations for clearing up the osteogenetic problems. This brings us to the next epoch of research, the experimental-surgical epoch.

5. *Experimental-surgical investigations.*

It was *Heine* who as long ago as in 1836 introduced partial resection into osteogenetic research. His experiments (costa resections) were performed on dogs and calves, and his con-

clusions were that periosteum plays the leading part in fracture healing, that the endosteum has the same properties but to a lower degree, whilst the osseus tissue itself had nothing whatever to do with bone formation.

In the 1920's the resection experiments were resumed systematically by inter alia *Lexer, Bier, Martin, Rohde, Partsch, Koch* and *Willich,* who made resections of periosteum, periosteum-compacta, compacta-marrow, etc., etc. However, the main outcome of these experiments was a discussion on the importance of the various layers to osteogenesis, a discussion to which callus microscopy as already stated made a contribution, and indeed transplantation experiments as well.

One school, the most common view, holds that bone formation mainly, but not exclusively, originates in the periosteum (*Cruveilhier, Flourens, Ollier, Syme, Virchow, Lexer, Pochhammer, Koch* and *Asada,* etc.); another (*Billroth, Berg & Thalhimmer, Bast, Sullivan & Geist,* etc.) credits osteoblasts from the Haversian canals with great importance, whereas others again (*Bergmann, Partsch, Willich, Bull* etc.) maintain that osteoblasts from the "endosteum" of the marrow are of just as much importance to osteogenesis as those from the periosteum and the Haversian cells, unless their vascular supplies are affected by the fracture.

Ollier's systematic transplantation experiments (1858-68) introduced periosteum-bone transplantation into experimental surgery and therapy; nevertheless, less extensive and more sporadic experiments[1] (*Merrem, v. Walter, Heine* and *Flourens*) had preceded *Ollier*'s, but had failed to attract much attention.

Ollier transplanted entire bones with periosteum and marrow and also isolated periosteum-compacta- and marrow tissue. Of the isolated transplants only the periosteum formed new osseus tissue (autologous experiments on young rabbits), whereas marrow tissue and compacta necrotized and were absorbed. On the other hand he considered the total transplant

[1] For the historical examination of these experiments, which mainly comprise animal trepanations with retransplantation of loosened cranial fragmina see *J. Wolff:* Arch. f. klin. Chir. *4*, 183-294, 1863.

of periosteum-bone-marrow to be extremely suitable material for transplantation and in fact states that under favourable circumstances all the layers in a living bone containing periosteum and marrow will survive transplantation.

Ollier's method, total and partial transplantation, has been widely employed in experimental osteogenesis research, but the results are not all consistent. In explanation of the formation of bone in the case of transplantation, three entirely different osteoblastic theories have been advanced, besides the osteocyte theory and the doctrine of metaplasia:

1) *Ollier*'s theory as referred to above, according to which periosteum, bone and marrow can survive transplantation.

2) *Barth's* theory (1893) that all layers die and that it is exclusively the recipient's osteoblasts that grow around and into the transplant and substitute the bone. Among those who adhere to *Barth*'s view is *Imbert* (1918). As will be known, *Barth*'s theory, according to which the tranplanted bone plays a role partly as a medium ("osteoconductor") for osteogenesis and partly as a depot of calcium, forms the basis of the various actions in favour of preparations of dead bone, for example os purum.

3) Finally, *Radzimowsky* 1881, *Axhausen* 1908 and *Bonome* 1885 report that tranplanted osseus tissue necrotizes and is subjected to lacunar absorption, but, in contradistinction to *Barth,* that it is the periosteum and endosteum of the donor bone which under favourable conditions survive and regenerate the transplant.

In a large monograph the Norwegian *Bull* 1928 went deep into the questions of bone transplantation and regeneration. His results are a combination of the theories of *Axhausen* and *Barth,* for he claims to have demonstrated that the periosteum and endosteum of *both* donor and recipient bones are active in the regeneration (i.e. breaking down and rebuilding) of the transplant.

By boiling the transplant he eliminates the periosteum, endosteum and possibly specific cells in the Haversian canals, whereafter he studies the regeneration, which emanates exclusively from

the recipient. As a result of these experiments he was able to confirm *Barth*'s statement that bone is thereby formed, though osteogenesis was more sparse and proceeded more slowly than when living bone was transplanted. By transplanting to the musculature, on the other hand, he was able to preclude the recipient and thereby confirmed *Axhausen*'s theory of the osteogenetic power of the donor bone. He accordingly claimed to have proved that bone formation proceeds from the *periostal and endostal osteoblasts* of both the transplant and the defective bone, although elsewhere he himself states that in total transplantations it is impossible to arrive at a definite conclusion as to the origin of the osseous tissue which successively replaces the necrotic bone substance. For this reason he implants isolated periosteum, isolated marrow tissue, as well as bones deprived of periosteum and marrow; but all he succeeds in showing is actually what several earlier investigators showed, that all these components may be osteogenetic. Neither *Bull*'s nor any previous experiments of the same kind prove that it is the preexisting osteoblasts in these isolated transplants that form the starting point of osteogenesis.

When making subperiostal resections in adults *Mac Ewen* never saw osseous tissue regenerated, and 32 experimental transplantations of periosteum from adult animals did not cause bone formation in a single case, whereas such a formation was observed in practically all transplantations of solid bone deprived of the periosteum. In view of this experience *Mac Ewen* recommends multiple small fragments of diaphysis corticalis as transplantation material, rather than large coherent flakes covered with periosteum, his opinion being that the osteocytes, from which osteogenesis emanates according to his osteocyte theory, are more readily released by the fragments.

Gallie & Robertson, Trinci, Murphy, Baetzner and several others like *Mac Ewen* deny the periosteum a part in osteogenesis, periosteum transplantations in their hands having proved abortive, whereas *Berg & Thalhimmer, Riess, Bull* and other supporters of the osteoblast theory maintain that osteogenesis is achieved if only the deepest layer of the periosteum, the cambium layer, is included in the transplant.

Leriche & Policard agree with *Mac Ewen* in disassociating the periosteum from any specific osteogenetic properties and consider that the positive results of transplantations reported must be due to small particles of bone having been transplanted along with the periosteum.

Baschkirzew & Petrow (1912) were probably the first to attribute their experimental results to the metaplasia theory. To the gluteal musculature they transplanted bones complete with periosteum and marrow, and bones without them, and as the results were exactly the same in the two series, they concluded that periosteum and bone marrow cannot be the starting point of the bone formation which in all the experiments followed upon incipient absorption. In a third series they transplanted dead bones, i.e. boiled or incinerated bones; the results was merely a progressive absorption, no bone formation, and so the authors considered that living bones alone are capable of producing the irritant which causes the metaplasia of the connective tissue.

Instead of boiled bones *Nageotte* (1918) as his dead material employed transplants fixed in alcohol and formol. Ossification occurred in some cases around them, and therefore this author, in contrast to *Baschkirzew & Petrow*, states that there is no principal difference between the behaviour of living and dead bones.

Other French investigators besides *Nageotte* are adherents of the metaplasia theory, including *Régard, Simon, Leriche & Policard*, but they have made no experiments for the purpose of determining what is the irritant or the substance that causes metaplasia.

