The Bone Induction Principle

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Reprinted from Clinical Orthopaedics No. 53

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When cellular differentiation is attributed to the physicochemical effect of one tissue upon and in contact with another, the mechanism is known as *induction.* When a tissue transmits the *bone induction principle,* a complex entity of unknown character, it produces differentiation of osteoblasts, and the process is called *bone induction.* Like an embryonic induction system, a bone induction system is composed of inducing cells and responding cells. An inducing cell is a cell that has had contact with the bone induction principle and induces a responding cell to differentiate into an osteoblast. The responding cell usually is a perivascular hypertrophied mesenchymal cell, and it becomes an induced cell when it differentiates into an osteoblast, a chondroblast, or a hematocytoblast.

Once a bone induction system is established, one layer of induced cells may become the inducing cells for the next layer of responding cells. Induction occurs in two directions: centrifugally, to produce lamellar bone; centripetally, to produce new bone marrow cells. The process continues until undifferentiated cells produce a large population of newly specialized cells arranged in the form of an organ. Thus, a bone induction system is a mechanism of organogenesis, that is, the development of an ossicle filled with bone marrow.

New concepts of embryonic induction are presented in important books by Ebert,[18] Brachet,[6] Jacobson,[30] Saxen and Toivenin,[44] Grobstein,[22] Wright,[65] Waddington[60] and Spratt.[47] Skeletal tissue induction is discussed in articles by Lash,[35] Bassett,[5] Burwell,[12] Moss,[41] Ray,[43] Young[67,68] and others.[7,8,45,50-58] During the past year one observation—the fact that the bone induction principle persists in the organic matrix of bone tissue free of living cells—opened the way for some important advances. In previous years, bone induction was described in tissue cultures and transplants of living cells, but the main interest was in whether the new bone arose from progeny of preexisting bone cells or from emigrant mesenchymal cells.[43] In recent years, guided by the Waddington modification of the Jacob-Monod hypothesis[29] and basic concepts of

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This work was aided by contracts between the U.S. Army Research and Development Command (DA-49-193MD2556), the Atomic Energy Commission (AT-11-1)-34 and the University of California; and in part by grants-in-aid from the USPHS, National Institute of Dental Research (No. DE-02103-01), William H. Donner Foundation Inc., Ayerst Laboratories Inc., and Orthopedic Research and Education Foundation.

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cell macromolecular biology,[61] attention was diverted away from the question of cell origins. It has become more realistic to assume that, irrespective of its origin, a proliferating, young, undifferentiated connective tissue (mesenchymal) cell, in the body of a bony vertebrate, has all the essential genetic machinery (inherited capacity) to proliferate, differentiate and to specialize as a cartilage, a bone or a bone marrow cell.[56,57] How this capacity could have been elicited in postfetal life and what kind of experiments have been designed to determine the nature and the site of action of the enigmatic bone induction principle will be related in this article.

The bone induction principle is demonstrated by simple tissue implantation experiments. It is a specific entity only in the sense that it is more consistently demonstrable and more active in an implant of decalcified *lyophilized* bone matrix than any other tissue.[50] It is unspecific in the sense that other matrices derived from dentin, cartilage, urinary bladder epithelium, etc. (tissues developed from chorda mesoderm anlage) can produce similar effects with lower percentages of positive results and yields of new bone. It is unspecific, also, in the sense that it may produce differentiation of tissues other than bone, i.e., cartilage, bone marrow, adipose and fibrous tissues. Present knowledge of either its chemical nature or of its mode of action is unsatisfactory, but most of the evidence we have seen to date points to the conclusion that it directs *competent hypertrophic* mesenchymal cells into one or another line of specialization, like the channel selector on a television set. The channel, in the terms of modern embryology, is called the *pattern* or *field* of morphogenesis,[46,62] and it is observed ordinarily through the laboratory methods of classic transplantation biology.

LYOPHILIZED BONE MATRIX

An implant of devitalized, *decalcified,* lyophilized bone matrix produced new bone very consistently in amounts proportional to the volume of the tissue. The percentage of positive results was as high as over 90% whenever attention is given to the following details.

Methodology. Long bones, excised from adult rabbits, rats and other laboratory animals, were cut in lengths of 1.5 cm. and decalcified, unfixed, in 0.6N HCl 1.0 Gm. of bone per 100 ml. of solution for not more than 24 hours. The acid was removed by washing in sterile 0.15M NaCl or 70% alcohol. Then matrix was frozen in liquid nitrogen at —70° C. and dehydrated *in vacuo* in a lyophilizing apparatus. The chemical composition of a typical sample of freeze-dried matrix from a Belgian rabbit, in mM./Kilo, was approximately: total Ca, 4.4 ± 2.6; total P, 17.5 ± 3.0; Na, 16.3 ± 1.8; hexosamine, 41.4 ± 11.5 (mean and standard deviation); in percentage dry weight, total N is 4.4 ± 0.5.

In experiments performed in the laboratory and the operating rooms during the past 4 years, bone induction was observed in implants of autologous, isogeneic, allogeneic and xenogeneic matrix of such composition in: (1) a pouch in the belly of the rectus abdominus, the quadriceps, or the erector spinae muscles of approximately 500 rabbits, 100 rats, 10 mice and 5 guinea pigs; (2) a defect in the ulna in 10 rabbits or a bed of bone in the lumbar vertebrae in 3 dogs; (3) a defect in a bone in various skeletal system disorders in 21 human beings. Bone induction was always greatly retarded when the matrix was cryolysed (frozen and thawed 3 times) or contaminated with infectious bacterial organisms. Xenogeneic matrix produced an adverse immune response, as will be noted later. Implants of glass, rubber and various plastics always were negative in abdominal wall muscles in rabbits[58] observed for periods as long as 8 to 12 weeks.

Matrix decalcified with ethylenediamine tetraacetic acid (EDTA), or 1:1 formic and citric acids, produced osteogenesis in the same way as did matrix decalcified with HCl,

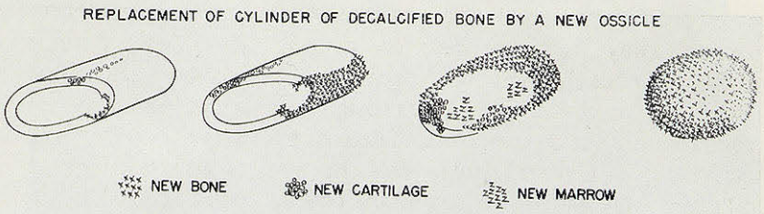


FIG. 1. Diagrammatic representation of process of bone induction by 0.6N HCl-decalcified lyophilized bone matrix. New bone appears at about 25 days; new bone and cartilage replace 30% of the volume of the implant at 4 to 5 weeks; hematopoietic bone marrow appears in the central cavity at 8 to 10 weeks; irrespective of the shape of the original implant, at 12 weeks it develops into a bone with the shape of a sphere filled with bone marrow. (Science 150:893, 1965)

but EDTA may have produced a slightly lower percentage of positive results; nitric or nitrous acids completely destroyed the bone induction principle; lactic acid (0.6N) did not remove all the mineral and also seemed to have adversely altered bone matrix, increased inflammation and prevented bone induction.

Sequence of Events Leading to Bone Induction. Within 1 hour after the operation, each implant of decalcified lyophilized bone matrix floated in a pool of serosanguineous fluid that persisted for a period of approximately 12 days. Except that the fluid was high in protein and leukocytes, the chemical composition was the same as that of an ultrafiltrate of mammalian plasma. Through this fluid, leukocytes and wandering histiocytes swam in gradually increasing numbers across the pool and into the marrow-vascular and microcanalicular channels to repopulate all the available spaces in the old matrix.

After the period from 10 to 12 days, the fluid was absorbed and replaced by an envelope of inflammatory connective tissue: i.e., wandering histiocytes or macrophages, large and small lymphocytes and fibroblasts. The old matrix became swollen and amorphous in appearance when stained with hematoxylin and eosin and was slightly metachromatic with toluidine blue. Collagen fiber bundles

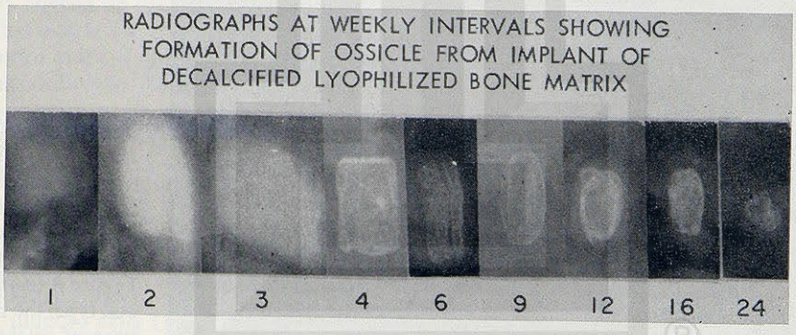


FIG. 2. Roentgenograms, showing various stages of bone induction by decalcified lyophilized matrix at various intervals from 1 to 24 weeks after implantation in the anterior abdominal wall of a rabbit. Between 1 and 3 weeks, the *radiodensity of the soft tissue* increases, owing to the ingrowth of cells of the host bed. Between 4 and 12 weeks, the implant is replaced by calcified new bone and bone marrow. At 12 weeks the new bone consists of a sphere-shaped ossicle. If it is induced by allogeneic matrix, the ossicle begins to be resorbed at 12 to 16 weeks and is steadily reduced in size over a period of 6 to 9 months.

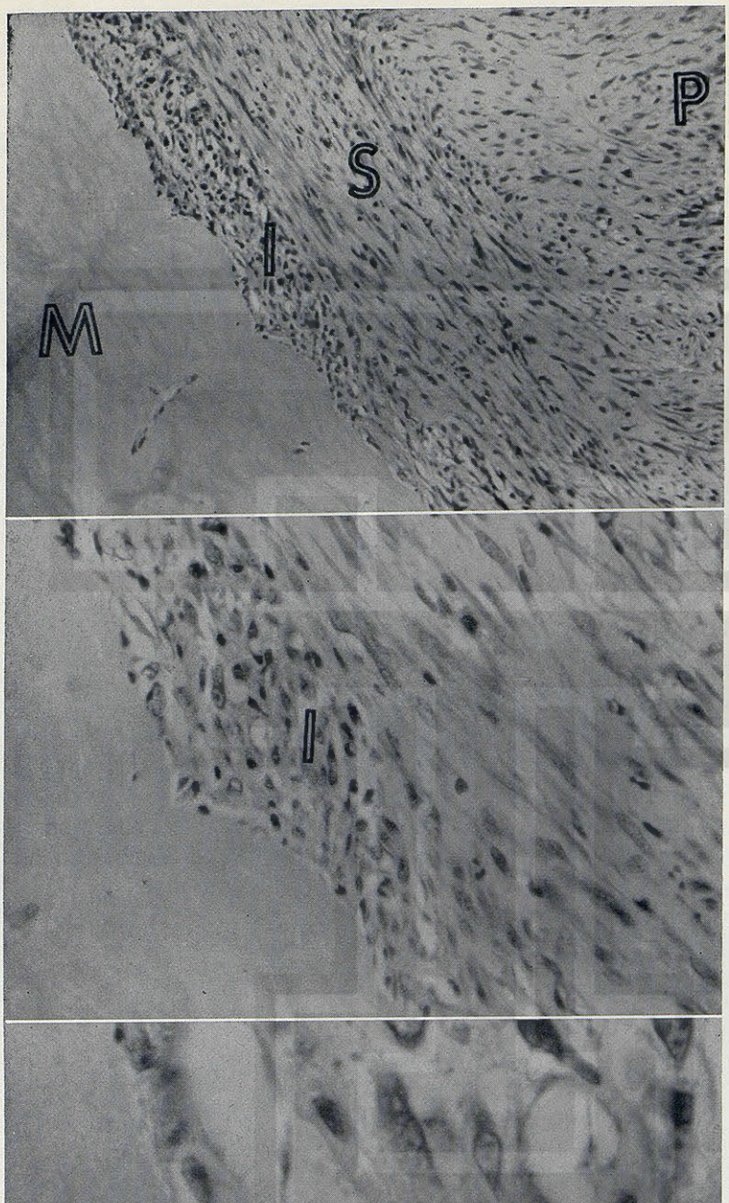


FIG. 3. (*Top*) Photomicrograph, showing tissues in the interior of an implant of 0.6N HCl-decalcified acellular bone matrix (M) 22 days after the operation. Note the stratification of the cells of the host bed: pool of mesenchymal cells, monocytes, lymphocytes, plasma cells and reticulocytes (P); layer of spindle-shaped connective tissue cells (S); zone of hypertrophied mesenchymal cells with basophilic cytoplasm (I). (Hematoxylin-eosin and azure stain)

(*Center*) Photomicrograph, showing zone of cell hypertrophy (I) in the interior of the implant shown in Figure 3 (*top.*) Note the random arrangement of the cells and the relatively large increase in the amount of cytoplasm.

(*Bottom*) Oil immersion lens, high-power photomicrograph, showing the 2 main cell types in the zone of cell hypertrophy (I) shown in Figure 3 (*center*). In one cell type, the ratio of volume of nucleus to cytoplasm is high, and the rate of cell division is rapid. In the other, the ratio of volume of nucleus to cytoplasm is low, and there is highly basophilic cytoplasm, indicative of accumulation of ribosomal membranes.

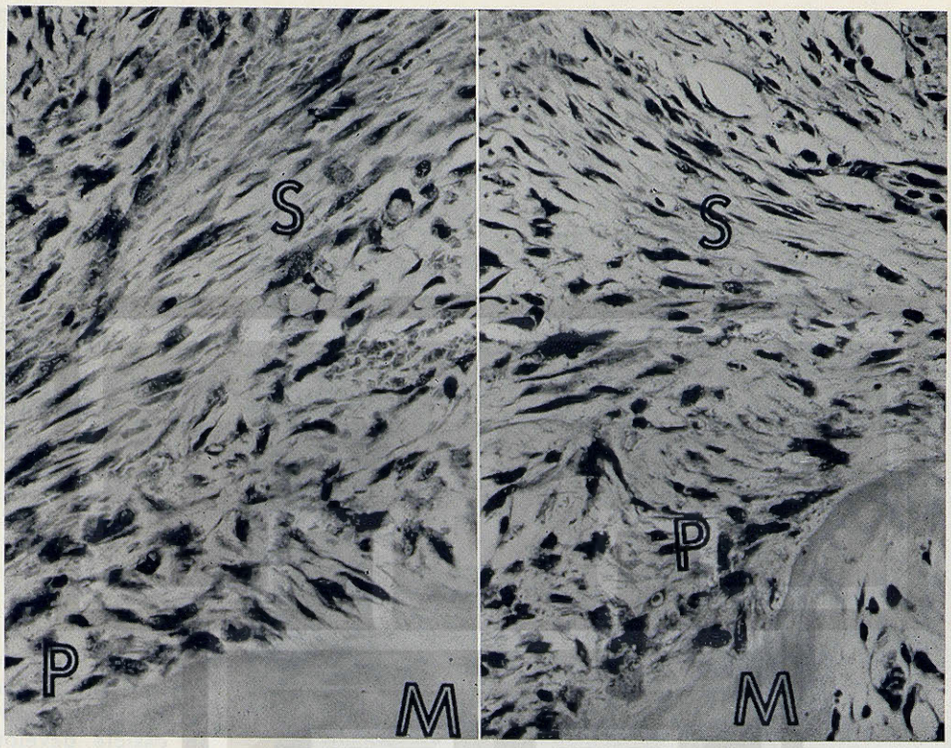
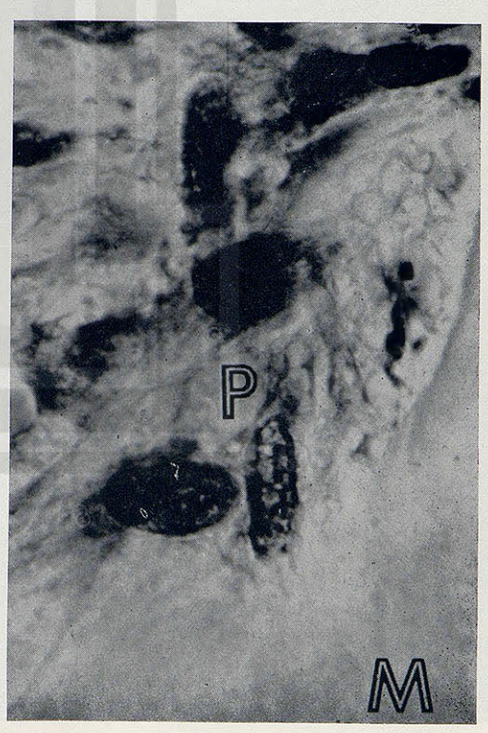


FIG. 4. (*Top, left*) Photomicrograph, showing early stages of bone induction in an implant of 0.6N HCl-decalcified bone 23 days after the operation. Note spindle-shaped fibrous connective tissue, blood vessels and rapidly dividing mesenchymal cells (S); palisades of densely basophilic cells (P) aligned on the surface of the old decalcified matrix (M); the palisaded cells are induced cells, if this picture is interpreted according to the theory of induction.

