Table 2. Methanol-forming enzyme in the pituitary gland of various mammalian species. Soluble supernatant fractions obtained from 10 mg of pituitary from various species were incubated and assayed as described in Table 1. Results are expressed as nanomoles of methanol formed per gram of tissue.

| Species | Pituitaries examined (No.) | Enzyme activity |
|---|---|---|
| Cattle, posterior pituitary | (5) | 80 |
| Cattle, anterior pituitary | (3) | 25 |
| Rabbit, whole pituitary | (2) | 105 |
| Rat, whole pituitary | (3) | 35 |
| Human, whole pituitary | (2) | 17 |

7.9, 50 $\mu$l of nonradioactive methanol and 5 ml of ethyl acetate were added. The mixture was dried with sodium sulfate and the methanol was converted to 3,5-dinitrobenzoate with 3,5-dinitrobenzoyl chloride and sodium carbonate. The methyl 3,5-dinitrobenzoate was recrystallized to a constant melting point and to constant specific activity. This material showed only one peak of radioactivity on thin-layer chromatography; this peak coincided with the one shown by authentic methyl 3,5-dinitrobenzoate. These observations were taken as evidence that the volatile product formed enzymatically from pituitary extracts and $C^{14}$-methyl-*S*-adenosylmethionine was methanol.

The other product formed from this reaction should be *S*-adenosylhomocysteine. To establish this, *S*-adenosylmethionine-2-$C^{14}$ (*7*) was incubated with a partially purified enzyme preparation. After incubation the enzyme mixture was applied directly to Whatman No. 1 paper and chromatographed. The radioactive metabolite formed corresponded in $R_F$ value on paper chromatograms to authentic *S*-adenosylhomocysteine in two solvent systems. In parallel experiments the relative conversion of $C^{14}$-methyl-*S*-adenosylmethionine to methanol and *S*-adenosylmethionine-2-$C^{14}$ to *S*-adenosylhomocysteine was found to be stoichiometric. These results demonstrate that the enzyme in the pituitary gland carries out the following reaction:

*S*-adenosylmethionine + $H_2O \rightarrow$
*S*-adenosylhomocysteine + $CH_3OH$

This reaction proceeds by methylation of water or by hydrolysis of *S*-adenosylmethionine.

The methanol-forming enzyme was assayed in pituitary gland (Table 2) and other tissues of various species by the procedure described in Table 1. In cattle the anterior pituitary had about 25 percent of the activity of the posterior pituitary. All mammalian pituitaries examined (human, rat, rabbit) contained enzyme activity. The methanol-forming enzyme was also examined in tissues of ten organs of the rabbit. Negligible activity was present in these tissues; the small amounts of methanol found might have resulted from further metabolism of methanol. Whole brain and skin of frog (*Rana pipiens*) contained the methanol-forming enzyme. An ectopic tumor of possible pineal or pituitary origin also contained large amounts of the methanol-forming enzyme (*8*), but no hydroxyindole *O*-methyltransferase, an enzyme found in tissues of pineal gland (*9*). This specificity of the enzyme indicates that this tumor was of pituitary origin.

Methanol is present in the normal breath of human subjects (*10*) and is believed to be formed endogenously. However, no pathway for the formation of methanol in animals has been demonstrated previously. It is possible that this alcohol might arise from the action of the methanol-forming enzyme on *S*-adenosylmethionine, in the pituitary gland. Although the role of the methanol-forming enzyme remains obscure, its localization in an organ of high physiological activity, the pituitary gland, suggests that it might have a function.

JULIUS AXELROD
JOHN DALY
*National Institute of Mental Health*
and *National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, 20014*

**References and Notes**

1. J. Axelrod, in *Transmethylation and Methionine Biosynthesis*, S. R. Shapiro and F. Schlenk, Eds. (Univ. of Chicago Press, Chicago, 1965), pp. 71–83.
2. J. Axelrod, *Biochim. Biophys. Acta* **45**, 614 (1960).
3. J. Daly, J. K. Inscoe, J. Axelrod, *J. Med. Chem.* **8**, 153 (1965).
4. J. Axelrod, *J. Pharmacol. Exp. Therap.* **138**, 28 (1962).
5. D. D. Brown, J. Axelrod, R. Tomchick, *Nature* **183**, 680 (1959).
6. H. M. Adam, in *Regional Neurochemistry*, S. S. Kety and J. Elkes, Eds. (Pergamon, Oxford, England, 1961), pp. 293–306; J. Axelrod, P. D. MacLean, R. W. Albers, H. Weissbach, *ibid.*, pp. 307–311.
7. We thank S. H. Mudd for supplying us with *S*-adenosylmethionine-2-$C^{14}$.
8. S. H. Snyder, J. Axelrod, O. Smith, unpublished observations.
9. R. J. Wurtman, J. Axelrod, R. Toch, *Nature* **204**, 1323 (1964).
10. S. P. Eriksen and A. B. Kulkarni, *Science* **141**, 639 (1963).