Leriche & Policard (1926) moreover maintain that metaplasia arises out of the matrix, whereas the cells take quite a passive part. It proceeds in three stages: First there is an oedematous infiltration into the connective tissue, accompained by a marked increase of the quantity of fibrils. Then there is infiltration of a special preosseous substance, and finally, calcification proceeds by means of the precipitation of Ca-phosphates and carbonates. This process is independent of cell func-

tion, "interstitial and humeral"; indeed, the authors hold that the cells "fight against" ossification and restrain it. They state that two factors must be necessary for the experimental induction of bone formation: I. an irritant, vascular, nervous, endocrine or pharmodynamic, which causes the transformation of the connective tissue into "preosseous tissue", and II. a supply of Ca-phosphates and carbonates in such a mixture and state (hitherto unknown) as enables them to be assimilated directly by the tissue. Neither of these factors is accessible as yet, and at present experimental ossification can be induced solely by the transplantation of osseous tissue which, through its autolysis, presents the calcium salts in a form assimilable by the tissues.

The Italian *Polletini* (1923) continues *Nageotte's* experiments with bones fixed in alcohol and formol, which he implants subcutaneously in rabbits, chiefly in the ears. In some cases the result was a formation of cartilage and bone, and the author interprets his experiments to mean that the bones contain an alcoholstable substance with an osteogenetic effect on fibroblasts.

The works of the Swede *Levander,* 1938, 1939 and 1940, deal with transplantation of isolated periosteum and marrow tissue. In contrast to earlier investigators he attaches most weight to microscopic examination shortly after transplantation, from the second day, for, he says, when bone formation is demonstrated weeks or months after transplantation one cannot draw justifiable conclusions regarding the initiation of the induced osteogenesis. In all his experiments he finds degeneration of the tranplanted tissue, ending in total necrosis, and the formation of young, vascular connective tissue which is afterwards ossified (as far as periosteum is concerned, but only when taken from young, growing animals). He considers that his experiments prove the incorrectness of other authors' reports of the survival of specific osteoblastie cells, as no cell division has ever been demonstrated in transplanted tissue, and that they support the theory of a humeral osteogenetic influence on the unspecific connective tissue emanating from the transplanted tissue elements.

Levander's countryman *Svante Orell* has also made weigthy contributions in support of the metaplasia theory by his transplantation experiments (1934, 1943, 1944). On transplanting fresh bone to the subcutis he found osteogenesis already after the 15th day. When he implanted dead bone, "os purum", treated by protracted boiling, potash lye, enzyme solutions, trichloraetylene, acetone, etc. he again found bone formation, but only after about three months. From these results he concludes that cells not belonging to the osseous tissue are converted by the influence of the implant into bone cells with the property of producing collageneous and calciferous connective tissue. According to these experiments fresh bone seems to contain larger quantities of the principle which induces osteogenesis than dead bone, a circumstance that could be utilized by the adherents of the osteoblast theory and the dualistic school in support of the theory of a survival of specific osteoblastic cells. *Orell* points out that the difference may also be a result of the treatment of the killed bone having destroyed much of the osteogenetic factor contained in fresh bone tissue, and therefore, in collaboration with *Engström,* he studied the killing of bone, periosteum and marrow cells in a manner less drastic to the osseous tissue itself.

According to *Luyot* and co-workers, it is possibly by refrigerating tissue—provided the process is kept slow, especially within the crystallization zone around -15°—definitely to kill the cells and leave the chemical substances more or less unchanged. *Engström* & *Orell* have utilized this finding; over a period of five days they refrigerate the bone transplants down to -190°, and with these they obtain osteogenesis after 30 days of transplantation to subcutis. This means that surviving cells cannot be necessary for osteogenesis when fresh bone is implanted.

On man, too, *Orell* has made subcutaneous implantations of bone which had spent 7 days in the refrigerator, with distinctly positive results after 28 days.

Thus several recent transplantation experiments, especially in Sweden, have provided important support to *Baschkirzew* &

Petrow's metaplasia theory; and simultaneously, as a logical consequence, physiolo-biochemical research has been started to find the osteogenetic principle that is necessary to metaplasia.

6. *Experimental Physiological Investigations.*

Levander (1935, 1938) takes a very important step farther than *Polletini* who, as already stated, in 1923 announced that osseous tissue contains an alcohol-stable substance with osteogenetic effect; the does not fix the osseous tissue, but extracts with alcohol and employs the resulting extract for injection in the soft parts. With *Levander*'s experiments, osteogenesis research proceeds from the histological and later experimental-surgical period into its third epoch: the experimental-physiological.

Originally (1935) *Levander* fractured rabbit fore-legs and amputated them a week later. After carefully dissecting the soft tissues, bone, marrow and callus tissue were thoroughly pounded, whereafter the bone pulp was extracted with alcohol. In order to avoid extensive muscle necrosis the extract was diluted with saline to the half, and thereafter the extract from the same rabbit was injected, 3 ml. every other day for a total of three or four times. In 14 out of 60 experiments, with an observation period of from 2 to 9 weeks, there was more or less pronounced cartilage or bone formation at the injection sites. In 60 control experiments with alcohol in a similar concentration there was not a single case of chondrification or ossification.

The experiments were supplemented later (1938) with the extraction of non-fractured bones with hydrochloric acid-alcohol (190 ml. alch. abs. + 10 ml. HCl 0.2 %). Of 10 experimental animals two had chondrification at the site of injection, whereas 20 control animals, injected with extracts of connective tissue and musculature, gave no sign of osteo- or chondrogenesis.

Annersten (1939, 1940) (a pupil of *Levander*'s), checked the original extraction method and experimented with various other extractors such as ether, benzole, oil, hydrochloric acid, lactic

acid and saline. The positive results were obtained overwhelmingly with alcohol, ether-alcohol and benzole, and in most cases —as in *Levander*'s experiments—with hydrochloric acid-alcohol, as a result of which *Annersten* has worked out a standard method of extraction with acid alcohol.

The tubular bones of a rabbit are dissected and pounded to a smooth pulp, which is divided into portions of 5-8 gr. Each portion is placed in a glass tube with 15-20 ml. hydrochloric acid-alcohol (100 alch. conc. + 5 HCl 1/10 n.) and then rotated continuously for 16-24 hours at 1°. It is then carefully dessicated to about 1/4 with a stream of hot air. With this concentrate *Annersten* obtained osteo- or chondrogenesis in more than half of the homologous experiments.

After evaporation to dryness and solution in physiological salt-water the extract is inactive, and in dialysing only the inner phase is active.

Besides autologous and homologous experiments with rabbits, *Annersten* made heterologous experiments extracting the extremity bones of freshly killed suckling calves with hydrocloric acid-alcohol and injecting the extract into rabbits. Positive results were obtained in 10 out of 39 experiments.

The behaviour of these extracts suggests that the osteogenetic factor is of lipoid character, and *Annersten*'s chemical analyses point in the same direction. For example, the N content varied about 0.05 %, which must mean than the protein content at any rate is very low, whereas the P content varied between 1.7 and 5.6 mg%; and all the analyses show that practically all phosphor was in the form of lipoid phosphor. In the benzole extract, which was also active, the phosphor content was very low, 0.1 mg%, for which reason *Annersten* considers that the osteogenetic substance rather has the character of a steroid than a phosphate. In this connection the reader is referred to the embryonic induction substances, which chemically come very close to the steroids. (See Chapter 9 for a discussion on *Annersten*'s work).