(*Top, right*) Photomicrograph of another area of the same section shown above (*left*), showing the changes in size, shapes, alignment, and staining characteristics of the cells on the surface of decalcified bone matrix (M). The zone (S) contains many capillary sprouts. The first layer of induced cells (P) is always in direct contact with surface of the matrix.

(*Bottom*) Photomicrograph; oil immersion, showing the coarse chromatin granules in the nuclei of the induced cells (P) and the intimate irregular line of contact of the cells with the old matrix (M). A loose network of new collagen fibrils, hranching through a foamy loose ground substance, attaches the induced cells to the old collagen of bone matrix.



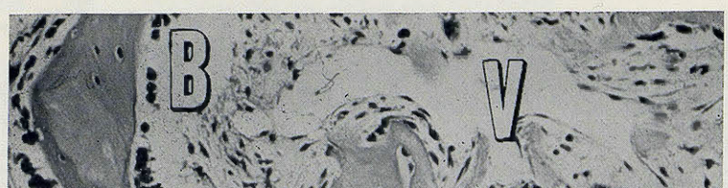


FIG. 5. (*Top*) Photomicrograph of an implant 4 weeks after the operation in a rabbit, showing palisades of osteoblasts and the first deposits of new bone (B) on the surfaces of old decalcified matrix. The marrow vascular spaces are repopulated with wandering and fixed histiocytes (H). Dilated blood vessels (V) are surrounded by spindle-shaped mesenchymal cells.

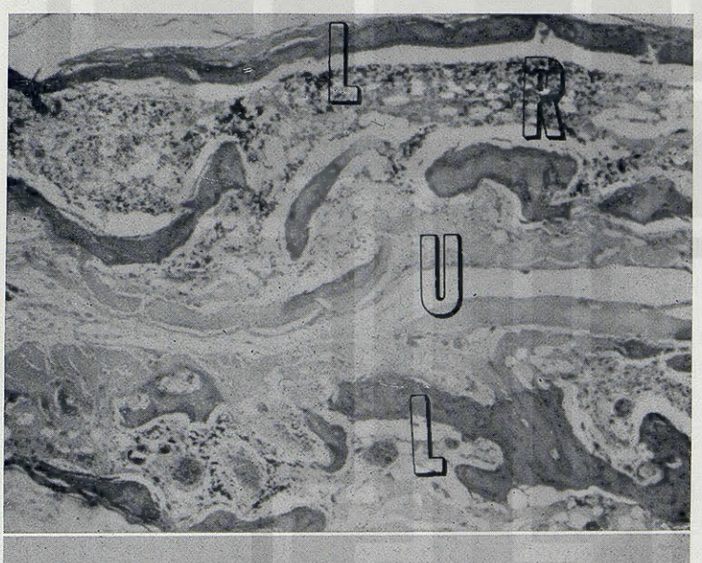


FIG. 6. (*Center*) Photomicrograph showing low-power view of an ossicle developed from an implant of decalcified bone matrix 8 weeks after the operation: new lamellar bone (L), red bone marrow (R), unabsorbed remanents of old matrix (U).

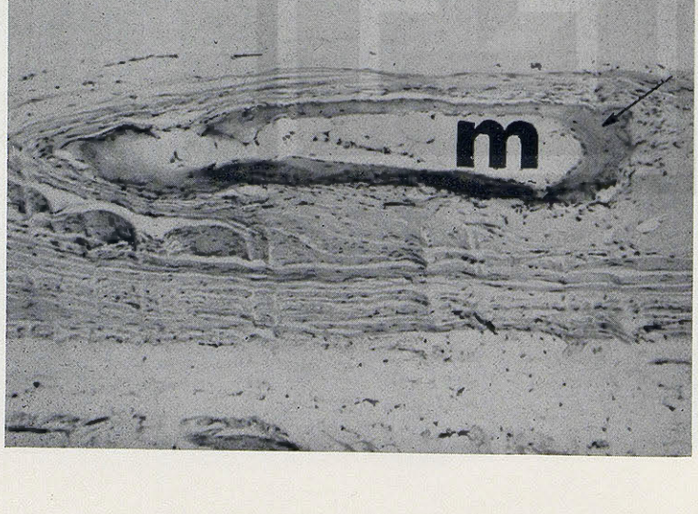


FIG. 6. (*Bottom*) Photomicrograph, showing ossicle developed from an implant of decalcified lyophilized allogeneic bone 10 months after the operation. Note compact bone (*arrow*) formed from remodeled lamellar bone and yellow fatty bone marrow (m) formed from red bone marrow.

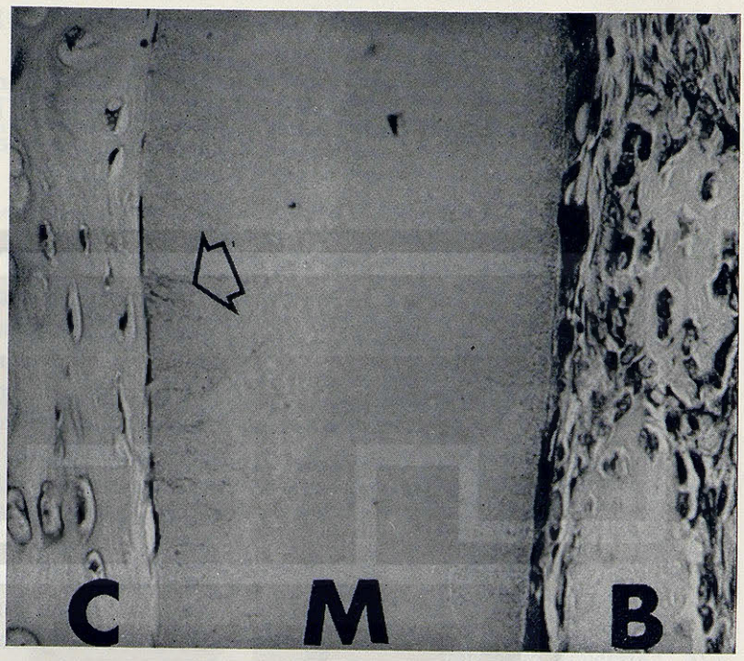


FIG. 6 (*Cont.*) Photomicrograph, showing bone induction by cartilage and/or chondro-osteoid (C) developed from a mixture of minced viable muscle and non-viable bone matrix. Note cartilage and chondro-osteoid (C) with cell extensions (*arrow*); millipore microweb filter 150 μ in thickness, pore size 0.45 microns; (M) new lamellar bone (B). (Hematoxylin-eosin-and-azure stain)

remained clearly distinguishable and surprisingly intact with Wilder's stain. The donor cells underwent autolysis, because the osteocyte lacunae were enlarged and empty, except for random dead cell remnants and nuclear debris. Such living cells as were found inside the implant were motile cells, derived from the host: i.e., leukocytes, wandering histiocytes and fixed histiocytes.

The emigrant cells were arranged in 3 population groups. One was swarming in excavation chambers produced by the proteolytic activity of macrophages, the gathering of foreign-body giant cells, the multiplication of young connective-tissue cells and the pressure of sprouting capillaries. Another was found between ribbons of disintegrating collagenous matrix and consisted of giant cells, inflammatory round cells, dilated blood vessels and fibrinous exudate. Another appeared in closed ends of smooth-walled, old, vascular channels of the decalcified matrix or between folds of compressed, softened matrix and sometimes developed nests of proliferating cartilage cells.

The earliest deposits of new bone appeared at 24 to 26 days from a single layer of mesenchymal cells that hypertrophied on the walls of a well-vascularized excavation chamber. The new bone was remodeled by osteoclasts and redeposited by new osteoblasts as soon as it was laid down. Bone did not develop in areas occupied by plasma cells, small lymphocytes, large dilated capillaries or multinucleated cells associated with inflammation. Wherever bone induction occurred, the induced field consisted of clusters of capillaries, surrounded by layers of mesenchymal cells, covered with palisaded, deeply basophilic cells with a high ratio of volume of cytoplasm to nucleus. At 21 to 24 days, before bone appeared, the matrix was ensheathed in hypertrophied cells *competent* to synthesize and secrete proteinaceous matrix. After 24 days, these cells deposited layers of collagenous matrix; the new bone was separated from the old, dead, decalcified matrix by a film of cement substance. The process continued until excavation chambers were filled with lamellar bone and bone mar-

TABLE 1. Efferent Time and Cell Population Relationships in Bone Induction in Rabbits

Days	Predominant Host Cells in Interior of Implant	Incidence of Bone Induction (Per cent)
0	No cells	0
5	Polymorphonuclear leukocytes, lumphocytes, old erythrocytes, wandering histiocytes	0
13	Lymphocytes, plasma cells, wandering and fixed histiocytes, giant cells, fibroblasts	0
21	Wandering and fixed histiocytes, sprouting capillaries, perivascular connective tissue cells, giant cells, progenitor cells, "osteoblastlike palisaded polarized" cells	0
24	Osteoblasts, osteocytes, new bone, chondrocytes, new cartilage	80
28	Same as above, plus osteoclasts and bone remodeling cells	94
42	Same as above, plus red hematopoietic bone marrow	98
112	Resorption and regression of bone tissue transformation of red to fatty yellow bone marrow	100

row and never extended outside the interior of the implant. Bone formation always was strictly confined within the periosteumlike envelope of fibrous tissue around the implant (Figs. 1 to 6).

Tables 1 to 16 summarize the results of some experiments dealing with factors that either permit or prevent bone induction by bone matrix. Arbitrarily, we classified as *efferent* those contributed by the recipient (Tables 1 to 6), and *afferent* those introduced with the implant of inducing tissue (Tables 7 to 16) and simply recorded the percentage of positive results obtained in implants of 10 to 100 samples of various forms of matrix in the anterior abdominal wall of rabbits or rats. By this means we were able to record the evidence of the presence or the absence of the bone induction principle in a wide variety of experimental conditions.

Cell Population

Table 1 illustrates the time schedule of the appearance of various cells that appeared in the marrow vascular spaces and the excavation chambers in an implant of 0.6N HCl-decalcified lyophilized matrix in rabbits. A large population of cells, with the competence to secrete collagenolytic enzymes and the competence to respond to the bone induction principle developed at 21 days. The first layer of bone cells differentiated at about 25 days, and the first deposits of lamellar bone were arranged as shown in Figures 1 to 6.

In order to determine whether transfer of the bone-inducing principle and development of competence depends on earlier tissue reactions, implants were excised and replaced with silastic sponge during the preosseous phase at various early intervals after the operation. In another experiment, the pool of serous fluid around the implant was aspirated at daily intervals to determine whether it played an essential role in the process. Removal of serous fluid did not prevent bone induction.

When the implant was peeled out at 5 days, when the recipient site contained inflammatory connective tissue cells or at 7, 10 and 15 when it contained rapidly dividing mesenchymal cells, at 30 days there was no bone induction. At 20 days, the implanted matrix was attached to the recipient so intimately that it was impossible to separate it out or disengage it from its core of reactive tissue. These results suggested that before 3 weeks the cells in the envelope of inflammatory and the proliferating tissue were either incompetent or uninduced to differentiate into osteoblasts. Apparently, only post-

TABLE 2. Transfilter Tests for Bone Inductian in Rabbits

Contents of Millipore Chamber	Purpose	Product After 4 Weeks
Warm viable autologous bone	To determine whether the cells of the host would be induced to produce bone on the outer side of the millipore membrane	Culture of small amount new bone only inside of chamber
Warm viable allogeneic bone	To determine whether the cells of the host would be induced to produce bone on the outer side of the millipore membrane	Culture of small amount new bone only inside of chamber
Viable xenogeneic bone (rat)	To determine whether the cells of the host would be induced to produce bone on the outer side of the millipore membrane	No new growth
Lyophilized 0.6N HCl decalcified allogeneic bone	To determine whether the cells of the host would be induced to produce bone on the outer side of the millipore membrane	No new growth
Lyophilized 0.6N HCl decalcified allogeneic bone in millipore chamber with a fracture of the membranes	To determine whether admission of host cells and contact with implanted matrix would produce bone inside the millipore chamber	Development of large deposit of new bone inside the chamber

mitotic cells with a hypertrophied basophilic reactive ergastroplasm were competent and responsive to the bone induction principle in the implant. To establish the validity of these results, these experiments were performed with implants heavily labeled with radioactive phosphorus, proline, glycine, uridine and other substances that conceivably might be transferred locally from matrix to competent cells.

Diffusion and Contiguity

Table 2 illustrates the results of 5 experiments with preparations of either normal viable spongiosa or nonviable, undecalcified and decalcified, lyophilized bone matrix, enclosed in millipore chambers and implanted in the anterior abdominal wall of the rabbit. The millipore membranes were HA hydrosal filters of 0.45 micron pore width and 150 microns in thickness. The chambers and the surrounding muscle tissues were excised at intervals of 4, 8 and 12 weeks after the operation. A small amount of new bone formed inside the chamber containing viable autologous bone. Some fibrous tissue and possibly also a very scanty deposit of osseous tissue grew in the chamber containing allogeneic bone. No growth of any kind formed in the chamber that contained xenogeneic bone.

There were no lymphocytes or plasma cells in the envelope outside the chamber to indicate that a soluble antigen diffused from the implant into the lymphatic system of the recipient. Neither was there a culture of xenogeneic cells inside the chamber to indicate that the body fluids of the recipient could promote differentiation other than that of autologous and allogeneic cells.

No bone cells were found in the millipore chambers containing lyophilized decalcified allogeneic bone matrix. In 2 instances there was a fracture in the millipore membrane; in these animals, the cells streamed through the opening to produce a deposit of new bone inside the chamber at points of coaptation of living cells and resorbed surfaces of lyophilized decalcified bone matrix. These results suggested that the bone induction principle was not soluble and diffusible through thick (150 micron) millipore membranes. The observation of Grobstein[22,23] and Cooper[13] that living inducing cells on

TABLE 3. Effect of Loading Macrophages (Wandering Histiocytes) on the Efferent (Host-mediated) Limb of the Mechanism of Bone Induction

Agent	Concentration (Per cent)	Route of Administration	Incidence of Bone Induction (Per cent)
Charcoal, bovine bone	2	Intra-arterial	60
Trypan blue	1	Intra-arterial	8
Thorium dioxide	24	Intravenous	0

one side of a membrane may transmit an inducer to embryonic responding cells on the other side of a thin (25 micron) millipore membrane would not exclude the possibility of interaction through cell secretions. Such preparations are now being examined with the electron microscope to search for desmosomes or attachment bodies across the pores of the membrane.

Bone Induction Across Millipore Membranes. From the calvaria of newborn isogeneic and allogeneic mice, Goldhaber[20a] and Post and associates[42a] were able to grow new bone inside of millipore diffusion chambers. On the external surface of the millipore membrane, directly opposing the layer of cultured bone, there was a second deposit of new bone. The membrane between the 2 deposits contained a feltwork of basophilic material, which all observers viewed as consisting possibly of pseudopodialike extensions of cytoplasm. In experiments in rabbits by K. Büring and Urist (in press, 1967) with millipore chambers filled with decalcified dead lyophilized bone matrix and live muscle mesenchymal cells, we noted new *cartilage induction* and new bone inside and bone induction on an opposing surface outside the millipore membrane. Where the new tissues were not in close contact with each other on opposing surfaces of the millipore membrane, there was no basophilic material in the wall of the membrane, and there was no bone induction.

Millipore chambers filled with decalcified bone matrix alone, or muscle mesenchymal cells alone, did not induce bone either inside or outside the millipore membrane. Electron micrographs may answer the question whether the feltwork of basophilic material is ground substance or pseudopodialike cytoplasmic extensions through which there is transmission of the bone induction principle. For the present it would seem that the bone induction principle is not freely diffusible and normally moves through ground substance only short distances along or between cell membranes, or extensions thereof (Fig. 6 c).

Phagocytosis

Table 3 illustrates the results of experiments in which the host received intra-arterial and intraperitoneal injections of colloidal suspensions of charcoal, trypan blue and thorium dioxide to load the histiocytes (macrophages) foraging in the interior of an implant of decalcified lyophilized bone matrix. The object was to determine, first, whether it was possible to label histiocytes and their progeny with phagocytosed particles; and, second, whether cell populations engaged in phagocytic activity simultaneously could phagocytose[59] or resorb[64] bone collagen and respond to the bone induction principle of bone matrix. The results demonstrated that a 2% suspension of bovine bone charcoal and a 1% solution of trypan blue produced a large population of phagocytes and retarded bone induction. From 2 to 6.0 cc. of a 24% suspension of thorium-dioxide injected intravenously almost prevented bone induction; cells loaded with particles of ThO_2 were everywhere inside and around all the old marrow vascular spaces of the implant.