9 August 1965

## Bone: Formation by Autoinduction

Abstract. *Wandering histiocytes, foreign body giant cells, and inflammatory connective-tissue cells are stimulated by degradation products of dead matrix to grow in and repopulate the area of an implant of decalcified bone. Histiocytes are more numerous than any other cell form and may transfer collagenolytic activity to the substrate to cause dissolution of the matrix. The process is followed immediately by new-bone formation by autoinduction in which both the inductor cells and the induced cells are derived from ingrowing cells of the host bed. The inductor cell is a descendant of a wandering histiocyte; the induced cell is a fixed histiocyte or perivascular young connective-tissue cell. Differentiation of the osteoprogenitor cell is elicited by local alterations in cell metabolic cycles that are as yet uncharacterized.*

New evidence in favor of the theory of induction can be gathered from the process of bone formation in the interior of an implant of acellular, devitalized, decalcified, bone matrix. Differing from previous demonstrations of induction systems, which produce scanty deposits and less than 30-percent positive results (*1*), decalcified bone yields new bone in an amount proportional to the volume of the implant; the percentage of positive experimental results is as high as over 90 percent. The results of some 70 experiments on approximately 300 animals are summarized below to introduce a hypothesis of postfetal osteogenesis by autoinduction (*2*).

Long bones, excised from adult rabbits and other laboratory animals, were cut in lengths of 1 to 2 cm and decalcified, unfixed, in 0.6*N* HCl. Samples of human cortical bone obtained from accident cases, excised under aseptic conditions at autopsy, were lyophilized and decalcified similarly in sterile solutions of 0.6*N* HCl for a period of 5 days. The acid was removed by prolonged washing in sterile 0.15*M* NaCl. The chemical composition of HCl-decalcified bone matrix, in millimoles per liter, per kilogram,

was 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 was 4.4±0.5.

The samples of HCl-decalcified homogenous diaphyseal bone were implanted into (i) a pouch in the belly of either the rectus abdominus, quadriceps, or erector spinae muscles of approximately 250 rabbits, 20 rats, 10 mice, and 5 guinea pigs; (ii) a defect in the ulna in 10 rabbits or a bed of bone on the lumbar vertebrae in 3 dogs; (iii) a defect in a bone in various skeletal system disorders in 21 human beings. In an effort to alter the chemically reactive groups associated with the structure of the matrix, samples of bone were (i) decalcified in a series of equimolar solutions of seven different acids; (ii) decalcified in HCl and treated with blocking reagents for carboxyl, $\epsilon$-amino, and nonterminal amino or sulfhydryl reactive groups; (iii) altered physically by alcohol fixation, heat shrinkage at 70°C, lyophilization, or denaturation and sterilization in $\beta$-propriolactone. The effects were assessed by correlated radiographic and histological methods and by determinations of the percentages of positive results in eight to ten implants of each preparation (Table 1).

The typical implant of homogenous HCl-decalcified matrix, 3 weeks after implantation in the anterior abdominal wall, was enveloped in loose, highly vascular, inflammatory, and fibrous connective tissue. The trabecular interstices and old vascular channels were infiltrated with wandering histiocytes or macrophages, large and small lymphocytes, and fibroblasts. The matrix was swollen and amorphous in hematoxylin and eosin and showed metachromatic staining with toluidine blue, but the collagen fiber bundles were clearly distinguishable and surprisingly intact with Wilder's stain. The osteocyte lacunae, however, were empty and often very much enlarged.

The invading cells of the host, chiefly wandering histiocytes, were first closely related to the process of resorption of dead decalcified bone matrix but later became arranged in three distinct population groups. One was found in excavation chambers produced by the pressure of sprouting capillaries, the proteolytic activity of macrophages, the gathering of foreign body giant cells, and the multiplication of young connective-tissue cells. 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 consisted of nests of proliferating cartilage cells. The earliest deposits of new bone appeared at 4 to 6 weeks from osteoprogenitor cells in the interior of well-vascularized excavation chambers. Later, between 8 and 16 weeks, bone formed from proliferating connective-tissue cells associated with vascularization, calcification, and replacement of the nests of cartilage by the typical route of endochondral ossification. Bone formation did not occur from foreign body giant cells, plasma cells, small lymphocytes, large dilated capillaries, or cell populations associated with inflammation. Wherever bone induction occurred there was a pool of stem cells, osteoprogenitor cells, and small capillaries, surrounded by palisades of deeply basophilic plump osteoblasts. By appositional formation of new bone, layers of osseous tissue with intermediate cement lines were deposited on the surface of decalcified dead matrix. The deposits were always enclosed in excavation chambers, either within the external surface or deep inside the old matrix; new bone never extended outside the implant. A periosteum-like outer envelope of fibrous tissue surrounded the dead matrix and confined the processes of bone and cartilage induction strictly to spaces created by resorption of the dead matrix (Figs. 1 to 3).