7. *Investigations into the Histochemistry of Ossification.*

The first precondition for a histochemical solution of the problems concerned with the mechanism of post-foetal ossification must of course be a thorough knowledge of the chemical structure of already-formed osseous tissue. We have nothing like this knowledge as yet, and the contributions hitherto made by biochemistry towards the clarification of the osteogenetic questions are therefore small; for this reason our present knowledge of the chemistry of the osseous tissue and ossification will be recounted quite briefly without going into controversial hypotheses.

In contrast to former opinions of osseous tissue as the most imperishable component of the organism, this tissue nowadays is regarded as one of the most labile in the whole body, subjected to a constant deposition and dissolving of calcium salts, varying according to the calcium supply and demand in the organism, subjected to hormonal regulation (parathyroids, adrenals), depending on the supply of D-vitamins etc.

Thus despite its durability the macerated skeleton represents merely a snapshot of the bones of the individual at the moment of death. This being so, it is not to be wondered at that different analyses of bone tissue have given varying results and cannot be directly compared, as according e.g. to *Marek, Wellmann & Urbanyi* (1935) they vary as a result af species, race, age, nutrition, and indeed according to the various layers in the bone.

Among the various analytical reports on the chemical composition of osseous tissue the following is according to *Leriche* (1939): Fresh bone: water 50 %, fat. 15.75, osseine 11.40 and calcium salts 22.85 %. Dried and defatted: osseine and proteids 30 %, inorganic comp. 70 %, whereas for ribs *Kramer, Yuska & Steiner* (1939) give:

Foetus 5th month:	water 31.10,	fat 4.11,	ash 64.50.
New-born:	» 32.30	» 3.57	» 57.75.
Child, 10 years:	» 32.80	» 0.08	» 61.50.
Adult, 29 years:	» 34.97	» 4.36	» 58.80.

For tibia the water content was nearer *Leriche*'s figure, about 45 %.

The *inorganic substance* consists mainly of calcium, phosphate and carbonate. Calculated from dry and defatted bone the values are according to e.g.

Shear & *Kramer* (1928) Ca 25 % P 10.6 and CO_2 4.66.

Morgulius & *Janecek* (1931) Ca 32.90 % P 15.75 and CO_2 2.32.

Toverud & *Toverud* (1933) Ca 21.65.

Kraemer, Ruska & *Steiner* (1939) Ca :21.96-22.74, P: 10.11-11.08, CO_2 2.38-3.37.

The remainder of the inorganic substance (less water) consists chiefly of oxygen (from the phosphates) and very small quantities of magnium (*Marek, Wellmann* & *Urbanyi:* about 0.7 %), sodium, potassium, fluorine, chlorine and iron.

The percentages given above refer to human bone and show clearly the great variations in the degree of calcification. Whereas it seems beyond doubt that the carbonate content of the bones increases with age, we may assume that there is an almost constant relation between "the residual calcium" (i.e. Ca not combined as CO_3) and total phosphate, regardless of the age and topography of the bone, i.e. about 1.94.

This corresponds to the tertiary calcium phosphate, $Ca_3(PO_4)_2$, and in fact one of the theories on the chemical state of bone calcium is that it is a mixture of $Ca_3(PO_4)_2$ and $Ca\,CO_3$ (*Marek* etc.). However, there are many other hypotheses (for details see *Kramer* etc. 1939), for example the dahlite or apatite theory, according to which the formula is $n[Ca_3(PO_4)_2]\,Ca(OH)_2$. $CaCo_3$ or $n[Ca_3(PO_4)_2]$, CaX_2, where $X_2 = CO_3$, $(OH)_2$, F_2, SO_4, or Cl_2.

The organic substance consists mainly of the co-called osseine fibrils. Osseine, which as a raw material is of great importance in industry, i.a. the manufacture of gelatine, is considered to be closely related to collagen, indeed, according to *Leriche,* it is perhaps identical to it, except that it is modified by the changed biological surroundings.

In addition to the manufacture of glue, decalcified bone ash gives the by-products albumoid and mucoid (osseoalbumoid and osseomucoid), and it is possible that it is "contamination" by these substances that gives the osseine fibrils their particular character. According to *Hisamuri* (1938), osseomucoid can be broken down into two fractions, one consisting chiefly of chondroitin sulphuric acid, the other of acetylchondrosamine and galactose.

Both the chemical structure of the organic components of the vital osseous tissue and the connection (chemical combination, adsorption?) between it and the inorganic components, for the time being must, however, be described as insufficiently elucidated, and that is the cause of the conflicting theories on the chemical mechanism of ossification. The more important of these theories will be referred to briefly below, without entering upon a motivation or criticism of the works on which they are based.

According to the "precipitation theory", calcium precipitation—at any rate in the initial stages—is the result of a local over-saturation of the blood-serum's calcium-phosphat and carbonate solution. According to *Hofmeister, Kleinmann* and others, oversaturation is the result of a locally reduced carbon-dioxyde tension with a shift of the reaction in an alkaline direction, whereas according to the "Kalksalzfängertheorie" (*Pfaundler, Klinke, Freudenberg* & *György*) the fixing of the calcium is due to the precipitation of the lime caused by a substance in the tissue, absorption effect of the performed osteoid tissue, or chemical combination with albumin components in the tissue.

Leriche & *Policard* deny that there is an active cell function having an influence on the fixing of calcium in the osteoid tissue; they consider the process to be purely humeral, whereas *Röhmann, J. C. Watt* and others regard it as purely cellular, and the calcium salts combined with the tissue as a secretion of specific osteoblasts.

Between these extremes we have *Robison* and collaborators. In 1923 he demonstrated the enzyme phosphatase and its abundant occurrence in bone substance, especially in growing bone substance and in pure cultures of osteoblasts. The enzyme,

which *Robison* considers is formed by the osteoblasts, hydrolyses ester-combined phosphate, whereby a locally increased phosphate concentration and a consequent precipitation of calcium are made possible.

However, the mechanism of ossification was not cleared up by the discovery of phosphatase, as was at one time thought. The abundant occurrence of phosphatase in the liver, renal cortex, serum, leucocytes, intestinal mucosa, etc., and in fact in rachitic metaphyseal cartilage, which does not ossify, is alone sufficient to show that several unknown factors are active in the precipitation of calcium.

Among these factors particular mention should be made of the reaction shift in tissue and tissue fluid. According to *Leon Blum, Delaville* et *van Caulaert* (1925), it is the variation in p_H that regulates the dissolution or the precipitation of bone calcium, and *Annersten*, in large thorough-going works (1940, 1942), by means of electrometric and gasometric p_H determinations in fracture calluses of various ages demonstrated an alkalization moving parallel with the progressive ossification, the result of an increase in the content of bicarbonate.

8. *Explantation Experiments.*

Cultivation experiments in vitro so far made, like surgical, physiological and chemical investigations, have been unable to provide an unambiguous answer to the question of the causal genesis of osseous tissue.

Briefly, the results according to *Roulet, Fischer* and others may be summarized as follows: In explants of foetal (e.g. tarsal bone or tibia diaphysis), differentiation with enchondral and perichondral ossification continues as in vivo, unaffected by the new conditions in the tissue culture (*Fell, Fell & Robison*).

These experiments, involving several different kinds of tissues, are complex, however, and therefore unsuitable for closer analysis.