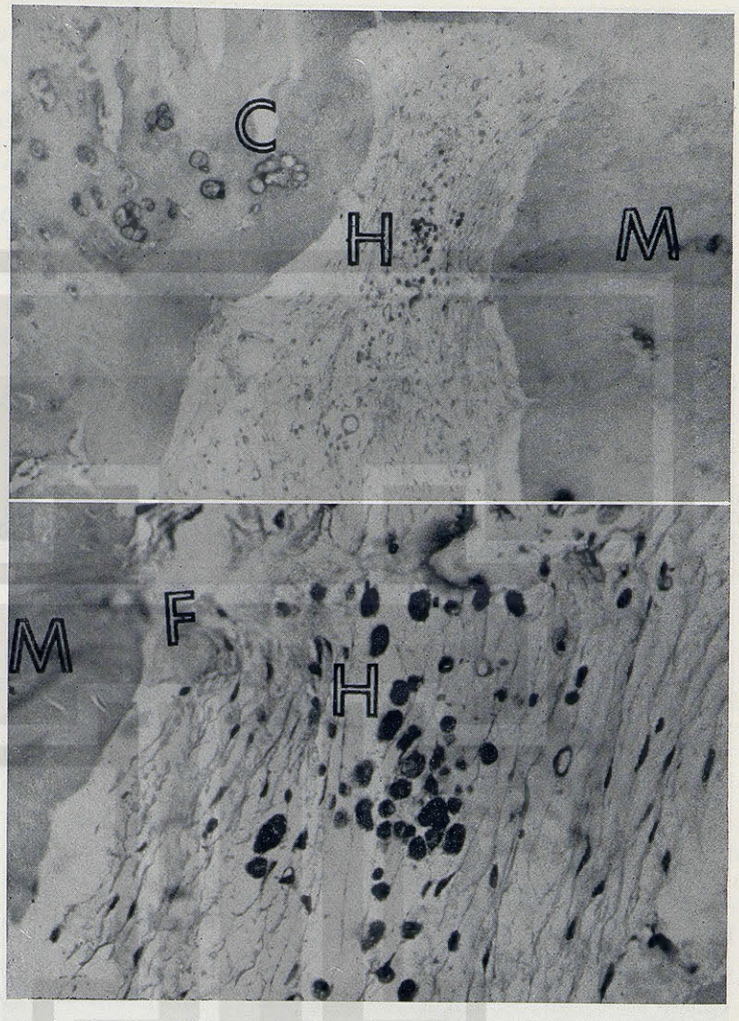


FIG. 7. (A, *top*) Photomicrograph, showing cross section of an implant of 0.6N HCl-decalcified cortical bone, 4 weeks after the operation, and 1 week after an intra-arterial injection of a suspension of charcoal particles. Note old decalcified bone matrix (M), old vascular channels partially filled with new cartilage (C), and old medulla filled with new connective tissue cells, including (H) macrophages loaded with particles of charcoal. The presence of cartilage and the absence of bone suggest that cartilage induction may occur before 3 weeks, but that bone induction (which generally occurs at 24 to 26 days in this model) is blocked by differentiation of mesenchymal cells into phagocytes instead of osteoprogenitor cells. Note also that there is little or no evidence of resorption of matrix or formation of excavation chambers for osteogenesis.

(B, *bottom*) Photomicrograph, showing high-power view of a collection of macrophages (H) filled with carbon particles in the new connective tissue in the center of interior of the implant (M). The peripheral cells consist of nonproliferating spindle-shaped fibrocytes (F). (Hematoxylin and eosin stain)

There were numerous multinucleated giant cells but relatively few sprouting capillaries and no excavation chambers in the old matrix. No evidence of new bone or new cartilage formation was found in 18 implants injected with ThO_2 at daily intervals between 3 and 5 weeks after the operation. In implants in animals injected with carbon particles there was some cartilage induction in isolated areas in old marrow vascular spaces in a few instances. Cartilage induction occurred without visible resorption of matrix and appeared to have begun in some implants before 21 days, the time of the first injection (Fig. 7 A to D).

RADIOISOTOPE UPTAKE

Table 4 illustrates the results of 7 experiments designed to label the ingrowing cells of the host bed with radioactive nucleoproteins and amino acids in order to delineate the cell sequences that lead to differentiation

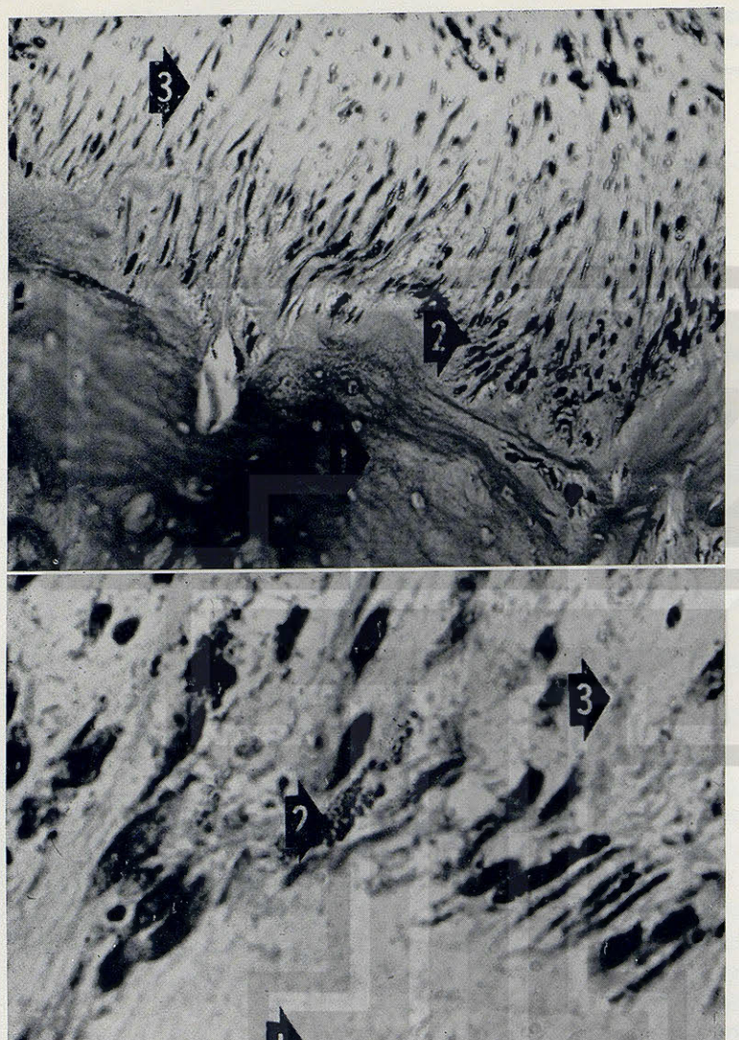


FIG. 7. (C, *top*) Photomicrograph showing ThO_2-loaded macrophages inside of an implant of decalcified lyophilized bone matrix (*arrow* 1). Note that the palisaded, polarized cells (*arrow* 2) present on and beneath the surface of the matrix are loaded with highly refractile particles of ThO_2. The pool of undifferentiated mesenchymal cells (*arrow* 3), unencumbered with particulate material, is relatively small.

(D, *bottom*) Photomicrograph in high-power magnification, showing old matrix, macrophages containing particulate ThO_2, and undifferentiated connective tissue cells labeled as shown in Figure 7 C.

of bone in the interior of an implant of decalcified allogeneic bone matrix. The object was to compare the cell forms in the orthotopic process of bone formation, as described by Young[67] and Owen,[42] with heterotopic bone formation by induction in implants in the abdominal wall. Six rats were injected with 100 microcuries of ^{3}H-thymidine 4 weeks after implantation of decalcified bone matrix in the rectus abdominus. Three were sacrificed at 72 hours after the injection, and 3 were sacrificed at 96 hours after the injection. All of the implants (6 samples in each rat) were positive for new bone formation. At 72 hours after the injection the nuclei of many of the perivascular connective tissue cells were labeled with ^{3}H-thymidine. At 96 hours after the injection the label was found not only in the perivascular connective tissue and the mesenchymal cells but also in the osteoblasts lining the lamellae of new bone tissue. There was also an occasional osteo-

TABLE 4. Distribution of Labelled Substances in Rats and Rabbits With Intramuscular Implants of Decalcified Bone Matrix

Line	Amino Acid	Specific Activity mc./mM.	Dose Per Animal μc.	Site of Injection	Species	Grain Count	Location
1	^{3}H-thymidine	360	100	Intra-peritoneal	Rat	Variable	In nuclei of proliferating cells
2	^{3}H-proline	50	75	Intra-peritoneal	Rat	High	In newly deposited bone matrix
3	^{3}H-glycine	194	50	Intra-peritoneal	Rat	Low	In newly deposited bone matrix
4	^{3}H-thymidine	360	390 x 6	Intra-medullary directly into implant	Rabbit	Variable	In nuclei of proliferating cells
5	^{3}H-cytidine	1000	390 x 6	Intra-medullary directly into implant	Rabbit	High	In nuclei and cytoplasm of all protein synthesizing cells including osteoblasts
6	^{3}H-proline	50	75 x 3	Donor	Rabbit	Low	Only old matrix; none in the new bone
7	^{3}H-glycine	194	50 x 3	Donor	Rabbit	Low	Only old matrix; none in the new bone

cyte with a labeled nucleus. These results demonstrated that the *responding cells* in the induction system for new bone formation in the interior of an implant of lyophilized matrix were perivascular connective tissue cells. There were few or no labeled nuclei among macrophages and multinucleated giant cells in areas unassociated with sprouting capillaries or proliferating connective tissue cells (Fig. 8).

Table 4 also illustrates the results of experiments in which rats were injected with ^{3}H-proline and ^{3}H-glycine daily for 3 days between the 32nd and the 34th days after the operation. The labeled amino acids were metabolized for synthesis of intercellular matrix by connective tissue cells, particularly osteoblasts, in the process of deposition of new bone. There was no uptake of the labeled amino acids by the old decalcified bone matrix. The labeled amino acid was utilized by heterotopic osteoblasts, just as by orthotopic osteoblasts, and produced bone in the femur within the same time intervals, in the same animal (Fig. 9 A and B). The results with ^{3}H-glycine were exactly the same as results of experiments with ^{3}H-proline but the latter was deposited in the matrix more densely and in larger concentrations.

Table 4, line 4, summarizes the results in 8 rabbits given injections of a single injection of 29 μc. of ^{3}H-thymidine, not systemically but locally or directly into the medullary cavity of the implant. Each rabbit had 6 intramuscular abdominal wall implants. One had injections in each implant on the 4th day and sacrificed on the 5th, and another was injected on the 4th day and sacrificed on the

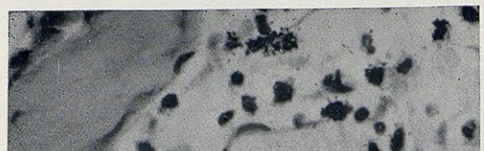
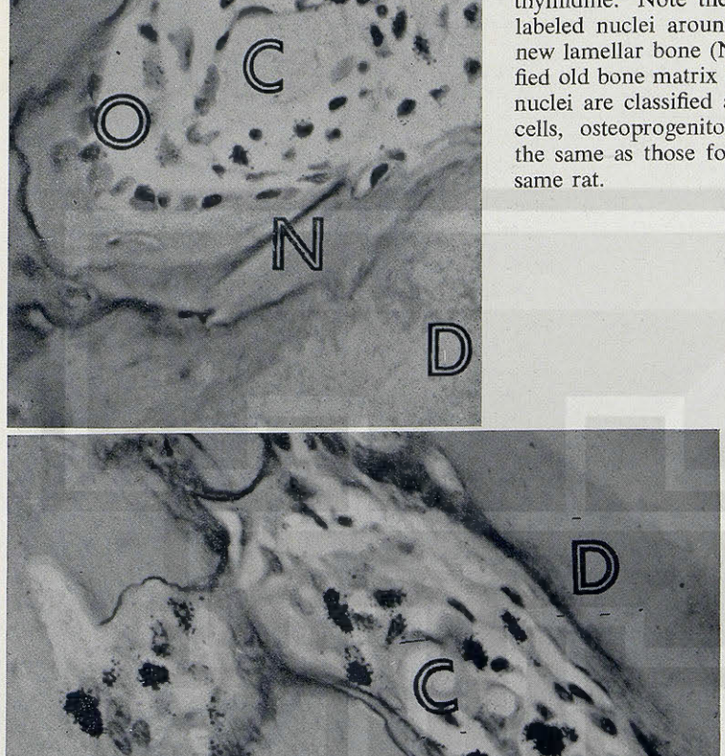
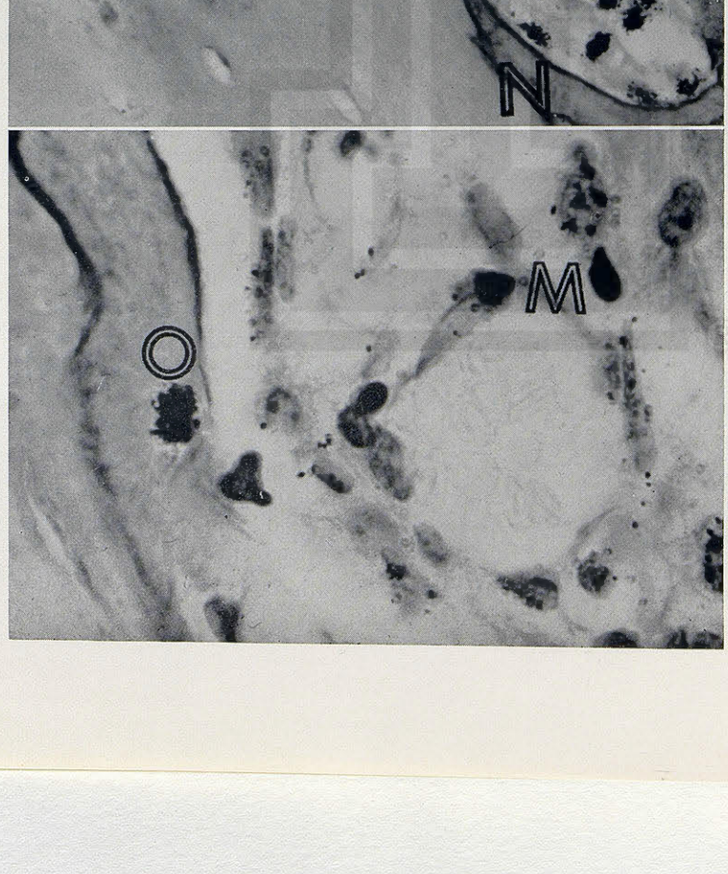


FIG. 8. (A, *top*) Autoradiograph of implant of 0.6N HCl-decalcified allogeneic cortical bone in a rat, 4 weeks after the operation and 72 hours after an injection of the host with 100 μc ^{3}H-thymidine. Note the distribution of cells with labeled nuclei around a central capillary (C), new lamellar bone (N), osteoblasts (O), decalcified old bone matrix (D). The cells with labeled nuclei are classified arbitrarily as mesenchymal cells, osteoprogenitor cells, or preosteoblasts, the same as those found in the skeleton of the same rat.



(B, *center*) Autoradiograph of an implant of decalcified bone in muscle in a rat 4 weeks after the operation and 96 hours after an injection of the host with 100 μc ^{3}H-thymidine. Note the excavation chamber in old decalcified matrix (D) with capillaries (C) in the center surrounded by labeled osteoprogenitor cells and osteoblasts, and lamellar new bone (N). A layer of cement substance separates the old, dead, from the new, living bone.



(C, *bottom*) Autoradiograph of another area of the section shown in Fig. 8 B, showing new osteocyte (O) with ^{3}H-thymidine labeled nucleus with a high grain count. Note capillary and circumferential, rapidly dividing mesenchymal cells (M), nuclei with low grain counts.