Living new bone was always easy to distinguish from dead, mineral-free matrix. It calcified rapidly almost as soon as it was laid down, and it was always separated from decalcified matrix by a thin line of cement substance. Areas of recalcification of dead matrix were few, and they rarely coincided with areas of osteogenesis. In one experiment the decalcified bone was primed in a solution of calcium chloride before implantation to initiate recalcification (*3*), but this treatment retarded resorption of matrix and hampered osteogenetic induction. Matrix decalcified with ethylenediamine tetraacetic acid (EDTA), mixed formic and citric acids, or acetic acid produced osteogenesis in the same way as matrix decalcified with HCl, but EDTA produced a slightly lower percentage of positive results. Lactic acid failed to remove all the mineral, and a diffuse deposit which remained seemed to increase inflammation and prevent osteogenesis. Heating to 70°C, sufficiently to produce shrinkage of the collagen fibers of the bone matrix, impeded but did not altogether prevent osteogenetic induction. Matrix decalcified with nitrous acid or nitric acid, and matrix sterilized with $\beta$-propriolactone and decalcified with HCl did not induce bone formation in a single instance. Inhibition also occurred from dinitrophenolation (FDNB) and iodoacetamidization (IAA) of matrix decalcified with HCl. Morphologically, nitrous and nitric acid, which deaminated tissue proteins, produced widespread inflammation and total disintegration of the decalcified matrix; FDNB, which blocks $\epsilon$-amino and other groups, and IAA, which blocks sulfhydryl and other reactive groups, dehydrated and shrank the decalcified matrix and appeared to prevent osteogenetic induction by retarding the cellular ingrowth and excavation of matrix. Toluidine blue, which blocks carboxylic acid groups and binding of calcium ions, prevented recalcification of matrix but did not inhibit osteogenetic induction. Neither did lyophilization or matrix fixation in alcohol (Fig. 4).

A series of experiments, designed to determine the fate of HCl-decalcified matrix in a host bed of bone tissue, demonstrated that HCl-decalcified bone is an excellent substitute for a bone graft. The dead, decalcified matrix was invaded by new blood vessels and resorbed rapidly; new bone was deposited in pockets or excavation chambers filled with proliferating osteoprogenitor cells. The process began within a few weeks and was complete within a few months. How much new bone could be attributed to the osteoconduction of cells growing in from the walls of the host bone, and how much could be accounted for by osteogenetic induction, was not apparent from experiments on normal, healthy bones. The most important tests were on decalcified matrix in adult human bones with avascular necrosis, large amounts of missing bone substance, or irregular, small, cortical surfaces like those of the lower lumbar vertebrae. Another important test was the capacity of decalcified bone to produce lumbar spinal fusion in a dog; in this area of the skeleton the mechanical system of the wagging tail acts to impede

osteoconduction from the host bed. In abnormal host bone beds, either mechanical or pathological, successful results were obtained chiefly in young individuals with active, rapidly proliferating osteoprogenitor cells. In cases in which there was a defect to fill, the most striking difference between undecalcified and HCl-decalcified bone matrix was in the radiographic picture and relatively rapid rate of replacement Undecalcified bone grafts were more radioopaque than ingrowing new bone and were replaced slowly over long periods of time, ranging from months to years. Decalcified bone implants were at first completely radiolucent but were replaced by low-density, new-bone tissue very rapidly over a period of weeks to a few months. Mechanically, decalcified matrix was unsatisfactory, inasmuch as it did not provide internal fixation during the preinduction phase and did not arthrodese the vertebrae of the moving lumbar spine of a dog (Fig. 5).