In contrast, *pure* cultures such as osteoblast cultures cannot be induced to differentiate into bone (*Policard & Bouchar-*

lat), and epiphyseal cartilage discs freed from their perichondrium do not grow at all in a tissue culture (*Fischer*). On adding a piece of cartilage to a pure culture of heart fibroblasts, however, *Albert Fischer* in 1931 succeeded in inducing chondrogenesis in the culture, and these facts: the absence of differentiation in pure cultures and *Fischer*'s cartilage induction in fibroblast culture, may perhaps be placed to the credit of the metaplasia theory.

However, *Törö* (1934) considers that *Fischer*'s chondrification was not due to a specific induction, as he was able to repeat the experiments with the same result when he added dessicated cerebral substance or retina powder to the fibroblast cultures instead of cartilage tissue.

B. OWN INVESTIGATIONS INTO POST-FOETAL OSTEOGENESIS

9. *Problem definition, criticism of earlier extraction experiments.*

The present author's investigations have their source in the extraction experiments of *Levander* and *Annersten,* which have shown that from rabbit and calf bones it is possible to make extracts which, on being injected into periosteum-free regions of rabbits, induce osteogenetic processes in about 20 to 40 % of the experiments.

This observation provides important support to the theory of metaplasia as the origin of post-foetal osteogenesis, but it leaves several problems obscure and therefore liable to contradictory interpretations.

1. It is for instance unsatisfactory that the osteogenetically-active extract takes effect in only about 20 % of the experiments, and even after concentration in only about half. One would expect of a steroid substance that it would be possible to produce more constantly active preparations by the concentration of sufficiently large initial material.

2. The occurrence of chondrification and ossification in a

number of the control experiments must be regarded as a weakness in the evidence of the specificity of the induced tissue reactions; *Annersten* observed metaplasia to cartilage or bone in one case among 13 experiments with tendon extract,

1 case among 16 experiments with muscle extract.

2 cases among 33 experiments with alcohol with Ca and P added,

1 case among 10 experiments with kidney extract, and

1 case among 6 experiments with dialysed urine with hydrochloric acid-alcohol added.

Annersten explains the negative finds in the main experiments as individual differences among the rabbits, and the positive finds among the controls from a theory of the circulation of the osteogenetic "hormone" in the blood and excretion through the kidneys, in analogy with the sexual hormones; but there is no excretion through the liver (6 negative experiments).

Though the difference between the results in the principal and the control experiments are given clear significance, the possibility of an unspecific reaction—perhaps characteristic of rabbits—can scarcely be precluded on the basis of present experience.

3. If thus the *biological* characterization of the active factor is a matter of difficulty, it is certainly no less in the chemical classification.

For example, *Annersten* states that the extracts are inactive when made in a thermostat or when evaporated to dryness and then dissolved, and therefore he recommends extraction in a refrigerator. Nevertheles, there were chondrification and ossification in 5 out of 14 experiments for which the extraction proceeded in the Soxhlet apparatus for four hours with alcohol, alcohol-ether and benzole, where the temperature must consequently have been much higher than in a thermostat.

As already stated, the majority of the positive extraction experiments were obtained with lipoid solvents, but 4 out of 9 experiments with pure hydrochloric acid extracts (n/1, n/10) were positive, as were 2 out of 6 with lactic acid extract.

If experimental osteogenesis research on these lines is to be continued the first object must therefore be a rational attempt at preparing a fairly constantly-active extract. The osteogenetic effect need not be particularly marked at first if only it is biologically well-defined, i.e. occurs in for example 80 to 100 % of the experiments. As long as we may reckon only with an effect in every second, third or fourth injection experiment, and in fact may expect to obtain positive results in control experiments, we have not got a biological method that is applicable in fractionating, concentrating and purifying experiments.

In theory, three possibilities may be imagined as the cause of the lack of constancy in *Annersten*'s experiments:

1. The extraction material may be irrational or inconstant.

If for instance the osteogeneous component is associated solely with the periosteum or the marrow, a constant initial material will be obtained only when the periosteum or the marrow is isolated.

2. The extraction method may be ineffective, and

3. The test employed, with the injection into alcohol-injured muscle tissue, may be unsuitable. *Annersten* himself states that in some cases the tissue reaction failed after the alcohol injection, whereas in other cases there occurred large necroses.

Thus the objects of our investigation are:

1. The separation of the morphological components of the bone, periosteum, diaphysis, corticalis, epiphyseal tissue and marrow, and the extraction of each of these components separately, and

2. Extraction by other mehods and with other solvents than those employed by *Levander* and *Annersten*.

3. An attempt to procure a better test for the injection experiments. Here perhaps young connective-tissue cultures in explantation experiments may be useful; but in particular one should examine whether a traumatic tissue lesion, e.g. forcipressure of rectus femuris, can ensure a more certain growth of young, undifferentiated connective-tissue cells than the injection of alcohol.

10. *A Comparison between Extracts of Total Bone and Extracts of isolated Diaphysis Corticalis, Marrow, Epiphysis and Periosteum.*

The osteogenetically-active principle must either occur ubiquitously in the osseous system or be associated with particular tissue elements within that system. According to the metaplasia theory, which in the case of fractures and transplantations counts on the release of a hormone or an enzyme-like substance, association with certain tissue elements must presumably be the most natural hypothesis. Nevertheless, the author knows of no previous experiments for the purpose of throwing more light on this question.

Annersten's supposition of the chemical relationship of the osteogenetic factor to the foetal induction substances, which presumably may be called steroids, cannot but direct attention particularly to the lipoid components of the bones, i.e. above all the *marrow,* whereas what we know of the continuous building up of the bones, the correlation between breaking-down and rebuilding processes, will perhaps rather point towards the osteocytes, i.e. *corticalis,* at the seat of osteolytic and osteogeneous substances.

According to clinical and roentgenological experience, however, it is difficult to deny the *periosteum* some importance in fracture healing—humeral or cellular, as the case may be; and finally, our knowledge of the growth and development of bone points first and foremost to *epiphyseal regions* and *Haversian canals.*

In the first series of experiments I kept almost solely to *Annersten*'s methods of extraction; but I prepared special extracts of diaphysis corticalis, marrow, periosteum and ephiphysis, having in mind the supposition already mentioned that more constant results from the injection experiments would presumably be obtainable if extraction were more rational, that it to say, from marrow tissue alone, if the osteogenetically-active component is to be found particularly in that tissue.

Bone pulp, periosteum and marrow tissue were not weighed, as quantitative experiments seem not to be indicated as yet; the bone was broken up in a mincer, an together with an estimated quantity of twice or thrice the volume of extraction fluid shaken at room temperature for 16 to 24 hours and then evaporated by hot air current to one-third or one-fourth the volume. The extracts were preserved in a refrigerator until used. The results will be seen from the following tables[1]:

I. *Total extract.*

No.	Pre-injection[2] with 40 % alk.	No. of extract[3] injections	Duration of[4] exper. Days	Result
1 l	+	1	29	÷
5 l	+	1	41	÷
7 r	+	1	49	+
8 l	+	1	20	÷
17 l	÷	1	24	±
22 r	+	1	18	+
24 r	+	5	29	(+)
24 l	+	1	29	(+)
40 l	+	1	39	+
42 l	+	1	52	÷
43 r	+	1	17	÷
43 l	+	1	17	÷

in all 12 experiments: 6 positive, 6 negative (50 %)

---

[1] The age of the rabbits varied between 3 and 6 months. r: right rectus femoris, l: left rectus femoris, rs: right shoulder musculature. No series sections were made as in *Annersten*'s experiments, but macro.-changed tissue was removed and several preparations made from it in paraffin sections. Staining: chiefly Hansen-haematoxylin, also v. Gieson-Hansen, and sometimes Mallory and mucicarmine.