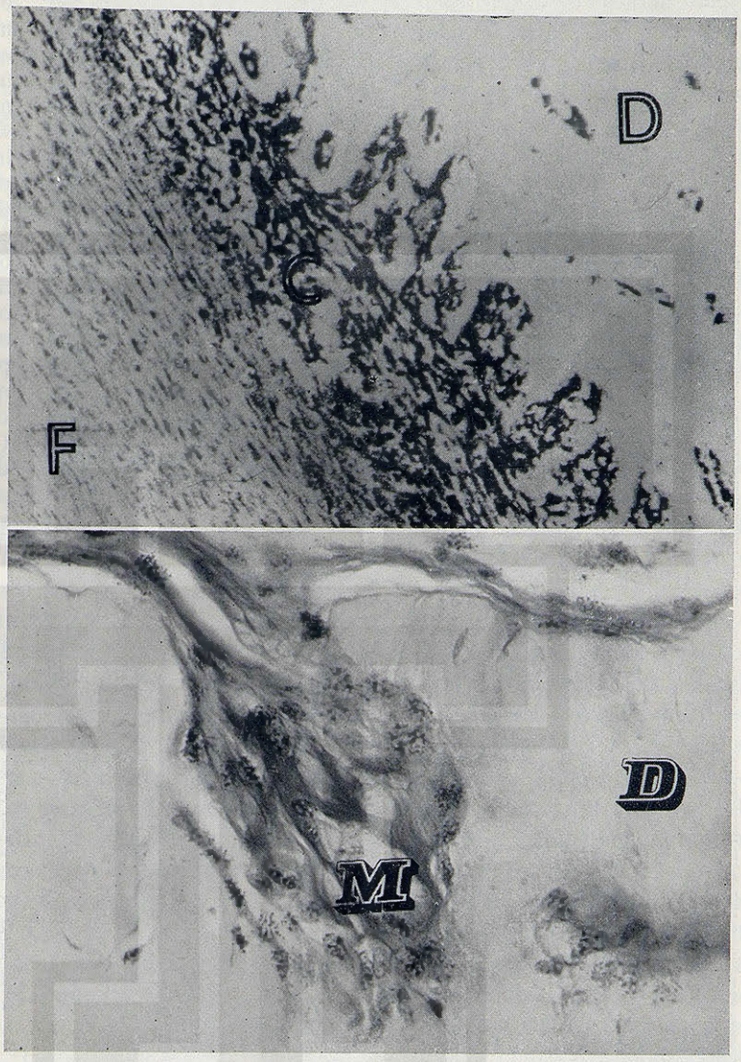


FIG. 8. (D, *top*) Autoradiograph of a decalcified lyophilized bone (D) after 20 days in the abdominal wall of a rabbit, and 24 hours after a single dose of 360 μc of ^{3}H-cytidine was injected *directly* into the medullary cavity of the implant. Note the pool of spindle-shaped proliferating mesenchymal cells with a low grain count (F), and the mesenchymal cells with hypertrophied cytoplasm with a very heavy grain count of ^{3}H-cytidine (C) filling every available crevice or opening into the interior of the old bone matrix.

(E, *bottom*) Autoradiograph, showing excavation chamber filled with mesenchymal cells injected topically with 390 μc of ^{3}H-thymidine during the same intervals as the implants shown in Figure 8 D. Note labeled nuclei of the ingrowing cells of the recipient; decalcified matrix (D); mesenchymal cells (M).

9th. The same procedure was followed on one littermate, injected on the 10th day and sacrificed on the 11th, and one injected on the 10th day and sacrificed on the 15th. Another set was injected on the 20th and sacrificed either on the 21st or the 22nd postoperative day. Another set was injected on the 20th day and sacrificed either on the 24th or the 27th. These procedures labeled: (a) inflammatory cells that had migrated into the implant by the 5th day; (b) histiocytes (mesenchymal cells) that developed inside the implant by the 9th day and the 11th day; (c) the population of various osteocollagenolytic cells developed in the area of the implant by the 21st and the 22nd days; (d) palisades of polarized, *osteoblastlike cells* that had been induced to differentiate by the 24th and the 27th days.

Table 4, line 5, illustrates the results of an experiment in which exactly the same procedure was followed on 8 rabbits, each with 6 intra-abdominal muscle implants, except that ^{3}H-cytidine was used to label the nucleolar and the ribosomal elements of the various populations of cells that are present before, during and after the establishment of the bone induction system.

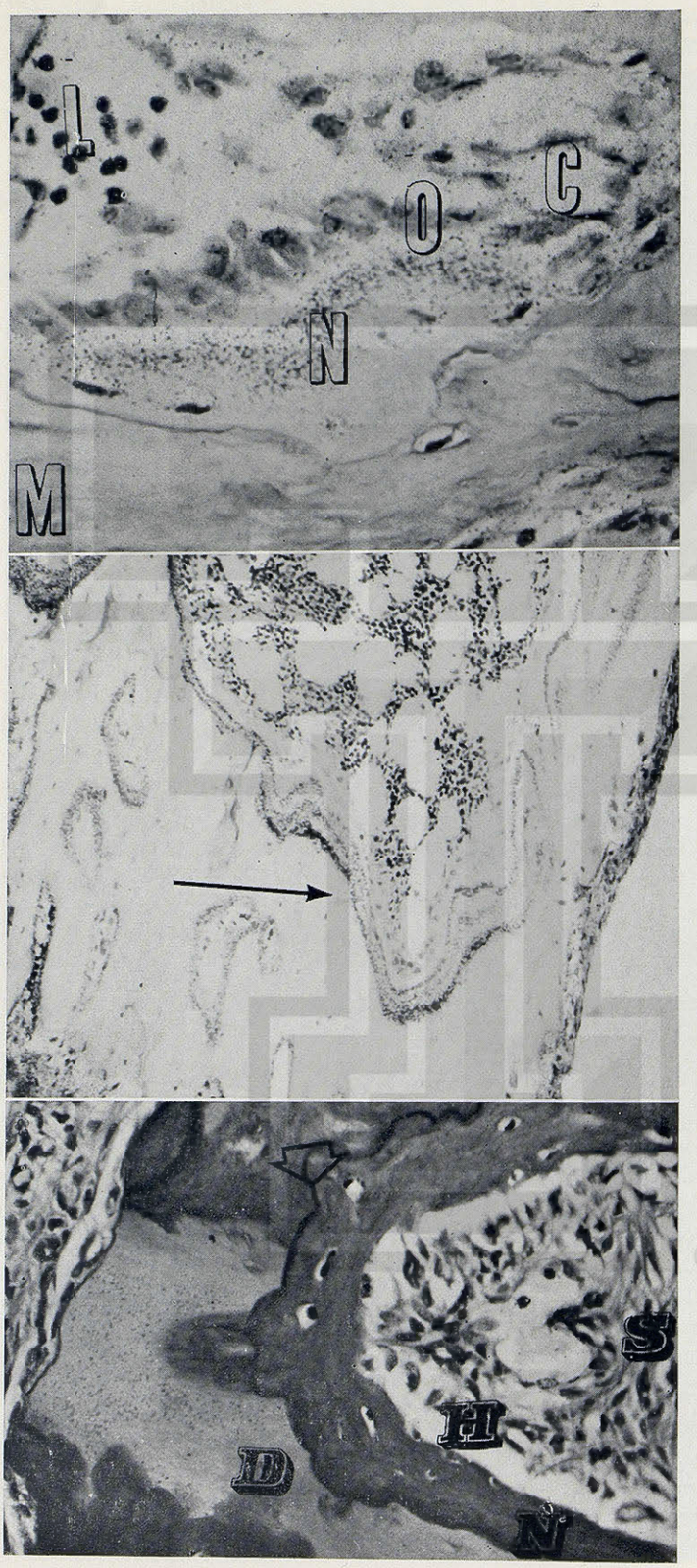


FIG. 9. (*Top*) Photomicrographs of an implant of 0.6 HCl-decalcified homogenous bone matrix, 35 weeks after implantation in the anterior abdominal muscles of a rat. The *host* was injected with 3 daily injections of 25 μc of ^{3}H-proline, from the 32nd to the 34th day, after the operation, to label the matrix of the new bone and to distinguish it from the matrix of the old bone. Note central pool of lymphocytes, plasma cells and mesenchymal cells (L), capillary (C), osteoblasts lightly labeled (O), and new bone (N), densely labeled with ^{3}H-proline but deposited on the surface of unlabeled, old decalcified matrix (M).

(*Center*) Photomicrograph of a section of the bone tissue of the lower end of the femur of the recipient of the implant shown in Figure 9 (*top*). Note the 3 lines of deposition (*arrow*) of ^{3}H-proline, corresponding to the 3 daily doses of 25 μc of the labeled amino acid. The implant shows one line and suggests that the layer of new bone had been formed sometime between the 33rd and the 34th days after the operation.

(*Bottom*) Autoradiograph, showing implant of ^{3}H-glycine labeled bone matrix (D), cement line (*arrow*), new bone (N), hypertrophied mesenchymal cells (H) and spindle-shaped mesenchymal cells (S). Note that radioactivity with a significant grain count is present only in the emulsion overlying the decalcified old matrix (D) of the donor, and there is no detectable radioactivity in the new bone produced by the cells of the recipient.

Inflammatory Cells. On the 5th day after the operation the areas inside and around the implant were infiltrated with leukocytes and wandering histiocytes. The nuclei of leukocytes rarely were labeled, but approximately 3% of the histiocytes had nuclei labeled with ^{3}H-thymidine, or both their nuclei and cytoplasm labeled with ^{3}H-cytidine. The structure of the implanted matrix was relatively unaltered and intact at these early intervals.

Histiocytes. On the 11th day after the operation, the cells in and around the implant were much more numerous, and approximately 10% were labeled heavily with ^{3}H-thymidine and cytidine. The label generally was in spindle-shaped, rapidly dividing connective tissue cells, generally classified as *mesenchymal cells, or wandering and fixed histiocytes*. They formed an envelope of loose connective tissue in which the implant was enclosed and still easy to extricate. Before 12 to 20 days, before the onset of some resorption of the old matrix, the implants contained only leukocytes and wandering histiocytes and never any of the more specialized forms, such as bone or marrow cells. A few implants had nests of new cartilage cells in deep recesses of old vascular channels as early as 20 days.

Osteocollagenolysis. On the 21st day after the operation, the cells in and around the implant consisted of densely packed, *hypertrophied mesenchymal cells*. More than 60% were labeled with ^{3}H-thymidine. The implant was attached firmly by a network of ingrowing capillaries and hypertrophied mesenchymal cells and was impossible to shell out of the host bed. Hypertrophied mesenchymal cells were identified by the high ratio of cytoplasm to nucleus and a prominent golgi vacuole. They produced numerous excavation chambers and always were associated with sprouting capillaries. The implants injected topically with ^{3}H-cytidine on the 20th day had almost 90% labeling with a very high grain count in the cytoplasm of hypertrophied mesenchymal cells. The coaptation of the labeled cells and implant was so close that the cytoplasm appeared to be spread out in all directions over the surface and in every exposed crevice in the old matrix. Because the location of these cells coincided with areas of resorption of matrix, it was reasonable to suppose that these cells were actively engaged in the protein synthesis, specifically induced to secrete osteocollagenolytic enzymes.

Bone Induction. Insofar as it was noted that ^{3}H-cytidine labeled the basophilic cytoplasm of hypertrophied cells, i.e., ribosomal membranes, immediately in contact with old matrix, and inasmuch as the label was very dense in the layer of cells that produced the first deposits of bone, circumstantial evidence indicates that this was the site of bone induction. The cytoplasm of these hypertrophied cells filled the microcanalicular system of the old matrix; this was not visible in ordinary histologic sections. When the microcanicular system of surface of the matrix became outlined by the cytoplasmic processes labeled with ^{3}H-cytidine, there was evidence of nearly complete contiguity of surfaces of new cells and implanted material. This degree of contiguity produced resorption of matrix and probably, also, transfer of the bone induction principle as well (Fig. 8 D).

Osteoblasts and Osteocytes. On the 27th day after the operation, the implant contained all of the cells noted at 21 days and, in addition, a special group of polygon-shaped cells, polarized or oriented in palisades on the surfaces of old matrix inside of the excavation chambers. In some excavation chambers these cells were aligned in rows on the surface of old matrix, the same as on lamellae of new bone. The implants injected with ^{3}H-thymidine showed heavy labeling (high grain counts >50) in the spindle-shaped cells, and light labeling (low grain counts <10) in osteoblasts and osteocytes. The implants labeled with ^{3}H-cytidine showed heavy labeling in both spindle-cells and osteoblasts, with very heavy labeling in osteoblasts and always relatively light labeling in osteocytes. These observations suggested that the uptake of ^{3}H-cytidine depended upon

TABLE 5. Effect of Histocompatibility Antigens Upon Bone Induction

Classification	Donor	Host	Incidence of Bone Incidence (Per cent)
Autologous matrix	Rabbit	Rabbit	98
Autologous matrix	Rat	Rat	96
Allogeneic matrix, lyophilized	Belgian rabbit	Belgian rabbit	98
Allogeneic matrix, unfixed	Belgian rabbit	Belgian rabbit	60
Allogeneic matrix, lyophilized	New Zealand rabbit	Belgian rabbit	94
Allogeneic matrix, second set	New Zealand rabbit	Belgian rabbit	90
Xenogeneic matrix, unfixed	Human	Rabbit	5
Xenogeneic matrix, lyophilized	Human	Rabbit	3
Xenogeneic matrix, unfixed	Bovine	Rabbit	16
Xenogeneic matrix, lyophilized	Bovine	Rabbit	2
Xenogeneic matrix, lyophilized	Rat	Rabbit	5
Xenogeneic matrix, lyophilized	Bovine	Rat	5
Xenogeneic matrix, lyophilized	Human	Rat	2
Isogeneic matrix	Rat	Rat	98*
Allogeneic matrix	Rat	Rat	90†

*Persistence of the ossicle for 1 year.

†Necrosis, resorption, remodeling, absorption of the ossicle after 6 months.

metabolic activity of hypertrophied cytoplasm (ribosomal membranes) of osteocollagenolytic and osteocollagenosynthetic cells. After the cell became an osteocyte and enclosed in matrix and no longer resorbing or depositing matrix, the grain-count of the ^{3}H-cytidine label always was relatively low.

Labeled Matrix. Preparations of the matrix labeled with amino acids were unhomogeneous and difficult to evaluate; but, wherever the labeled areas induced bone, it was not possible to observe local transfer of ^{3}H-glycine or ^{3}H-proline between implant and cytoplasm of induced cells.[66] In areas of ^{3}H-proline labeled old matrix, in the *intercellular* substance between collagenolytic mesenchymal cells (macrophages), not covered by cement lines or new bone, there was diffusely distributed radioactivity. Thus, dispersal of labeled amino-acid was into the area corresponding to the area of inducing not the responding or induced cells. There was no evidence of local transfer of radioactivity from old matrix to osteoblasts or osteocytes or new bone matrix. Moreover, this pattern of dispersal of radioactivity from the donor tissue was the same as that noted previously by others working with labeled cartilage[54]

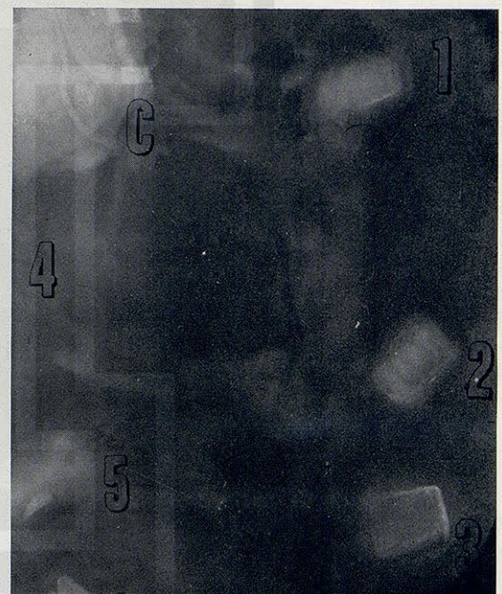


FIG. 10. (A) Roentgenogram showing 6 implants of decalcified lyophilized xenogeneic (calf) bone, fixed in 70% alcohol, after 12 weeks in the anterior abdominal wall of a rabbit. The numbers 1 to 6 indicate the area of the implants. Implant No. 3 is shown in Figure 10 B.