The area of the implant of dead bone matrix is vacant territory and immediately begins to attract wandering histiocytes. Within 3 weeks every accessible microscopic space is occupied by a new cell. The invading cells resorb matrix, release tropic chemical agents, and stimulate capillaries and perivascular connective tissue to excavate large chambers for further occupancy by their offspring and additional cell populations. When one wandering histiocyte (the inductor cell) and one perivascular connective-tissue cell (the responding or induced cell) divide and interact, cellular differentiation occurs by autoinduction to produce two additional cells, one responding cell and one specialized form, either an osteoprogenitor or a chondroprogenitor cell. The chondroprogenitor cell produces cartilage, which later becomes vascularized to set up a second or delayed induction system for bone formation. Whether the progenitor cell

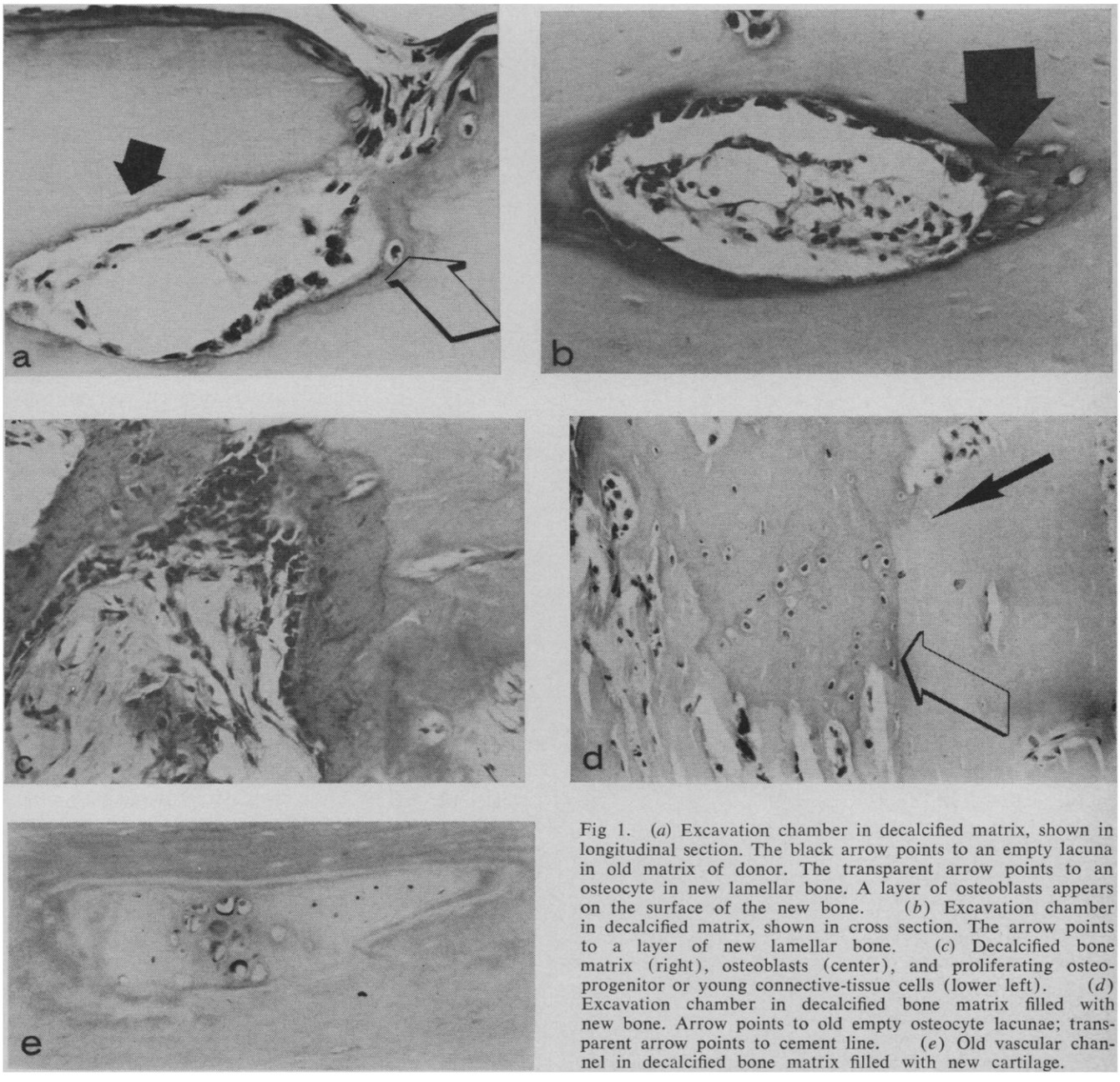


Fig 1. (*a*) Excavation chamber in decalcified matrix, shown in longitudinal section. The black arrow points to an empty lacuna in old matrix of donor. The transparent arrow points to an osteocyte in new lamellar bone. A layer of osteoblasts appears on the surface of the new bone. (*b*) Excavation chamber in decalcified matrix, shown in cross section. The arrow points to a layer of new lamellar bone. (*c*) Decalcified bone matrix (right), osteoblasts (center), and proliferating osteoprogenitor or young connective-tissue cells (lower left). (*d*) Excavation chamber in decalcified bone matrix filled with new bone. Arrow points to old empty osteocyte lacunae; transparent arrow points to cement line. (*e*) Old vascular channel in decalcified bone matrix filled with new cartilage.