[2] In the cases marked +, 4 c.c. alcohol was injected in the experimental region two to four days before the first extract injection.

[3] For the injections 2 to 5 c.c. of the extract were used, usually undiluted, occasinally diluted with equal parts of physiol. saline.

[4] Reckoned from the first extract injection till section autopsy day.

II. *Marrow.*

No.	Pre-injection with 40 % alk.	No. of extract injections	Duration of exper. Days	Result
2 l	+	1	21	+
7 l	+	1	18	+
9 r	+	1	24	÷
9 l	+	1	24	÷
13 r	+	1	48	+
16 l	+	1	40	+
16 h	+	1	40	+
21 r	+	3	39	+
21 l	+	3	39	+
23 r	+	1	21	+
23 l	+	1	21	+
29 r	÷	2	28	+

in all 12 experiments: 10 positive, 2 negative (83 %).

III. *Corticalis.*

3 l	+	1	21	+
6 r	+	1	18	÷
6 l	+	1	18	÷
10 r	÷	1	24	÷
10 l	+	1	24	÷
13 l	+	1	47	÷
15 r	+	1	26	÷
15 l	+	1	26	÷
19 r	+	1	24	+
19 l	+	1	24	+
22 l	+	1	17	+
29 l	+	2	27	+

in all 12 experiments: 5 positive, 7 negative (42 %).

The tables show that cartilage or bone development was found in 29 out of 60 experiments. The changes, however, were as a rule limited to small islands or streaks in the excised tissue; they are sparse, but still very characteristic. Apart from one or two cases (figs. 2, 3), however, the cartilage found was not

IV. *Epiphysis*[1].

No.	Pre-injection with 40 % alk.	No. of extract injections	Duration of exper. Days	Result
12 r	+	1	32	÷
12 l	+	1	32	÷
12 l	÷	1	32	÷
14 rs	÷	1	26	÷
14 r	+	1	26	÷
14 l	÷	1	26	(+)
17 r	÷	1	23	+
20 r	+	3	31	+
20 l	+	3	31	+
80 r	÷	1	46	÷
80 l	÷	1	46	÷
82 r	+	1	19	÷

in all 12 experiments: 4 positive, 8 negative (33 %).

V. *Periosteum.*

30 r	+	1	21	+
30 l	+	1	21	+
31 r	+	1	35	÷
31 l	+	1	35	+
32 r	÷	3	35	÷
32 l	÷	3	35	+
44 r	+	1	31	÷
44 l	+	1	31	÷
53 l	+	1	39	÷
77 r	+	1	39	÷
77 l	+	1	39	÷
82 l	÷	2	14	÷

in all 12 experiments: 4 positive, 8 negative (33 %).

"mature", largecelled, but small-celled of foetal type with connective-tissue-like cells (fig. 1). Nevertheless, this atypical chondrifications does not prevent the cartilage here and there

[1] The bone extremities were cut off just below the metaphysis. Thus epiphysis and head form part of the extraction material.

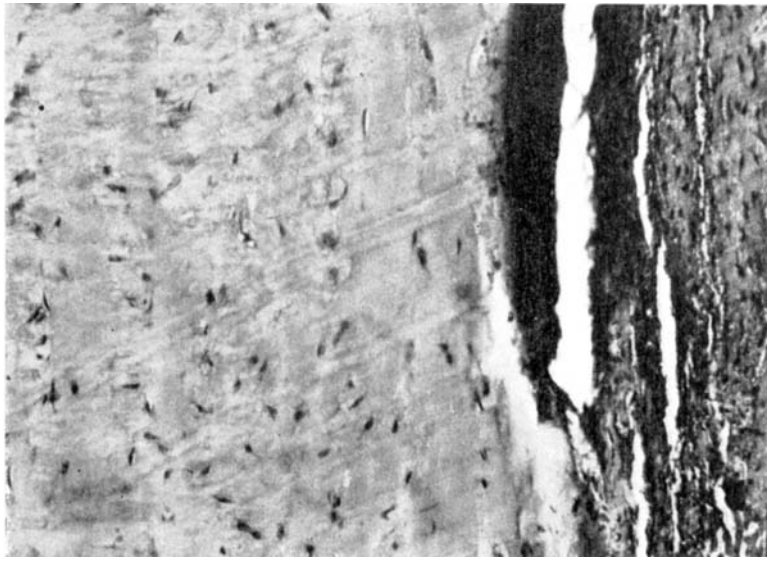

Fig. 1.
Small-celled cartilage. The cells resemble those of connective tissue.

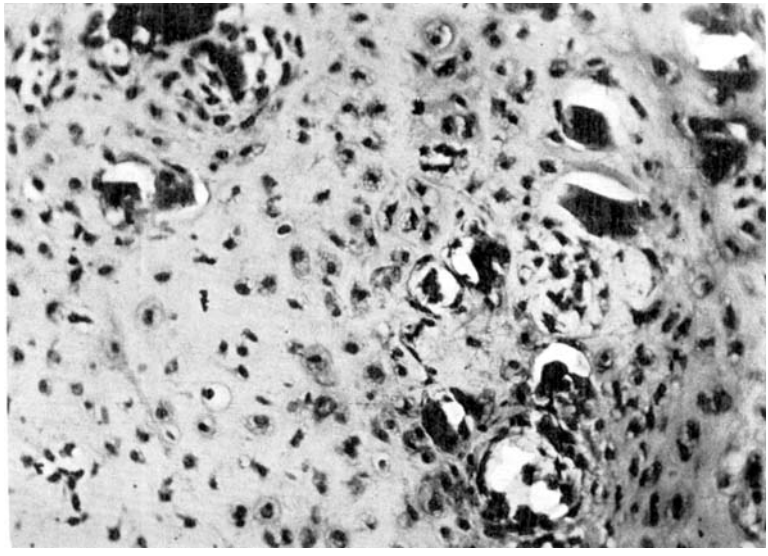

Fig. 2.
Cartilage, with islands of necrotic musculature. More distinct "cartilage capsules".

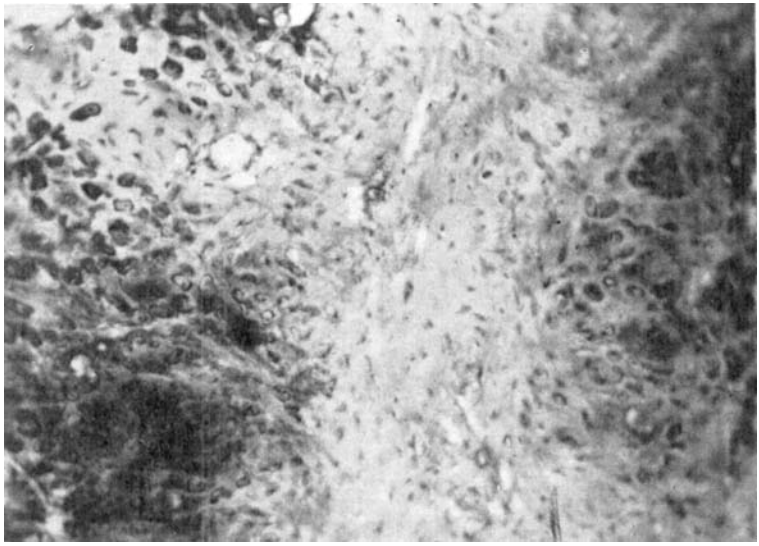

Fig. 3.
Mature, large-celled cartilage with streaks of connective tissue and cartilage of foetal type among the cartilage islets proper.