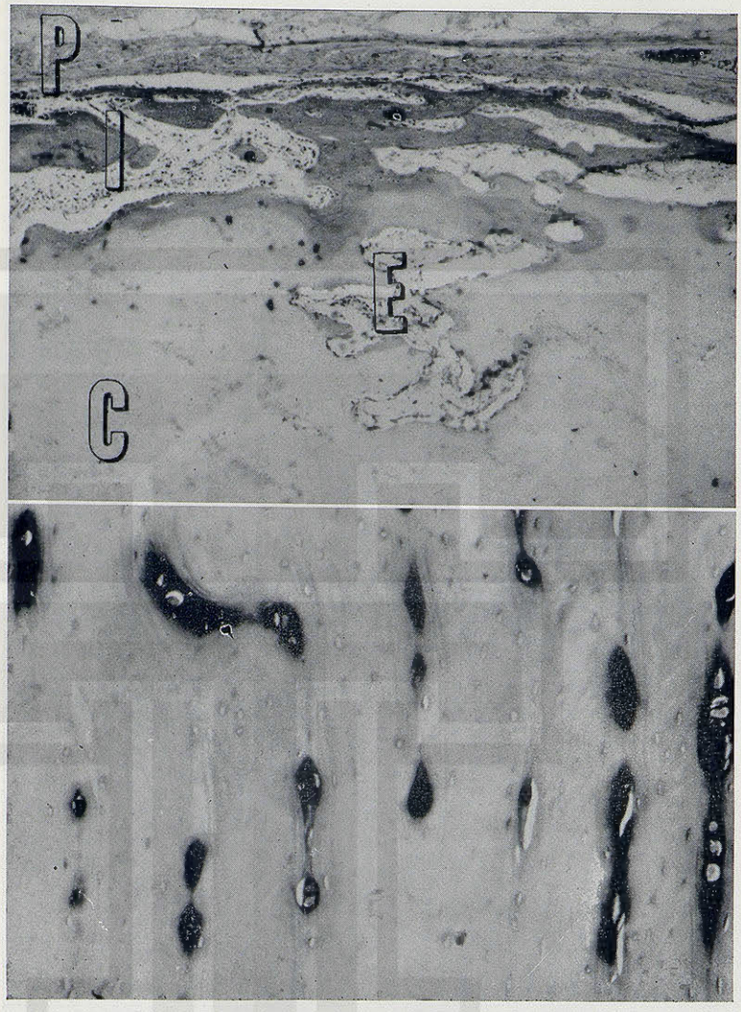


FIG. 10. (B, *top*) Photomicrograph of Implant No. 3, illustrated in Figure 10 A. Note: fibrous connective tissue capsule (P), induced bone (I), excavation chamber filled with capillaries and connective tissue cells (E), and old decalcified calf bone matrix (C). The surrounding muscle outside the area of the implant is not affected and does not enter into the system for bone induction.

(C, *bottom*) Photomicrograph, showing cartilage induction in the interior of an implant of decalcified lyophilized calf bone matrix after 8 weeks in the anterior abdominal wall of a rat.

and spinal cord,[21] and various other nonosseous tissues. Grobstein[21] noted transfilter movement of radioisotope from embryonic mouse spinal cord labeled with 17 different amino acids. These observations were not interpreted to indicate that amino acids *per se* were inducers but only labeled elements of a system synthesizing macromolecules, which constitute tissue surfaces with inductive activity.

HISTOCOMPATIBILITY ANTIGENS

Table 5 illustrates the results of experiments designed to determine the effect of the immune response of the recipient upon the mechanism of bone induction in an implant. Resorption of an implant of undecalcified bone, compared with an implant of decalcified lyophilized bone, was relatively slow and must have liberated histocompatibility antigens in proportionally small doses. Decalcified matrix was resorbed rapidly, and the recipient must have received a relatively large dose of the so-called histocompatibility or H-antigens that incited a prolonged lymphocyte-plasma—cell-reticulocyte immune reaction in the host bed. When the matrix was not lyophilized, fixed in alcohol, or extracted, the immune response appeared to reduce the incidence and the yield of bone induction.

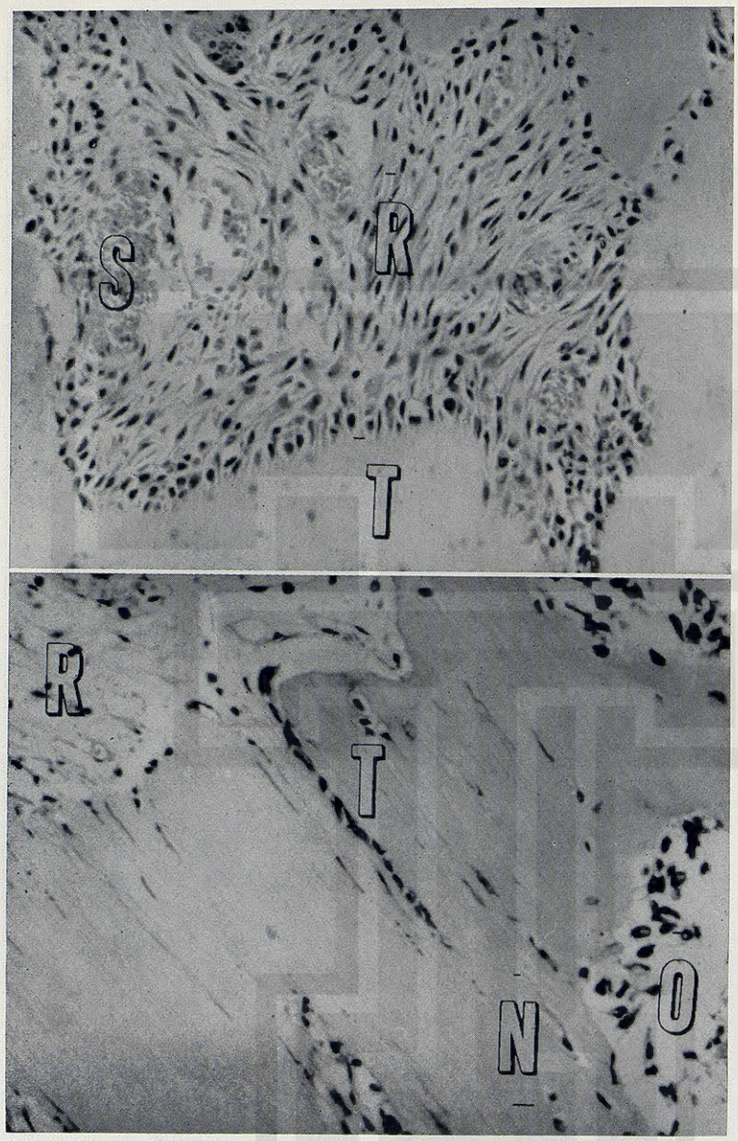


FIG. 11. (A, *top*) Photomicrograph, showing excavation chamber in an implant of decalcified lyophilized allogeneic dentin in the anterior abdominal muscles of a rat. Note: row of cells with basophilic deep-staining cytoplasm polarized and oriented in the direction of the dentinal tubules (T); sprouting capillaries (S); mesenchymal cells (R). (Hematoxylin-eosin and azure stain)

(B, *bottom*) Photomicrograph of a high-power view of dentinal tubules (T), filled with polygonal osteoblastlike cells with strongly basophilic and metachromatic cytoplasm, in an implant of decalcified lyophilized dentin matrix. Note new bone (N), osteoblasts (O), mesenchymal cells (R). (Hematoxylin-eosin and azure stain)

When the implant was lyophilized before implantation, the immune response was lower, and the incidence of bone induction was high. A second set of implants of lyophilized allogeneic matrix in recipients of a different strain induced bone almost as well as the first set.

Xenogeneic matrix introduced a large dose of very potent histocompatibility antigens and rarely induced bone formation. Moreover, when the results were positive, bone induction occurred late, and the yield usually was scanty. Xenogeneic matrix always was infiltrated with and eveloped by great masses of inflammatory cells, lymphocytes, plasma cells and reticular cells, including large volumes of serous fluid and many dilated venules. Cartilage induction often occurred in microcanalicular channels containing wandering histiocytes in some xenogeneic implants in rats.

The above observations demonstrated that allogeneic histocompatibility antigens, but *not* xenogeneic antigens, were altered signifi-

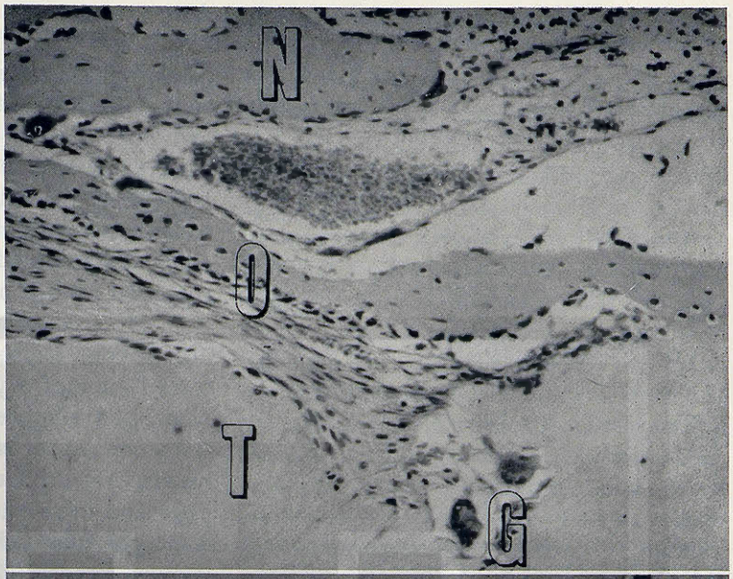


FIG. 11. (C, *top*) Photomicrograph, showing formation of deposits of new lamellar bone on the wall of an excavation chamber in an implant of decalcified lyophilized dentin. Note: dentin (T), osteoblasts (O), new bone (N) and multinucleated giant cells (G).

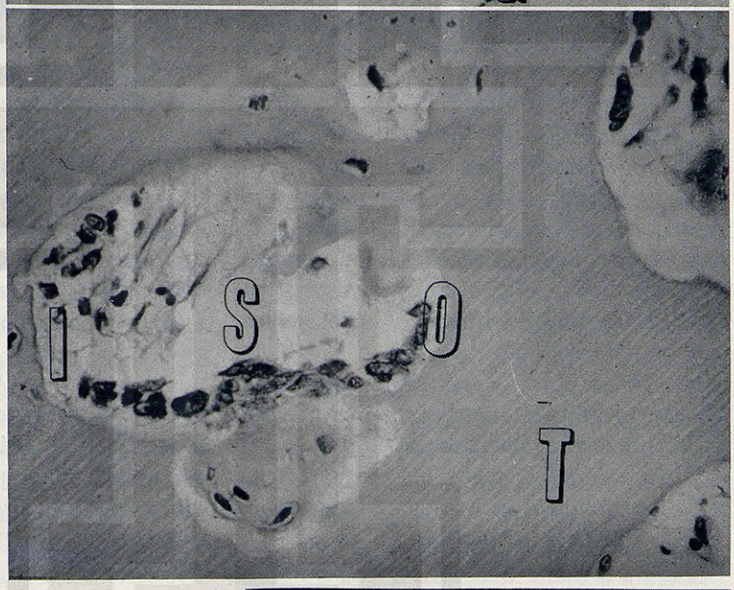


FIG. 11. (D, *bottom*) Oil-immersion, high-power photomicrograph, showing excavation chamber in an implant of *undecalcified* lyophilized dentin matrix, 12 weeks after the operation. Note: dentinal tubules (T), osteoblasts (O), capillary sprouts (S), initial deposit of bone (I).

cantly by lyophilization, and that bone induction was inhibited by the systemic and local manifestations of the immune response to a nonviable foreign tissue (Figs. 10 and 11). Whether the bone-inducing factor was also an autoantigen, a substance which has weak antigenic activity even when derived from the tissue protein of the same individual, is a question that needs to be investigated now by quantitative immunochemical methods.[40]

In order to determine whether the bone induction principle is an antigen, and whether

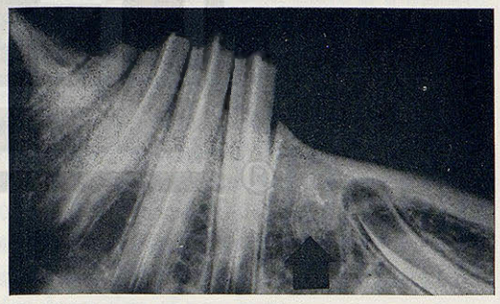


FIG. 11. (E) Roentgenogram, showing ossicle of new bone (*arrow*) formed from an implant of dentin in a tooth extraction socket in the mandible of a rabbit 8 weeks after the operation.

TABLE 6. Effect of Particle Size and Shape of Lyophilized Bone Matrix Upon Efferent Factors in Bone Induction

Particle Size	Shape	Incidence of Bone Induction (Per cent)
1.0 x 2.0 cm.	Cylinders*	94
1.0 x 2.0 cm.	Plates	90
1.0 x 2.0 cm.	Spirals	90
1.0 x 2.0 cm.	Triangles	90
1 cu. mm.	Cubes	96†
420 microns	Cubes	1
250 microns	Cubes	1

*Implants of the cylinders of glass, rubber and plastic of the some size and shape did not induce bone formation.[58]

†Induction of new hyaline cartilage was widespread and in amounts approximately 10 times greater than noted in 1.2 x 2.0 cm. size implants.

the differentiation of osteoblasts depends upon an immune response to allogeneic protein, 10 rabbits with bone implants were treated with 6-mercaptopurine. This drug was injected daily to blockade the immune response. The results demonstrated that the appearance of bone induction was delayed as long as 2 weeks, and the yield of new bone was relatively low. Whether this was due to effects upon mitosis, matrix-protein synthesis, or immunosuppression was not clear.

Table 5 also illustrates the results of experiments designed to investigate the possible relationships between histocompatibility antigens and survival of the newly induced bone and bone marrow cells. The question is whether the bone induction principle is transferred from the old matrix to the new cells, and whether it sensitizes the new cells to the antibodies produced by the host. The results suggested that the new bone induced to form from isogeneic matrix survived, went on producing bone marrow cells and was not resorbed for over a year. Similar implants of lyophilized allogeneic matrix induced bone formation almost as consistently as isogeneic matrix, but the induced osteoblasts, osteocytes and even osteoclasts appeared to become pyknotic and possibly injured by the immune response of the host.

Ossicles formed from allogeneic matrix became necrotic, slowly resorbed over a period of 6 months and replaced by a fibrous tissue scar. The observation that the recipient eventually would recognize and destroy his own cells if they had adsorbed something allogeneic (possibly a macromolecular bone induction principle), albeit derived from nonviable tissue, was most remarkable and demands further experimental work.

Particle Shape and Size

Table 6 illustrates the effect of the shape of the implant and the particle size on the mechanism of bone induction. Provided that the implant was larger in volume than 0.5 to

TABLE 7. Uptake of Total Ca, P and N by Implants of Lyophilized Decalcified Bone Before and After Bone Induction

Days Postoperative	mM./Kg. Ca	mM./Kg. P	mgN/Gm.	mg./Hexosamine/Gm.
0	25 ± 5	8.12 ± 2	136 ± 18	4.0 ± 1.0
2	—	12.10 ± 5	142 ± 10	4.5 ± 1.0
5–7	30 ± 6	15.80 ± 5	156 ± 10	4.0 ± 1.2
18–20	26 ± 6	147.00 ± 10	140 ± 12	9.0 ± 2.0
23	335 ± 15	416.00 ± 25	130 ± 10	9.8 ± 2.2
28	1369 ± 29	1039.00 ± 40	124 ± 15	10.3 ± 1.8
35	1382 ± 29	1073.00 ± 28	109 ± 10	10.6 ± 2.1

TABLE 8. The Effect of Freezing and Lyophilization Afferent Factors in Bone Induction

Temperature	Other Treatment of Implant	Incidence of Bone Induction (Per cent)
28° C.	0.6 HCl	60
28° C.	0.6 HCl + 70% ethanol	70
0° C.	0.6 HCl + 70% ethanol	70
−30° C.	0.6 HCl + 70% ethanol	80
−70° C.	0.6 HCl + lyophilization	96
−70° C.	0.6 HCl + lyophilization + 70% ethanol	90
Cryolysis, −30° to +28° C.	Thrice frozen and thawed	5

1 cu. mm., the size and the shape of the implant had no effect on the percentage of positive results. As a rule, when the matrix was cut into small particles (0.1 to 0.3 cu. mm.) there was a smaller amount of bone induction and a larger amount of cartilage induction; the latter was found particularly inside of old marrow vascular channels with smooth walls and closed ends. Cartilage induction also occurred in small spaces between opposing surfaces of small particles of bone.

When the matrix was ground in a Wiley mill, through 40- and 60-mesh screens, to produce particles measuring from 250 to 420 microns in diameter, cartilage as well as bone induction was greatly inhibited. The particles of matrix were engulfed in large multinucleated giant cells and resorbed rapidly. Such observations suggested that the products of resorption by giant cells may differ from those liberated by mononucleated collagenolytic cells and sprouting capillaries. They also suggested that, unless there was coaptation of old matrix and layers of competent cells developing from proliferating and sprouting capillaries, the conditions for bone induction were not present.

There was no visible structural characteristic to distinguish competent from incompetent connective tissue cells; and, except for the above-described forms of cell aggregation and disaggregation in relation to the implant of matrix, there was no clue to the time that cells first acquire competence. However, this arrangement of competent cells is the same as that described by Cooper,[14] working with Robinson and associates, in which they noted a gradual transition of cell forms without any sharp lines of demarcation in ultrastructural characteristics in the cytoplasm of endothelial, mesenchymal and osteoblastic cells in normal orthotopic bone formation. Factors associated with aggregation and disaggregation of cells in nonosseous induction systems are described by Mascona[39] and Weiss.[63]

The Chemical Composition of the Implant Before and After Bone Induction

Table 7 summarizes the results of chemical analyses of the 6 to 10 samples of decalcified lyophilized allogeneic bone matrix in rabbits, excised at various intervals of zero to 35 days after the operation. The implants were excised with as little as possible of the surrounding muscle and dried to constant weight for analysis by the methods of Eichelberger *et al.*[19] The results demonstrated that, until the onset of bone induction, there was relatively little uptake of calcium or phosphate. Between 3 and 4 weeks, associated with collagenolysis of the implant, there is a marked decline in total nitrogen possibly because at 4 weeks the collagen of the matrix of compact bone was being replaced by mesenchymal cells, new spongiosa or marrow cells containing fat and a large amount of water. At the same time there was a precipitous rise in the amount of hexosamine, possibly reflecting the appearance of islands of cartilage induction and the formation of cement lines for deposition of new bone.

Freezing and Freeze-Drying

Tables 8 to 16 summarize the results of

TABLE 9. The Effect of Heat Upon Afferent Factors in Bone Induction

Temperature	Incidence of Bone Induction (Per cent)	Volume of New Bone, cm.^{2}
25° C.	60	1.8
37° C.	80	1.5
40° C.	80	2.0
50° C.	90	2.0
60° C.	70	1.5
70° C.	60	1.5
80° C.	30	1.0
90° C.	30	0.5
100° C.	0	0.0
Autoclave	0	0.0

TABLE 10. The Effects of ^{60}Cobalt Radiation Upon Afferent Factors in Bone Induction

Dose in Millions of r	Reaction	Incidence of Bone Induction (Per cent)
0	New bone, high yield	90
1	New bone, low yield*	90*
2.3	Fibrous tissue only*	0†
10.0	Fibrous tissue only*	0
22.0	Fibrous tissue only*	0
37.0	Fibrous tissue only*	0

*Disorganized structure; cells fail to polarize and palisade.

†Foreign-body giant cells were numerous.

the experiment designed to determine the nature of the bone induction principle, the afferent factor of the mechanism of bone induction. Table 8 summarizes the results of experiments illustrating the effect of cold upon the bone induction principle in matrix. The highest percentage of positive results was obtained by freezing at —70° C. in liquid nitrogen. When employed for cold sterilization of the implant, 70% alcohol did not have deleterious effects on bone induction. How long lyophilized bone induction principle would retain its activity at room temperature was not determined; positive results have been obtained with samples fixed in alcohol and stored as long at 6 to 9 months. Cryolysis, thrice freezing and thawing, as noted above, destroyed bone induction completely and immediately. The bone induction principle in sterile decalcified nonlyophilized bone matrix stored in sealed containers at room temperature deteriorated within a period of 3 months.

TABLE 11. The Effect of Extraction of Lipid and Lipoprotein Upon Afferent Factors in Bone Induction

Extractant Solution	Incidence of Bone Induction (Per cent)
Ether	98
Alcohol 70%	90
Acetone	96
3% Hexochlorophene	98
5% Sodium dodecyl sulfate	96
Tween 80	94

Heat

Table 9 summarizes the results of experiments designed to determine the effect of heat denaturation on the bone induction principle in untreated or unlyophilized matrix. Heat denaturation, like lyophilization, promoted bone induction. Shrinkage of the bone collagen occurred at temperatures above 60° C., but it did not prevent bone induction until the matrix was transformed into gelatin. Temperatures approaching 100° C. completely destroyed the bone induction principle.

Radiation

Table 10 summarizes the results of experiments designed to determine the effect of denaturation of lyophilized decalcified bone by gamma rays of radioactive cobalt. The results with samples irradiated before implantation demonstrated that exposure to levels above 2 million roentgen-equivalent physicals (REP) inhibited bone induction. Resorption of the *irradiated denatured* matrix induced the formation of a mass of fatty fibrous tissue, never new bone. Further observations are

TABLE 12. The Effect of Action of Various Enzymes Upon Afferent Factors in Bone Induction

Enzyme	Concentration (Per cent)	Substrate	Incidence of Bone Induction (Per cent)
Alkaline phosphatases	0.1	Organic phosphates	96
Acid phosphatase	0.5	Organic phosphates	94
Elastase	0.05	Elastin and other proteins	50
Collagenase	0.1	Collagen	5
Papain	1.0	Proteins, nonspecifically	0
Ficin	2.0	Proteins, nonspecifically	0
Bromelin	2.0	Proteins, nonspecifically	0
Chymopapain	0.1	Proteins, nonspecifically	0
Hyaluronidase	0.1	Proteins, nonspecifically	96
Trypsin	0.1	Peptide bonds	5
α-chymotrypsin	0.1	Peptide bonds	0
Pronase	0.1	Peptide bonds	0
Ptyalin	0.1	Sugar	0
RNAase, pancreatic	0.1	RNA	50
DNAase, pancreatic	0.1	DNA	50

*The sources of the above enzymes were as follows: phosphatases from calf intestine, Calbiochem, Los Angeles; elastases pancreatic, Calbiochem; collagenase, bacterial, Burroughs Wellcome Co.; papian, African papaya, Calbiochem; ficin fig latex; bromelin, pineapple stem, Calbiochem; chymopapain, crystalline, Sigma Co., St. Louis; hyaluronidase, bovine testes, Calbiochem, Los Angeles; trypsin, bovine pancreas (grade B, IX crystal), α-chymotrypsin, bovine pancreas, Calbiochem; pronase, streptomyces, grisens, Calbiochem; ptyalin, human salivary; RNAase bovine pancreas; DNAase, bovine pancreas, from Calbiochem.

necessary to determine the exact levels of radiation that will reduce antigenicity[9] and enhance bone induction activity, including cell regeneration.[49]

Lipid

Table 11 summarizes the results of experiments designed to determine the effect of extraction of various lipids and lipoproteins from samples of decalcified lyophilized bone. The results suggested that extraction of components of bone matrix with ether, alcohol, acetone, hexachlorophene, common detergents and Tween 80 had little or no effect and may even have enhanced bone induction.

Enzymatic Degradation

Table 12 summarizes the results of experiments designed to eliminate one or another of the components of decalcified lyophilized bone by enzymatic digestion or degradation of the exposed surfaces of the matrix in vitro before implantation. The results demonstrated that hydrolysis of matrix organic phosphates by acid or alkaline phosphatases, and mucopolysaccharides by hyaluronidases, did not inhibit bone induction. Proteolytic enzymes, either of plant or of animal origins, degraded or digested the proteins of the decalcified bone matrix sufficiently to inhibit bone induction. Experiments with embryonic tissues treated with RNAases and DNAases have been reported to suggest that it is the protein and not the nucleic acid that is the inducer.[6] The proteins of small amounts of cell debris retained in the implant could account for the specificity of the bone induction by dentin, bone, cartilage and urinary bladder epithelium. In embryonic induction systems, it is noted often that only traces of a specific protein macromolecule may be sufficient to produce induction.[6,44,60]

Bone Collagen

Table 13 summarizes the results of experiments designed to degrade and fractionate the

TABLE 13. The Action of Various Preparations of Bone Matrix Collagen

Preparation	Particles	Incidence of Bone Induction (Per cent)
Acetic acid, 0.6N	Cell debris and acid-soluble proteins	0
Dialyzed in normal saline	α particles + acid-soluble proteins	0
5M NaCNS, 2° C., dialyzed in normal saline	α and β particles	0*
5M NaCNS, 2° C., dialyzed in normal saline	Residue + β and γ particles	70
$CaCl_2$, 500 mM./L. + 0.6N HCl, extracted collagen	Bone collagen fibers	90

*Calcified collagen fibers.

various components of allogeneic lyophilized decalcified bone matrix by the methods of Eastoe and Eastoe,[17] Eastoe and Courts[16] and Glimcher and associates.[20] Bone matrix prepared by simple extraction of the mucoproteins and the lipoproteins with calcium chloride induced both chondrogenesis and osteogenesis. The acid-soluble protein fraction, the alpha particles and the very small amount of alpha and beta particles that could be obtained from mammalian bone matrix calcified in some places but *did not* produce bone induction. However, the residue, consisting of the gamma particles and *undissolved bone collagen, did induce bone formation* in 70% of the implants.

Extraction of bone matrix with calcium chloride has been employed commercially for

TABLE 14. The Effect of Metal Ion (25 mM./L.) Upon Bone Induction

Salt	Reactive Site	Incidence of Bone Induction (Per cent)	Remarks
KCl	Carboxylic acid	98	Extensive ossification
$CaCl_2$	Carboxylic acid	98	Recalcification and resorption
$CaCl_2$ + HCl to remove Ca^{++}	—	90	Extensive assification
$KMnO_4$	Carboxylic acid	16	Recalcification and resorption
$SrCl_2$	Carboxylic acid	80	Extensive ossification
$ZnCl_2$	Carboxylic acid	90	Extensive ossification
$CuCl_2$	Chelation of carboxylic acid and amino groups	80	Extensive ossification
$CrCl_3$	Chelation of carboxylic acid and amino groups	84	Extensive ossification
$AlCl_3$	Chelation of carboxylic acid and amino groups	90	Extensive ossification
$PbCl_2$	Carboxylic acid group	96	Large amounts of unabsorbed matrix; inhibits osteaclastic activity and bone remodeling
$BeCl_2$	Carboxylic acid group	0	Inhibits cellular activity and absorption of all old matrix

TABLE 15. The Effect of Organic Blocking Reagents Upon Bone Induction in Lyophilized Bone Implants

Agent	Reactive Groups	Incidence of Bone Induction 12 to 14 Weeks	Remarks
Methyl alcohol	None	+	Large deposits
10% methyl alcohol + HCl, .2N solution	Carboxylic acid	±	Delayed induction
Merthiolate (thimerosal)	Amino groups	+	Large deposits
FDNB, 2%	Epsilon amino and terminal amino	±	Delayed bone induction
0.6N HNO_3	Deamination	0	Resorption with many foreign body cells
0.6N HNO_2	Deamination	0	Resorption with many foreign body cells
Formalin, 10%	Terminal amino groups	+	Small deposits of new bone
Orange G, 10%	Amino	+	Large deposits of new bone
Safranine O	Amino and hydroxyl group of mucopolysaccharides	+	Large deposits of new bone
Zephirin, 5%	Amino and hydroxyl group of mucopolysaccharides	+	Small deposits

preparation of bone collagen. Such preparations were implanted before and after washing to remove bound calcium ions. Unwashed implants recalcified, and the new apatite deposits retarded resorption and delayed the appearance of bone induction.[2,58] When the calcium was removed before implantation by washing with 0.6N HCl for 1 hour, it induced osteogenesis in 90% of the implants.

Metal Ion Complexes

Table 14 summarizes the results of implantation of decalcified lyophilized matrix, treated for 24 hours at room temperature in 10 different salts to determine the effect of formation of metal ion complexes with carboxylic acid groups of the bone proteins. The results demonstrated that extraction of neutral salt-soluble proteins and formation of metal ion complexes with divalent and trivalent cations did not inhibit bone induction. Potassium permanganate, a powerful oxidizing agent, produced *recalcification* and marked inhibition of bone induction. Beryllium complexes in matrix produced only loose edematous fibrous tissue and no bone induction, possibly because of the toxic effects of Be^{++} on the ingrowing cells of the recipient.

Organic Blocking Reagents

Table 15 summarizes the results of experiments to determine the effect of various organic reagents commonly used for blocking the various reactive groups of proteins. Methyl alcohol fixation did not inhibit bone induction, but methylation in acidic solutions produced denaturation or gelatinization of a large part of the implant and produced only small deposits after a delay of 6 to 8 weeks. Thimerosal, fluoro-dinitrobenzene, formalin, orange G, safranine O, and zephirin all had more or less ontoward effects but did not entirely prevent bone induction. Deamina-

TABLE 16. Effect of Complex Formation With Various Antibiotic and Antimetabolic Agents Upon Afferent Factors of Bone Induction

Agent	Concentration (Per cent)	Incidence of Bone Induction (Per cent)
Oxytetracycline	1	98
Erythromyecin	1	96
Streptomyecin	2	96
Chloromyecin	2	96
Penicillin	2	75
Actinomyecin D	0.004	25
Control, 0.9 saline	—	80
Control, 0.9 saline; ascorbic acid	0.2	80

tion by nitric acid or nitrous acid was a powerful inhibitor and stopped bone induction consistently and completely.

Antibiotics and Antimetabolites

Table 16 summarizes the results of experiments designed to sterilize and modify the composition of the bone matrix by treatment in vitro with solutions of various antibiotics and antimetabolites. Actinomycin D was adsorbed or bound to the bone matrix in very small concentrations in some unknown way and was the only compound that inhibited bone induction in a significantly high percentage of the implants. Whether it was transferred to inducing cells locally in amounts sufficient to inhibit synthesis of messenger RNA was a matter of speculation. Puromycin, a substance known to inhibit protein synthesis, introduced into the system in the same way as Actinomycin D, did not inhibit bone induction. If the sites of action of Actinomycin D were specific for m-RNA synthesis, these results and those noted below from systemic treatment of the recipient would suggest that the target for the induction principle was the genome, and that the inductive mechanism involved a feedback reaction between the cytoplasm and the nucleus of the cell, as proposed by Watson,[61] Brachet[6] and Waddington.[60]

The following drugs were used also for systemic treatment during the interval from 18 to 30 days after the operation: actinomyecin, 7.5 μg per kilo to inhibit m-RNA synthesis and new matrix formation; heparin, 5,000 units per kilo to increase the rate of resorption of matrix; penicillamine, 90 mg. per kilo to block the transformation of soluble tropocollagen units into insoluble collagen fibrils; oxytetracycline, 100 mg. per kilo, to chelate and decrease the availability of metal ion activators of enzymes used for protein synthesis; vitamin A, 12,500 USP units per kilo to increase the fragility of lyosomes and release mucoproteinases and other degrative enzymes.

Actinomycin inhibited bone induction. Heparin had little or no effect. Neither penicillamine nor oxytetracycline inhibited bone induction. Vitamin A produced equivocal results. The effects of these antimetabolites and drugs on cells palisaded or polarized in relation to the surfaces of implants of bone matrix are under investigation with the use of the electron microscope.

DENTIN

The bone induction principle was as active in dentin as in bone itself. When mature allogeneic dentin was separated from enamel and tooth pulp cells, decalcified and lyophilized, it induced bone formation within 4 weeks after implantation in the anterior abdominal wall of rats and rabbits.[3] Undecalcified dentin, similarly prepared and lyophilized, produced the same results, but the process did not begin until 8 to 12 weeks after the operation; the presence of the mineral apparently impeded the process of resorption of the matrix, retarded the formation of excavation chambers and delayed the onset of bone induction. The end-product of bone induction by dentin was an ossicle filled with bone marrow, the same as bone induction by bone matrix.