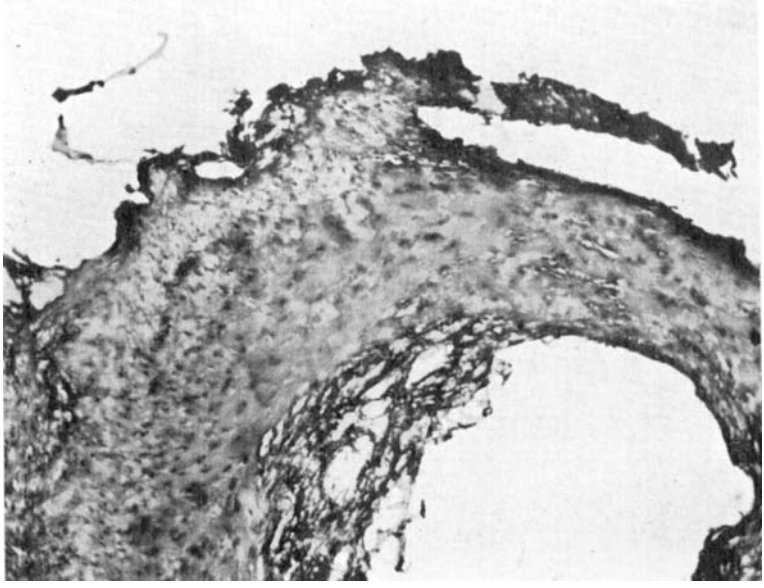

Fig. 4.
Osteoid tissue and osseous tissue.

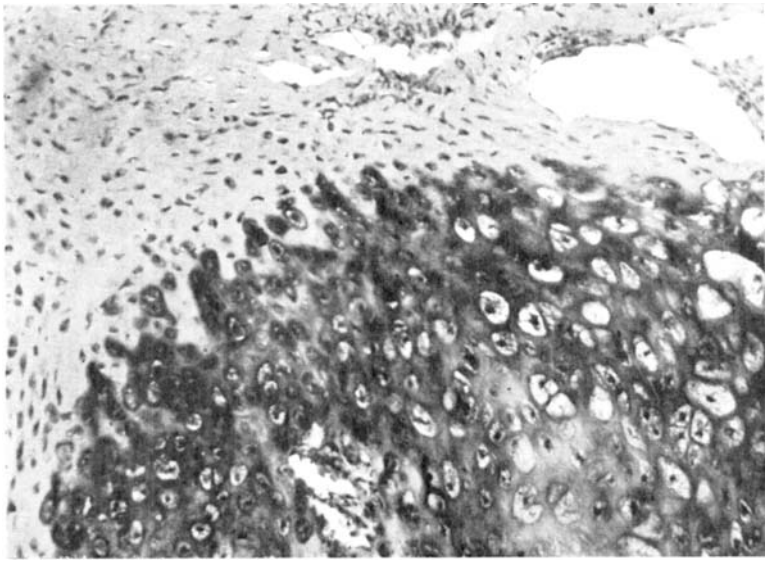

Fig. 5.
Cartilaginous exostosis.

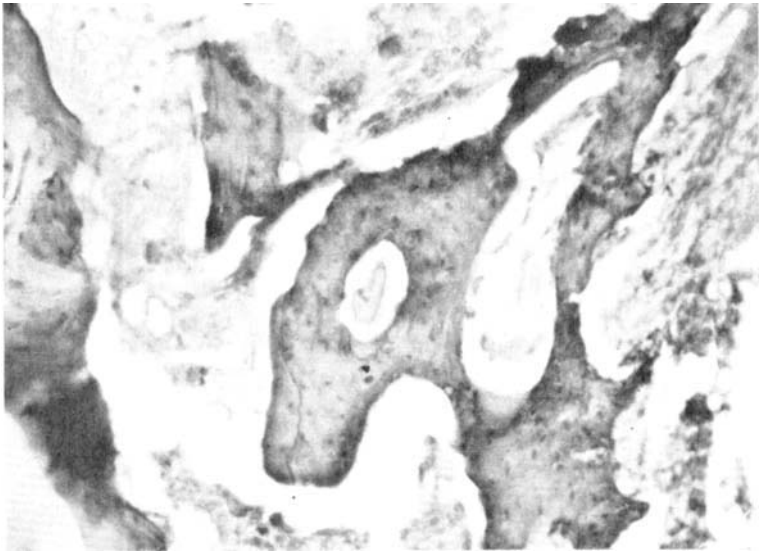

Fig. 6.
Osseous tissue appearing after injection of liver extract.

from passing into osteoid tissue or bone lamellage (fig. 4). It will thus be seen that on the whole the metaplasia conforms more to *Leriche & Policard*'s description than to *Annersten*'s, as the changes take place preponderantly in the matrix, whereas the cells are more or less unchanged. (*Annersten*'s pictures reveal fine large-celled islets of cartilage).

These tables show the much higher frequency with which it was possible to obtain osteogenesis with marrow extract than with extracts from total bone, corticalis, periosteum and ephiphysis.

As it seems justifiable to conclude that *the osteogenetic factor is at any rate chiefly associated with bone marrow, it would be reasonable to assume that the factor is contained rather in the lipoid components than in the albumoid and mineral components.*

In 10 experiments the extract injection was repeated two to five times as in *Levander*'s original experiments. *In 8 of these the result was positive.*

In 13 experiments the pre-injection of 40 % alcohol was omitted. Of these, 6 had a positive result, 7 a negative. The frequency of positive results is thus of the same order as in the total experimental material, which means that *Levander* and *Annersten*'s pre-injection cannot be assumed to be of decisive importance to the result. The mesenchymal reaction can be obtained with the extract alone, which contains sufficient alcohol to cause the requisite lesion in the tissue. In three of this group's positive experiments the extract injection was repeated twice, a fact which must be taken into consideration when appraising this observation.

The *length of the experimental period*[1] in each case is shown in Tables I-V. Reckoned from the first injection of the anticipated osteogenetic extract until the day of autopsy the period varied between 14 and 42 days. For the negative results the period averages 30 days, for the positive 29 days. Accordingly,

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[1] In *Annersten*'s experiments the period was between 8 and 34 days, except for 2 experiments, when the observation period was 80 days.

within the selected experimental period the length of the period of observation does not seem to have any bearing on the result.

11. *Control experiments.*

The following experiments were instituted:

Alcohol 40 %: 7 experiments, all negative (1 r, 2 r, 3 r, 4 r, 5 r, 14 ls, 16 ls); cp. *Levander*: 60 experiments, all negative.