Xenogeneic human dentin induced a scanty deposit of bone in less than 5% of implants in rats. Orthotopic allogeneic implants of decalcified, lyophilized implants in extraction tooth sockets and drill holes in the mandible in rabbits produced ossicles of new bone

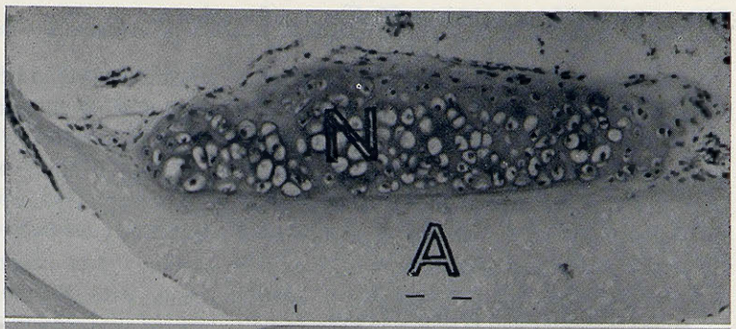
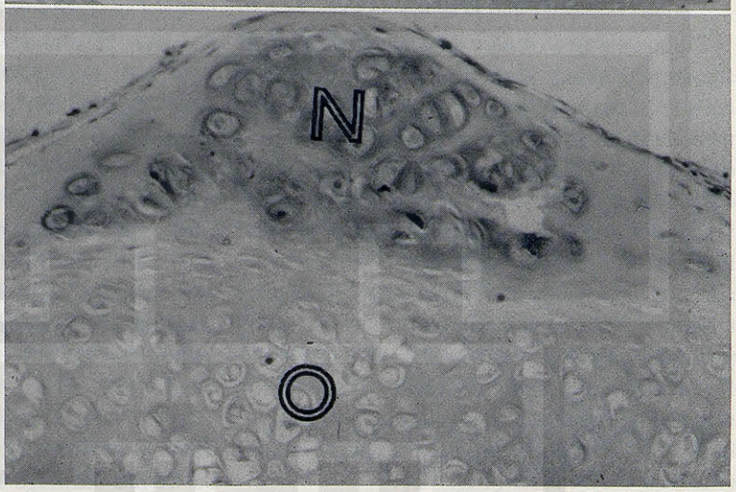
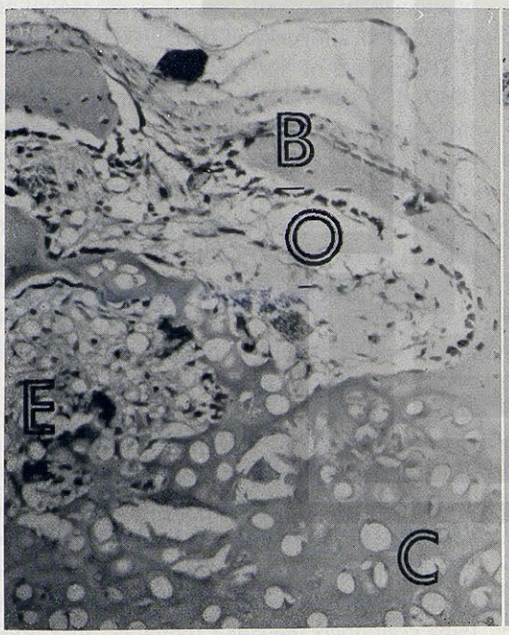


FIG. 12. (A, *top*) Photomicrographs, showing cartilage induction by an allogeneic implant of 0.6N HCl-decalcified lyophilized articular cartilage. NOTE: differentiation of new hyaline cartilage (N) from mesenchymal cells in contact with the surface of old acellular matrix (A).



(B, *center*) Induction of new hyaline cartilage (N) by the lyophilized xyphoid cartilage (O) with a product the same as shown in Figure 12A.



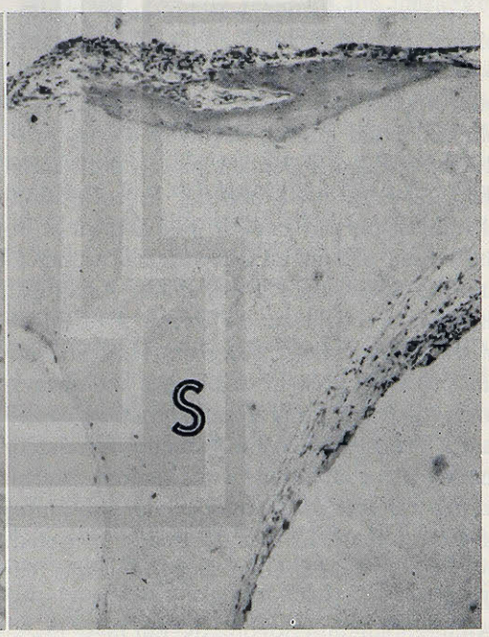


FIG. 12. (C, *left*) Induction of new bone (B) from highly vascular mesenchymal cells and osteoblasts (O) inside of an excavation chamber (E) in old acellular matrix of lyophilized costal cartilage (C).

(D, *right*) Bone induction (*top*) and almost complete refilling of an excavation chamber in old matrix of lyophilized decalcified nasal septum cartilage (S).

within the host bone.[66] These observations were of interest to students of phylogeny of the skeletal system, because they suggested the hypothesis that the appearance of dentin-like hard tissues of the ancient Heterostraci may have enduced the development of bone tissue with true osteocytes (Fig. 11).

CARTILAGE INDUCTION

Differentiation of new cartilage occurred in the embryo from interaction of mesenchymal cells with many different kinds of neural and mesodermal tissues.[13,35,36,48] In postfetal life, differentiation of new hyaline cartilage occurred from contact of mesenchymal cells with the structural components of decalcified lyophilized articular, nasal, xyphoid, tracheal and costal cartilages. Fibrocartilage tissue in tendon attachments and fracture callus also produce cartilage induction. The process frequently (although not always) set up a secondary induction system that was indistinguishable from the typical process of endochondral ossification (Fig. 12).

Cartilage induction was produced by implants of decalcified lyophilized cartilages, but the percentage of positive results was only approximately 10%. Decalcified lyophilized bone matrix, as noted above, produced cartilage induction more consistently than cartilage itself but not in excavation chambers, only in smooth wall cavities or in the closed end of an old vascular channel, and the yield was relatively small. As a rule, there was no evidence of a proteolytic effect of the cells or their precursors. Instead, the cartilage cells grew in small nests and always some distance away from sprouting capillaries. Thus, cartilage induction was related mainly to coaptation with smooth, unresorbed surfaces of the intercellular substance of the implant. In living cartilage, as noted by Holtrop[25] and Urist and Adams,[54] the chondrocytes can also dedifferentiate and redifferentiate into osteoblasts, but these reactions obviously were characteristic only of living epiphyseal chondrocytes of young animals. Cooper,[13] employing both direct and transfilter tests, noted that either flattened or hypertrophic chondrocytes induced chondrogenesis, but round encapsulated cells were generally ineffective.

The capacity of living and devitalized HCl-decalcified articular cartilage to induce new cartilage was observed recently in experiments with ^{3}H-thymidine and ^{3}H-glycine-labeled implants.[54] The stratified, flattened chondrocytes and dense arcades of fibrous matrix of the gliding surface produced one kind of indnction, while round-paired chondrocytes of the epiphyseal side produced another. A thin slice of articular gliding surface induced differentiation of new cartilage and no bone in 1/12th of the samples. A thick slice, which included some epiphyseal cartilage, produced not only cartilage but also bone induction, and the reaction occurred in 9/12th of the samples within 3 weeks after the operation. Nonviable implants produced a lower yield and then only after 6 to 8 weeks. Cryolysis completely destroyed cartilage induction, irrespective of whether the implant consisted of viable or lyophilized allogeneic tissue.

Extraction of the ground substance with $CaCl_2$, 500 mM, or degradation of protein polysaccharides in samples of articular cartilage in solutions of 1% papain-cystein, or 0.1% hyaluronidase, had unpredictable and highly variable effects. Our observations corroborated the view that the cartilage induction principle is an intercellular-macromolecular substance, or a surface-pattern, and noted that it is acid insoluble, presumably a highly complex protein.[13,26,35,62,63]

Fracture Callus

Fracture callus contained many inducing cells[56] and induced cells (chondroblasts and osteoblasts) and much more nucleoprotein and cytoplasmic protein than was present in matrix of compact bone. If intracellular proteins contained the bone induction principle, lyophilized callus should have produced a bone induction in a high precentage of implants. Instead, the percentage of positive

results was relatively low compared with bone or dentin matrix. These results suggested that intracellular nucleic-acid-protein-complexes of nonviable tissue were probably not as good as extracellular matrix proteins as a source of the bone induction principle.

MUSCLE

Alcohol-fixed muscle, implanted under the kidney capsule by Bridges and Pritchard,[8] produced new cartilage and bone, but implants of alcohol-fixed or lyophilized muscle, implanted both in the anterior chamber of the eye and the anterior abdominal wall, were uniformly negative in approximately 50 isogeneic and allogeneic samples in rabbits and inbred rats.[50,58] Negative results also were obtained with implants of alcohol-fixed or lyophilized kidney parenchymal tissue. Injections of alcohol into muscle produced only 8% positive results, and the bone developed only as scanty deposits near blood vessels.[45] Blunt mechanical injuries of muscles in rabbits, and in young men with athletic injuries, produced bone only in young growing individuals and mainly from the periosteum that overlay an area of contused bone.[69] These systems were in no way comparable with bone induction in implants of bone matrix; the muscle tissue surrounding an implant of decalcified matrix was unaltered and showed no deleterious changes. More experimental work needs to be done on muscle to determine how bone induction occurs in either myositis ossificans traumatica or in myositis ossificans generalisata.

TENDON

Buck[10] demonstrated, in an excellent experimental investigation of the process in rats, that bone induction occurs in tendon only in relation to areas of fibrocartilage in the tissue. Unlike the rat, the rabbit rarely has fibrocartilage or cartilage rests in the tendo achilles and does not produce bone following tenotomy. Unlike turkey tendon, which develops resorption cavities and induces cartilage and bone formation in the process of maturation,[31] rabbit tendon does not ossify. De la Sierra and Urist[15] were unable to produce bone induction in lyophilized decalcified tendon implanted in the abdominal wall of the rabbit. When the tendon was lyophilized and implanted in a rabbit muscle without preliminary decalcification, it calcified but did not ossify over a period of 16 weeks. Decalcified unlyophilized, unfixed tendon was absorbed rapidly and did not recalcify. Lyophilization may split noncovalent bonds[20] and make carboxylic acid-reactive groups available for binding and for accumulating calcium ions in sufficient amounts of calcium to transform collagen fibers into a calcifiable matrix.[52] These observations suggest that implants of lyophilized tendon did not induce either cartilage or bone formation but would calcify, particularly in the rabbit, a species with physiologic hypercalcemia.

THE BONE INDUCTION PRINCIPLE IN EPITHELIUM

Kagawa[32] recently reviewed the literature on bone induction by epithelial tissues of urinary bladder, ureter, renal calyx, gallbladder and fundus of the stomach. He also made a survey of the activity of 13 different enzymes in allografts of urinary bladder mucosa and concluded that the histochemical patterns in induced bone were essentially the same as in normal bone. Species differences, histocompatibility antigens, pericystic hyaline formation around the epithelial cells have been investigated in bone induction by transplants of living cells of autografts and allografts of urinary bladder, but the process has not yet been characterized in biochemical terms.

Devitalized, 0.6N HCl-decalcified or acid-extracted lyophilized allogeneic rat-bladder tissue induced bone formation. In the rat, the results were approximately 20% positive with lyophilized bladder implants either in the anterior chamber of the eye or in the anterior abdominal wall. In the rabbit, a species supposedly susceptible to heterotopic bone formation, there was rarely bone induc-

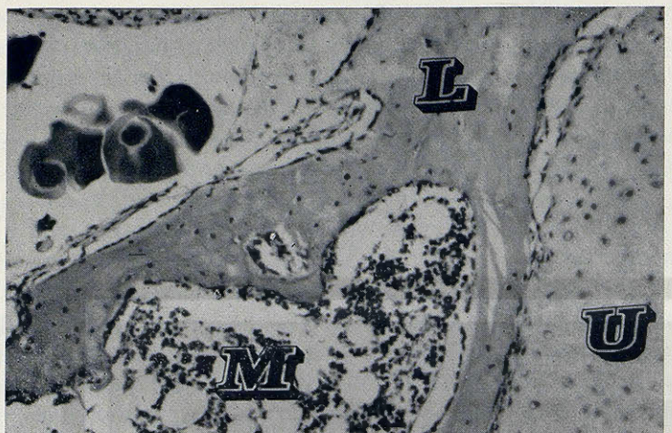


FIG. 13. Photomicrograph, showing implant of devitalized urinary bladder epithelium (U), ossicle of new lamellar bone (L) surrounding a lake of hematopoietic bone marrow (M) growing in the anterior chamber of the rat's eye. (Hematoxylin-eosin and azure II stain)

tion by bladder, either from living or from lyophilized dead tissues. The morphologic features or bone induction by the living bladder transplants in dogs, cats and guinea pigs have been described in detail[27]; differentiation of osteoblasts occurred from new proliferating, ingrowing cells of the host bed. Similar to bone induction in implants of dead decalcified lyophilized bone matrix, the process of induction was associated with encystment and resorption of the dead tissue. Mesenchymal cells invaded and repopulated the area of the implant, produced excavation chambers in the tissue, interacted with perivascular connective tissue cells and differentiated into new bone. Thus, the development of a population of competent responding cells of increasing complexity of organization, and of progressively increasing number, was critical.

Although nothing is known of the chemistry of the inducer, the action of the inducing tissue was not unspecific or mechanical; similarly treated implants of lyophilized tendon, muscle, liver, spleen and other tissues did not produce bone induction. The fact that the end-product of bone induction by bladder in rats was organotypic and consisted of a complete ossicle, as shown in Figure 13, indicated that the bone induction by nonviable cells was comparable with that previously observed in viable, autologous or allogeneic bladder-mucosal transplants in dogs and guinea pigs.

DISCUSSION

Differentiation of bone cells occurs very early in fetal life in the human after less than 7 weeks of gestation and begins in different parts of the body in cartilage models at various times that are predetermined genetically or epigenetically. If we apply the theory of Spemann,[46] as summarized in 1938, we assume that induction not only initiates osteogenesis but also sustains it throughout the life of the individual. Conceptually, induction is defined by Jacobson[30] as differentiation of a new cell form from interaction of inducing cells and responding cells; in the absence of one or the other, new more specialized tissues do not develop. Explaining bone formation by the theory of induction, we may assume that an inducing cell could be a cell that has recently resorbed or had direct contact with the matrix of dentin, bone, cartilage, urinary bladder, gallbladder or gastric epithelium. A responding cell would be a proliferating, perivascular connective tissue cell. In some situations, a cell may act either as an inducing cell or a responding cell; but if there are differences between the two, they can be defined at this time only by spatial relationships in the tissue.

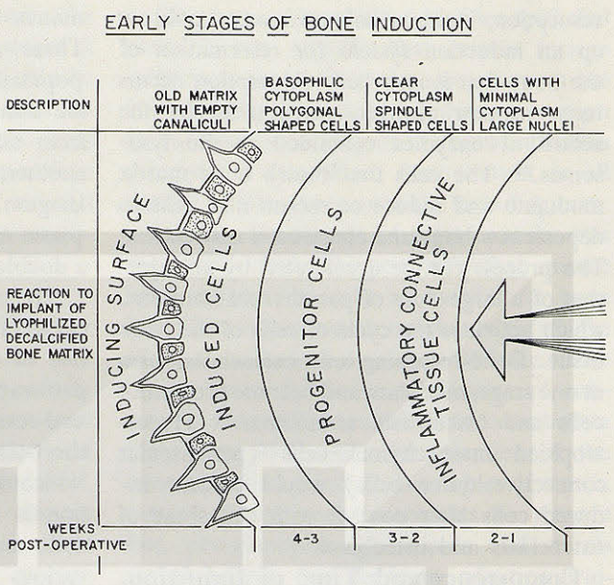


FIG. 14. Diagrammatic representation of the cell morphology and time relationships in bone induction by an implant of decalcified lyophilized bone matrix. The arrow indicates the direction of inductive activity, including the spatial relationships. The bone induction principle is transferred from the acid-insoluble fraction of the matrix that is in close contact with competent cells with basophilic cytoplasm; it is also transmitted to the adjacent layer of mesenchymal or progenitor cells. A layer of competent or progenitor cells responds to the inducing principle in the post-mitotic stage during the period of enzyme synthesis for secretion of extracellular protein. When the inducing principle is transferred from hyaluronomucoproteins of connective tissue, there is fibrous tissue induction; from chondromucoproteins of cartilage matrix, there is cartilage induction; from bone matrix proteins, there is bone induction. Whether the induction principle is a pattern or template of surface macromolecules, or a diffusible small protein that acts as the substrate for an enzyme are matters of speculation, but the intracellular reactions that follow may develop from feedback reactions from either cytoplasm to gene, or double cycle reactions from cytoplasm to cytoplasm, as outlined in Waddington's[60] modification of the Jacob-Monod hypothesis. The *transmission* of the inductive activity would depend on epigenetic systems (outside the genes) of both inducing and responding cells.

Bone develops in different locations from similar cells but always in one of two different arrangements. For example, bone forms in membrane in the vault of the cranium from the interaction of primitive mesoderm and perivascular connective tissue cells. Bone forms in the axial and appendicular skeletal from interaction of degenerating or dedifferentiating cartilage cells and perivascular connective tissue. Both of these induction systems, one in membrane and one in cartilage, begin early in fetal and continue in postfetal life. Both depend upon cell interactions and the maintenance of an equilibrium between bone resorption and bone formation that persists throughout the entire life of the individual.

When cells of one tissue interact with the cells of another and produce differentiation of the same product, it is described as *homeoinduction*. When cells of one tissue interact with the cells of another to produce a third tissue, the process is termed *heteroinduction*. In the case of an implant of dead, decalcified bone matrix, in which the cells that repopulate the implant and interact are both of mesodermal origin from the same organism, the process has been termed *autoinduction*.[50] Bone formation occurs by autoinduction with implants of decalcified, devitalized, lyophilized bone, dentin, cartilage and urinary bladder epithelium but not tendon or muscle tissue.

Resorption. Resorption of intercellular substances generally precedes bone induction. Resorption means destruction of the elements of intercellular matrix, and it puts a complicated structure into solution in such a fashion that it disappears—its end-products entering into the blood stream. The products of bone

resorption, in some unknown way, could set up an induction system for reformation of the bone that is resorbed. Resorption occurs in resorption cavities, presumably by the action of enzymes contained in the lysosomes.[34] The cells that resorb bone matrix modulate and induce or recruit new cells to deposit new bone and construct a new osteon. The process can be accelerated by an injection of a large dose of parathyroid hormone, which activates the cycle of cells of the bone tissue. Bone-resorbing cells, osteoclasts, form at one stage, modulate and become progenitor cells and osteoblasts at another.[67] Hypertrophied mesenchymal cells (perivascular connective tissue cells) would become induced cells after contact with cell slime of osteoclasts and osteoblasts.

Competency and Time of Induction. Numerous mitotic divisions occur over a period of 24 days to produce a large population of competent hypertrophied mesenchymal cells in the interior of an implant of bone matrix. An equally large population of mesenchymal cells develops within 24 days inside of an implant of silastic sponge, which does not induce bone. One very fundamental question is whether competency is coded in the nucleus at an earlier stage or developed in the cytoplasm by contact with bone matrix. If induction involves only postmitotic cells with cytoplasmic enzymes capable of synthesizing proteins of any one of several different kinds of matrices,[26] bone induction is a cytoplasmic reaction, and the period of time required to transform a mesenchymal cell into an osteoblast would be only a matter of hours or days. Observations with the use of tritiated thymidine-labeled nuclei suggest that the time required for transfer of the bone induction principle from matrix to an inducing cell, and then to an adjacent, undifferentiated responding cell, and thus produce an osteoblast, is less than 3 days.

The label appeared in osteoblasts at 3 days and osteocytes at 4 days, after the time of topical injection of a 3-week implant of bone matrix containing only mesenchymal cells. These observations suggest that, when a population of competent cells is in the area, the transfer of the bone induction principle from matrix to cells, and from one cell to another, could produce osteoblasts by Waddington's[60] second cycle, by which the cytoplasm affects the sites of protein synthesis by a double feedback loop.

Hypothetically, the control mechanism for embryonic induction systems is regarded in one of two ways: one in which the whole pathway from the beginning reaction to the end-result is triggered and determined by a short-term initial stimulus; the other, in which only the first step of a long chain reaction is activated, and in which it must be followed by secondary interactions between various tissue components. Observations upon some 200 different induction systems indicate that a single step process is most unlikely.[22,30,44] Nevertheless, we cannot be certain about the time of the beginning of bone induction. On one hand, we note that the time of induction coincides with the beginning of resorption of matrix. On the other hand, we note that cartilage induction does not require resorption of the implant, only contact between the first layer of mesenchyme and the surface of the old tissue.

Cooper,[13] from extensive tests with direct and transfilter inductive activity, noted that flattened but not round chondrocytes induced chondrogenesis, and that hypertrophied cytolysed chondrocytes induced osteogenesis. He observed that inductive activity is highest when cells are synthesizing or secreting extracellular matrix, and that "stage specificity" is critical in all induction systems. As in bacterial systems, the inducer would not produce new microscomal particles—only cause the cell to elaborate new messenger RNA, which would change the specific activity of the protein-synthesizing particles already in existence. Bone induction occurs after mitotic processes are concluded, and when the ergastoplasm of mesenchymal cells would be increasing in volume. The cause of the hyper-

trophy of the ergastoplasm is not the bone-inducing principle because it occurs in mesenchymal cells in implants of plastic which produce only fibrous tissue and no bone cells. Only the layer of hypertrophied mesenchymal cells in contact with the surface of the implant or a layer of newly induced mesenchymal cells, differentiates into osteoblasts.

The Composition of the Bone Induction Principle

The physicochemical composition of the bone induction principle is not known and presents a major challenge to students of vertebrate life processes. Extracellular fluid, lipid, polysaccharide, proteins are suspect and now being investigated by histochemical and analytical methods.

Extracellular Fluids, Mucous and Hyaline Substances. During the initial inflammatory reaction, in the period from 2 to 14 days after the operation, implants of bone matrix are always surrounded by a serous fluid. Physically, as well as chemically, this resembles edema fluid and has a high content of protein with many leukocytes in suspension. Whether it also contains chemical substances derived specifically from bone matrix, including the bone induction principle, is doubtful. If it is aspirated daily and withdrawn from the system, bone induction is neither enhanced nor inhibited. In any case, such experiments yield no evidence of a diffusible or soluble chemical inducer.

The bone-inducing principle is either cell bound or closely related to responding cells and secreted only during specific stages of cytodifferentiation. Mucoid substances,[22] cyst fluids,[27] hyaline[38] or extracellular matrix substances[13] are secreted only in spaces between interdigitating cell membranes when induction takes place. Thus, bone or cartilage induction systems appear to require continuous, prolonged, close contact between specific inducing surfaces or cells and a layer of competent mesenchymal cells with hypertrophied cytoplasm. Bone induction by a few implants of lyophilized bladder epithelial tissue in rats suggests that certain foreign substances may act in the same way as the bone induction principle, but this is no reason to disregard specificity of the bone induction principle in lyophilized bone matrix in all species of laboratory animals, including man.

Lipid. The matrix of bone contains less than 0.5% lipid. The possibility that a tissue lipid or steroid may be responsible for bone induction is suggested on the basis of the effect of acid-alcohol extracts of bone tissue injected into the belly of muscles of rabbits,[1,37] but we[45] have failed to corroborate this view. Moreover, the retention of the bone induction principle in samples of matrix extracted with various solvents to remove fats, phospholipids, glycolipids and sterols would indicate that the bone induction principle is not a lipid.

Polysaccharide. The polysaccharides iu bone consists of soluble and insolube protein-bound moieties firmly linked to collagen and represent approximately 5% of the dry weight of the matrix.[23] The possibility that polysaccharide may be the bone induction principle may be eliminated by the positive results that are obtained with samples extracted with neutral salt solutions, hyaluronidase, papain and chymopapain.

Protein. The possibility that the bone induction principle is a derivative of one of the acid-insoluble proteins in the bone tissue deserves serious consideration. The matrix of cortical bone consists of a highly cross-linked (gelatin-yielding) form of collagen (89.2%), mucosubstances (5%), a nongelatinous protein that resists solution in hot water (4.9%), chondroitin sulfate A (0.25%), a sialoprotein (0.15%), lipid (0.4%) and very small amounts of a soluble collagen and a citric-acid protein complex.[23] The cellular elements, of course, contain nucleoproteins and will be discussed briefly below. The bone induction principle may exist in one of the acid-insoluble protein fractions; but how it is mobilized from the dense mass of bone collagen by the process of bone resorption,

altered and absorbed by cells is obscure.

The cement lines of bone consist of a metachromatic, periodoic-acid-Schiff-reagent-positive amorphous, nongelatinous, condensed, glycoprotein. Its chemical composition is not known, but the possibility exists that the protein moiety may include the bone induction principle. If it does, it would explain how the inducer is conveyed from one layer of cells to the next and why lamellar bone is always deposited between films of cement substance. Moreover, it would account for the feltwork of metachromatic amorphous material that seeps through the 0.45 micron pores of 150 micron Millipore membranes and causes bone induction outside of Millipore chambers. Thus cement substance could have a dual function: (1) as the transmitter of the bone induction principle, and (2) as the adhesive material that binds the bone collagen fiber bundles together.

Osseocollagenolytic Enzymes. When a mass of decalcified bone matrix is implanted in the body, it is always resorbed by the cells of the recipient. The cells are macrophages, fibroclasts and multinucleated giant cells, which carry collagenolytic enzymes to the substrate.[28,34,64] The substrate of bone matrix is chiefly osseocollagen and accordingly resorbed by the action of cells that elaborate osseocollagenolytic enzymes. Cells that resorb tendon produce tendocollagenolytic enzyme.

That there are major differences in the solubility of collagens of bone matrix and tendon is demonstrated in recent observations of Glimcher and associates in systems in vitro.[20] Therefore, it is necessary to assume that cells that resorb bone matrix produce significantly different enzymes from those cells that resorb tendon. If this assumption is valid, cells that synthesize osseocollagenases at one stage may be the cells that perform an osteocollagenosynthetic function at a later stage. This would account for the specificity of the bone matrix for bone induction and tendon matrix only for fibrous tissue and not bone induction. This would account for the normal relationship between bone resorption and bone formation in resorption cavities, and the occurrence of bone induction only in excavation chambers in bone matrix.

Histocompatability Antigens. In implants of autologous and isogeneic lyophilized bone matrix, in which histocompatability antigens are almost nil, bone induction occurs with an incidence of over 96%. In implants of lyophilized, heated, or irradiated allogeneic bone matrix, in which histocompatability antigens are greatly reduced in potency, bone induction occurs with an incidence of at least 90%. Untreated allogeneic matrix produces bone induction in only 60% of implants; xenogeneic implants, even lyophilized, produce only 2% positive results. Such observations suggest that the bone induction principle may be a protein, which itself may or may not also behave as a histocompatability antigen. Hence, while bone induction is suppressed by the immune response of the host, it is possible that it may be an integral part of an autoimmune reaction, in which the inducing protein forms a macromolecular complex with a circulatory antibody of the recipient. It is also possible that present methods of detection of autoantigens and circulating antibodies from nonviable allografts are relatively insensitive, and cannot exclude antigen-antibody reactions in bone induction. It is not clear why particles of bone matrix, sizes 250 to 420μ, are absorbed rapidly and produce only a reticulocyte-plasma cell-macrophage reaction. Perhaps small particles introduce a large dose of antigen rapidly and incite an immune response, and possibly even antibodies, to coat the bone particles, and thereby prevent induction.

The Burnett[11] clonal selection theory invites some speculation about antigen-antibody reactions in cartilage induction. An implant of lyophilized cartilage would induce cells of the recipient to produce antibodies to cartilage-matrix antigens. The induced cells would multiply in the form of a nodule

or clone of cartilage cells adjacent to the site of origin of antigen. The clone is derived from a cell, which contains an allele that controls the synthesis of the antibody protein. The evidence against this line of investigation is first that cartilage induction occurs without a visible change or resorption of the matrix of lyophilized cartilage; and second, that inductive activity is increased by lyophilization, alcohol fixation, heat, and other agents that reduce the antigenic potency of the proteins. Waddington[60] contends that the genes of somatic cells are not labile enough to react in this way, and that it does not allow for epigenetic action systems of cells.

Organogenesis. Embryonic mesodermal cells contain genetic blueprints for a differentiation and growth of cartilage and bone cells, according to a prepattern or model of each bone as an organ. As an organ, a bone has a species-specific size and shape. Except that induction or cell interaction produces first the cartilage model and, second, the bone, the organizational process is visible only by biologic experiments and *a posteriori* morphologic evidence. One sees that a competent embryonic mesodermal cell reacts to the inducing agent, in accordance with its genetic constitution, including the specifications, and develops into the shape of a bone, such as a femur or a tibia. One assumes that a succession of inducing fields and patterns must determine whether there is full development of the potential of a cell for construction of all the details of the cartilage model. Therefore, the inducer must be less specific than the cell's reaction to it. Consequently, when one sees bone cells form in the inducing field of an implant of nonviable decalcified matrix, they must differentiate from mesodermal cells which carry the prepattern for development of a simple spherical bone with a central cavity for bone marrow. In postfetal life, mammalian mesodermal cells do not have the genetic specifications for development of a bone of any other shape than that of a simple sphere; the volume of the sphere is determined by the volume of the implant of inducing material. Only in lower vertebrates, i.e., salamanders, mesodermal cells of adult individuals have the genetic blueprints, as well as the competence to develop or regenerate whole bones of the shapes and sizes that are characteristic of the species.[4,60]

Practical Applications of the Bone Induction Principle

An induction system for osteogenesis can arise not only in sites where bone normally is found but also in pathologic conditions such as myositis ossificans, ankylosing spondylitis, osteoarthritis and tumors. An induction system may also be produced in sites of autologous and allogeneic bone-grafts for disorders of injury, aging and disease. More experimental work on the bone induction principle may produce new advances in the knowledge of pathogenesis and treatment of many orthopaedic conditions of unknown etiology.

Bone-Grafts. HCl-decalcified bone can be used to initiate osteogenesis in a bone-defect in a young animal, but it is not equivalent to a bone-graft in an adult.[24,58,66] A bone-graft is more than a source of inducer. When a bone-graft is placed in, or across, a bone-surface, it performs a homostructural as well as an osteogenetic function, that is, it persists to perform a mechanical function. When decalcified matrix is used for arthrodesis, it may produce a mass of new bone without fusion of the joint. The principal difference between a graft of undecalcified and an implant of HCl-decalcified bone matrix is immediately evident in the roentgenogram; an undecalcified bone-graft is densely radiopaque and remodeled and replaced by less dense new bone, relatively slowly, over periods ranging from months to years. Decalcified bone implants are completely radiolucent but are replaced rapidly within weeks by low-density, new-bone tissue. Mechanically, decalcified matrix is unsatisfactory, inasmuch as it does not provide internal fixation during the preinduction

phase, lacks homostructural function and cannot be expected to provide internal fixation.[50,51]

SUMMARY

1. However enigmatic induction systems looks in the living animal, biologists are getting closer and closer to a better understanding (including some practical applications) of a group of highly complex chemical substances identified collectively as the induction principle.

2. The bone induction principle is a derivative of specific proteins of the intercellular matrix of dentin, bone, cartilage; it is also a derivative of relatively nonspecific proteins of the epithelium of the fundus of the stomach, the gallbladder and the urinary tract (particularly the renal pelvis, the ureter and the urinary bladder).

3. Because proteins are ubiquitous constituents of all induction systems, and because only very small quantities of inducing material may be sufficient to induce bone formation, utmost caution should be exercised in attributing the origin of the bone induction principle to any single component of tissue. Tentatively, we may postulate that the bone induction principle is: (a) one or more acid-insoluble proteins of macromolecular dimensions, (b) associated with but not inseparable from antigenic activity, (c) transmitted short distances but not free in solution in extracellular spaces, (d) destroyed by amounts of heat, cryolysis, radiation, cytoplasmic poisons that denature proteins, (e) an integral part of cell slime and condensed glycoproteins of cement substance.

4. Both negative and positive experimental evidence demonstrates the existence of the bone induction principle; implants of lyophilized tissue that do not contain it produce proliferation of only macrophages, giant cells and fibrous connective tissue cells. Implants of lyophilized tissues that do contain the bone induction principle may produce the same cellular reactions, including all the microenvironmental requirements for oxygen saturation, CO_2 tension, pH, etc., but are different in that they transfer the macromolecular specifications for cellular differentiation of osteoblasts or chondroblasts.

5. Bone induction systems depend upon mesenchymo-mesenchymal cell interactions and upon the action of extrinsic controls upon intrinsic mechanisms of cell specialization. The extrinsic controls are transmitted by products of collagenolysis by motile mesenchymal cells herein referred to as inducing cells. Competent mesenchymal cells become responding mesenchymal cells only after contact with motile collagenolytic mesenchymal cells. Responding cells differentiate into bone cells in areas of sprouting capillaries. Responding cells in contact with similar motile mesenchymal cells but located in nonvascularized areas, unassociated with visible collagenolysis, differentiate not into bone tissue but into a clone of cartilage. There is no self-differentiation, only cellular differentiation in response either to coaptation with: (a) the *macromolecular structure of tissue containing the bone induction principle,* or (b) *surfaces of the newly induced cells which can transmit* and transfer it to layers of new responding cells.

6. Implants of bone matrix labeled with ^{3}H-glycine and ^{3}H-proline disclose radioactive isotope-labeled fixable degradation products of bone collagen dispersed throughout the extracellular substance of responding mesenchymal cells. But wherever new bone cells differentiate, the radioisotope-labeled material appears neither intracellular nor extracellular in the new bone deposits. Hence, inductive cell interaction is clearly a mesenchymal tissue extracellular process; it appears to be independent of pinocytosis, phagocytosis of fragments of bone collagen, or *intracellular* imbibition of the bone induction principle. Indeed, phagocytic or multinucleated giant cell activity generally occurs in areas not associated with bone induction.

7. Current concepts of cell inductive interactions suggest that osteogenesis may involve: (a) a positive feedback mechanism through

molecular assemblage conveyor belts, (b) physiologic coaptation-contact through cell secretions, (c) autoantigen-antibody complex effects, (d) tissue-specific bone mRNA for intrinsic control of biosynthesis of calcifiable matrix, (e) morphogenetic field formation or chreod for organ development, (f) unknown or as yet undiscovered species-and-tissue specific nucleo-cytoplasmic relationships.

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PRINTED IN U.S.A.