Hydrochlorid acid-alcohol: 10 experiments, all negative (28 l, 28 r, 34 l, 35 l, 35 r, 36 r, 36 l, 37 r, 37 l): cp. *Annersten:* 7 experiments, all negative.

In one of my cases, No. 36 l, a handsome exostosis appeared on the bone, presumably owing to a lesion of the periosteum, but there was no metaplasia of the muscle-connective tissue (fig. 5).

Muscle extract: 5 experiments, all negative. (38 r, 38 l, 39 r, 39 l, 40 r); cp. *Levander*: 20 negative experiments, and *Annersten:* 1 positive and 15 negative.

Liver extract: 9 experiments (41 r, 41 l, 42 r, 50 l, 51 r, 51 l, 52 r, 53 r) of which one was distinctly positive, with fine cartilage, bone, and even marrow formation at the injection site in the musculature (fig. 6). *Annersten* made 6 negative experiments with liver extract.

Bile extract: 2 experiments (66 r, 66 l), both negative.

Suprarenal extract: 2 experiments (67 r, 67 l), both negative.

Brain extract: 2 experiments (68 r, 68 l), both negative.

Ergosterin and D-vitamin (100,000 u., 150,000 and 200,000 u.): 4 experiments (69 r, 69 l, 70 r, 70 l), all negative. Cp. *Annersten:* 12 experiments with phosphatides and cholesterin, all negative.

Thus among 41 control experiments there was one positive case of metaplasia. Positive finds among the control experiments are not inconsistent with the *Levander-Annersten* theory, as already pointed out; but to *Annersten*'s supposition of a circulation of the osteogenetic substance in the blood and excretion through the kidneys we must, having regard to the present observation, add the possibility of excretion through liver and bile. Another

theoretically possible explanation of this curious find of pronounced osseous metaplasia after the injection of liver extract may lie in the chemical constitution of the osteogenetic factor. If the active principle is a relatively simple phosphatide or steroid, it must presumably be liable to occur in lipoid-containing organs. Tentative experiments with suprarenal and brain extracts gave no positive result, however.

12. *Experiments with other Methods of Extraction and Concentration.*

By means of extraction by the *Annersten* method I succeeded only in obtaining weakly-active osteogenetic extracts, the induced changes—including those in the marrow experiments—being quantitatively slight and local. This suggested and endeavour to employ a more effective extraction and a higher concentration of the extract obtained.

Tentative investigations with *ether extraction* of isolated marrow were not encouraging, as 4 experiments (4 l, 8 r, 11 r, 1 l) were all negative. In 2 experiments the mixing of equal parts of *acid alcohol and ether* gave a weak reaction (18 r, 18 l), whereas 2 experiments with 2-normal *lactic acid* (33 r, 33 l) were both negative.

I have also tried a more effective *acid-alcohol extraction* of the raw material than *Annersten*'s method, as follows:

After being broken up the tubular bones of 2 rabbits were treated with 150 ml. alcohol + 50 ml. acetic acid; this material stood for 24 hours, with frequent shaking. It was then centrifuged and treated thereafter with 150 ml. alcohol alone for 24 hours, followed by centrifuging, the liquid then being pipetted off. The sediment was distilled in a Soxleth apparatus with 96 % alcohol for 5 hours; the distillate and the pipetted extract were mixed, where after alcohol and acetic acid were distilled off with the addition of water, whereafter the volume was 75 ml., which was neutralized by titering with NaoH. 12 experiments (54 r, 54 l, 55 r, 55 l, 56 r, 56 l, 57 r, 57 l, 58 r, 58 l, 59 r, 59 l) were all negative.

An attempt to obtain a more powerfull effect by further *con-*

centrating the acid-alcohol extract than by evaporation to about 1/4 was quite negative. We evaporated 130 ml. extract prepared by *Annersten*'s method in vacuum to 18 ml. and, after pre-injecting alcohol 40 %, divided the concentrated extract into 6 experiments (45 r, 45 l, 46 r, 46 l, 47 r, 47 l). Autopsy was made after 25 and 60 days, but in no case were there osteogenetic processes in the injected musculature.

Another 130 ml. was evaporated to drynes and then dissolved in aqueous alcohol. 5 experiments (48 r, 48 l, 49 l, 50 r) were all negative.

The last three series of experiments were performed in conjunction with the chemist *Dr. Tage Astrup*. Their totally negative results are remarkable and can be explained only in two ways:

1) Either *Annersten*'s hypothesis of an osteogenetic factor associated with simple steroid or phosphatide components in the bone substance must be wrong, or

2) Besides the *Annersten* factor the extracts must contain other necessary components, which are inactivated by the methods of concentration and extraction employed.

These components may be of a protein-like nature, or perhaps enzymes (bone phosphatase etc.), or chondroitin compounds and calcium salts, which are precipitated during the processing of the extracts. On the other hand, simple phosphatide or steroid components cannot be expected to be destroyed or inactivated by the processes employed, though perhaps this may happen to lipoid compounds with long side-chains or labile double compounds.

The experimental results published in Chapter 10, showing that experimental chondro- og osteogenesis can be obtained with much greater constancy by the acid-alcohol extraction of marrow tissue than of corticalis, argue *in favour* of *Annersten*'s hypothesis and against the assumption of an unspecific reaction, caused by the addition af calcium- or chondroitin-sulphuric acid to the newly formed connective tissue.

On the other hand, Annersten's observation of chondrification and ossification after non-alcoholic hydrochloric acid and

lactic acid extracts, which cannot be supposed to contain lipoids, but quite possibly chondroitin sulphuric acid and lime salts, might be quoted as supporting this theory of an unspecific genesis of experimental cartilage and bone formation.

In any case, there must be more factors than *Annersten*'s lipoid components, as otherwise concentration would be possible; further investigations into this aspect of the matter are very desirable.

13. *Experiments with other test methods than alcohol-injured musculature.*

The object aimed at by *Levander* and *Annersten* with their injection of alcohol in Rectus femoris is a tissue lesion and the consequent mesenchymal reaction.

However, it must surely also be possible to induce the growth of young undifferentiated connective tissue in other ways, for example by a lesion of the tissue.

This was essayed in the experiments 60 r, 60 l, 61 r, 62 l, 63 r, 63 l, 64 r, 64 l, 65 r, 65 l, 71 r, 71 l, 72 l, 73 h, 73 l, 74 r, 74 l 75 r, 75 l, 76 r, 76 l, in which through a small incision in the soft parts of the thigh I performed *forcipressure of Rectus femoris,* whereafter, two or three days later, the injured tissue was injected with marrow, corticalis, epiphysis and periosteum extracts, prepared in the same manner as in the chief experiments (Chapter 10). In none of these 24 experiments was there the slightest trace of chondro- or osteogenesis, whereas there were strong mesenchymal reactions.

This observation confirms the supposition expressed above that in experimental osteogenesis by the methods of *Levander* and *Annersten* several more factors are at work than the effect of these authors' supposed extractable osseous-tissue inductor. The present experiments make it probable that the alcohol effect itself on the intact muscle tissue in some way or other has a bearing on the positive result of the experiments, but they make no contribution to the explanation of this curious phenomenon.

Of other experiments—also negative—for the purpose of procuring a new test method for the appraisal of the osteogenetic effect of the extracts I shall just mention experiments with tissue cultures. Cultures of heart-fibroblast were employed, and a single drop—or some few drops—of the extracts found to be active in the rabbit experiments was added. All the cultures died in the course of 3 to 5 passages, and microscopy revealed nothing but degenerative changes.

Presumably the alcohol and acid in the extracts killed the cultures, and consequently, similar experiments can only be resumed if it should prove possible to produce extracts devoid of components that are poisonous to the protoplasm.

Finally, in 2 experiments marrow and corticalis extracts respectively were injected into the liver, where the supply of phosphatase is presumably considerable (77 and 79). The preparations showed neither chondogenesis nor osteogenesis.

14. *Summary and Conclusions from Own Investigations.*

The present investigations were promoted by the experiments made by *Levander* and *Annersten*, who have demonstrated that from rabbit and calf bones it is possible to obtain extracts which in periosteum-free regions in rabbits induce osteogenetic processes in 20 to 40 % of the experiments.

Of 60 experiments with acid-alcohol extraction by *Annersten*'s method there was chondrification or ossification in 29.

12 experiments were made with extracts of total bone, 12 with marrow extract, 12 with extract of corticalis, 12 of epiphysis and 12 of periosteum.

Within these groups the positive results number 6, 10, 5, 4 and 4 respectively, that is to say with a distinctly higher frequency in the experiments with marrow extract. Among 41 control experiments one was positive; in this case the extract was from liver tissue.

In the case of Soxleth extraction with acid-alcohol (12 experiments) and concentration in vacuum (11 experiments), all

the results were negative, as were 24 experiments in which by means of forcipressure of Rectus femoris a strong mesenchymal reaction had been induced prior to the injection of the extract.

It is thus possible to confirm *Levander*'s and *Annersten*'s reports on the production of osteogenetically active preparations by extracting osseous tissue with hydrochloric acid-alcohol, and to supplement this observation with the main result of the present investigation: that marrow extracts are more constantly active than extracts of the other bone components.

The fact that a concentration by evaporation in vacuum, as also a protracted extraction in the Soxleth apparatus, involves constantly negative experimental results, nevertheless argues against *Annersten*'s hypothesis of an inductor substance of the stearine group as the (sole) active factor in the extracts.

Presumably it is necessary to reckon with several concurrent factors, all essential to metaplastic osteogenesis, in which connection the author suggests i.a. chondroitin compounds and calcium salts dissolved by the acid content of the extraction fluid.

Zusammenfassung und Konklusion Eigener Untersuchungen.

Die vorgelegten Untersuchungen nehmen ihren Ausgangspunkt in *Levander*'s und *Annersten*'s Versuchen, durch welche der Beweis erbracht worden ist, dass sich aus Kaninchen- und Kalbsknocheln Extrakte herstellen lassen, die in periostfreier Region bei Kaninchen in 20—40 % der Versuche osteogenetische Prozesse hervorrufen.

In 29 von Versuchen mit Säurealkoholextraktion nach *Annersten*'s Methode wurde Knorpel- oder Knochenbildung erlangt.

12 Versuche wurden mit Extrakten von ganzen Knocheln ausgeführt, 12 mit Markextrakt, 12 mit Extrakt von Corticalis, 12 von Epiphyse und 12 von Periost.

Die positiven Resultate verteilen sich in diesen Gruppen mit 6—10—5—4—4, d.h. mit einer augenfällig grösseren Häufigkeit in den Versuchen mit Markextrakt.

Unter den 41 Kontrollversuchen war einer positiv. Es handelte sich um ein Extrakt von Leber.

Bei Soxleth-Extraktion mit Säurealkohol (12 Versuche) und bei Konzentrierung in Vakuum (11 Versuche) waren sämtliche Versuche negativ, wie auch 24 Versuche, in denen man durch Forcipressur von Rectus femoris eine kräftige mesenchymale Reaktion hervorgerufen hatte, alle negativ ausfielden.

Man kann somit *Levander*'s und *Annersten*'s Mitteilungen von der Möglichkeit, osteogenetisch-wirksame Präparate durch Extraktion von Knochengewebe mit Salzsäurealkohol herzustellen, bestätigen und diese Beobachtung mit dem Hauptergebnis der gegenwärtigen Untersuchung ergänzen: Die Markextrakte sind konstanter wirksam Extrakte als den übrigen Bestandteilen des Knochens.

Der Umstand, dass eine Konzentrierung beim Eindampfen in ein Vakuum ebenso wie eine länger andauernde Extraktion im Soxlethapparat konstant negative Versuchsresultate ergibt, spricht indessen gegen *Annersten*'s Hypothese von einem Induktorstoff der Stearingruppe als (einzigen) wirksamen Faktor in den Extrakten. Vermeintlich muss man mit mehreren zusammenwirkenden Faktoren rechnen, die alle für die metaplastische Osteogenese notwendig sind, und man hat in diesem Zusammenhang u.a. auf Chondroitinverbindungen und Kalksalze, die mit dem Säuregehalt der Extraktionsflüssigkeit in Auflösung gebracht wurden, hingewiesen.

Résumé et Conclusions se basant sur des recherches personelles.

Les recherches dont il est rendu compte ici ont leur point de départ dans les essais de *Levander* et d'*Annersten* qui ont démontré que l'on peut, des os de lapins et de veaux, produire un extrait susceptible de provoquer chez les lapins, dans les régions dépourvues de périoste, des processus ostéogénétiques dans 20 à 40 % des cas d'essais.

Sur les 60 essais pratiqués à l'acide-alcool d'après la méthode d'*Annersten*, on a obtenu la formation de cartilage ou d'os dans 29.

12 essais furent effectués avec des extraits d'os entier, 12 avec de l'extrait de moelle, 12 avec de l'extrait de substance corticale, 12 avec de l'épiphyse et 12 avec du périoste.

Les résultats positifs obtenus dans ces différents groupes se répartissent comme suit: 6—10—5—4—4, ce qui revient à dire que les essais pratiqués avec l'extrait de moelle donnent le plus grand nombre de résultats positifs.

Parmi les 41 cas de contrôle, un était positif. Il s'agissait d'un extrait de foie.

Les essais pratiqués par extraction-Soxleth à l'acide-alcool (12) et par concentration au vacuum (11) furent tous négatifs, de mêne que 24 essais dans lesquels on avait provoqué une forte réaction mésenchymale par forcipressure du rectus femoris eurent aussi un résultat négatif.

On peut ainsi confirmer les communications faites par *Levander* et *Annersten* sur la possibilité de produire des préparations ostéogénétiques actives par l'extraction à l'acide-alcool de tissu osseux et de compléter cette observation par le résultat principal des présentes recherches, à savoir que les extraits de moelle ont une efficacité constante que les extraits d'autres éléments osseux.

Le fait que la concentration par l'évaporation au vacuum et que l'extraction de plus longue durée dans l'appareil Soxleth donnent des résultats d'essais négatifs constants, parle toutefois à l'encontre de l'hypothèse d'*Annersten,* selon laquelle une matière inductive du groupe des stérines serait le (seul) facteur actif des extraits. Il est probable qu'il existe plusieurs facteurs qui agissent de concert et qui sont tous nécessaires à l'ostéogenèse métaplastique et, à cet égard, on a attiré l'attention entre autres sur les combinaisons de chondroïtine et les sels calciques dissous par l'acide du liquide d'extraction.

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