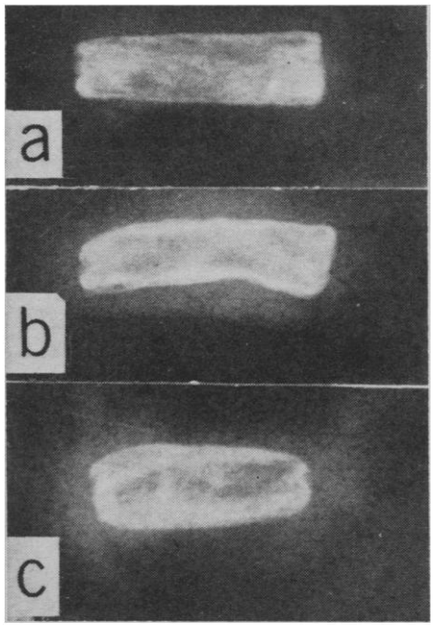


Fig. 2. Radiographs of cylindrical decalcified bone implants in belly of rectus abdominus muscle of rabbits: (*a*) at 6 weeks after the operation; (*b*) at 9 weeks; (*c*) at 12 weeks. Note the transformation from the cylindrical shape of the implant at 6 weeks into the spherical shape of medullary cavity of the ossicle at 12 weeks.

produces bone or cartilage is determined by microenvironmental or local factors. Bassett (*4*) prepared tissue cultures and reviewed the literature to point out that compaction of cells with low oxygen saturation produces cartilage; compaction with high oxygen saturation produces bone; stretching the tissue, or mechanical tension, produces only fibrous tissue. Whether chemical by-products of cell metabolism and bioelectric effects are secondarily produced are matters for future investigation.

Except that the repopulation of the implant with mesenchymal cells (both wandering and fixed histiocytes) is intimately associated with the process of absorption of the collagenous material, the mechanism of removal of decalcified matrix is not known. Foreign body giant cells (multinucleated cells larger than osteoclasts) are found in close contact with the cut edges of the trabeculae, but their numbers are too few to account for all the collagen that is removed. Wandering histiocytes (macrophages) appear in very large numbers between the fibers of disintegrating collagen but do not contain particulate material identifiable as phagocytosed matrix. The evidence at present is that colloidal material of ultrastructural, rather than microscopic, dimensions is phagocytosed. It is reasonable to suppose that the cells that make bone-matrix collagenase carry the enzyme to the substrate and break the fibrous proteins down to subultrastructural or even amino acid size particles. Collagenases are not stored and are not extractable from fresh tissues; they accumulate in the media of certain cells in tissue culture (*5*).

How the giant cells differ from osteoclasts and engage in resorption is still a mystery. They are found in pits similar to Howship's lacunae in the surface of decalcified and partially recalcifying surfaces indiscriminately, but they are larger and more numerous around bone decalcified with nitric acid, which neither recalcifies nor induces osteogenesis. Irving's (*6*) work on resorption of normal and decalcified dead bone matrix demonstrates that the amounts of cytochrome oxidase, acid phosphatase, succinic dehydrogenase, malic, lactic, and glutamic dehydrogenases, diphosphopyridine nucleotide diaphosphorase, and isocitric dehydrogenases are virtually the same in foreign body giant cells as in osteoclasts. Inasmuch as rachitic osteoid does not elicit foreign body giant cell formation, does recalcify, and is not resorbed, and insofar as decalcified normal bone matrix does attract multinucleated cells, Irving concludes, first, that the inorganic phase of bone plays only a minor role in causing osteoclasis; second, that matrix from a nonrachitic animal contains something that makes it resorbable; and third, that there is a tropism for the ends of the implants where the bone had been cut. Irving also described decalcified bone matrix, prepared in EDTA, 0.5*M* at *p*H 7.0, and implanted subcutaneously for 8 weeks, but did not mention bone induction.

The migration of wandering histiocytes into the decalcified bone implant resembles Weiss's patterns of movement of cells during tissue reconstruction as described by Mascona (*7*). Undifferentiated motile cells are guided by tissue-specific factors that control migrations, swarming, translocations, and regroupings. It is assumed, from studies on cell exudates, that cells elaborate an extracellular material that has the dual purpose of binding them

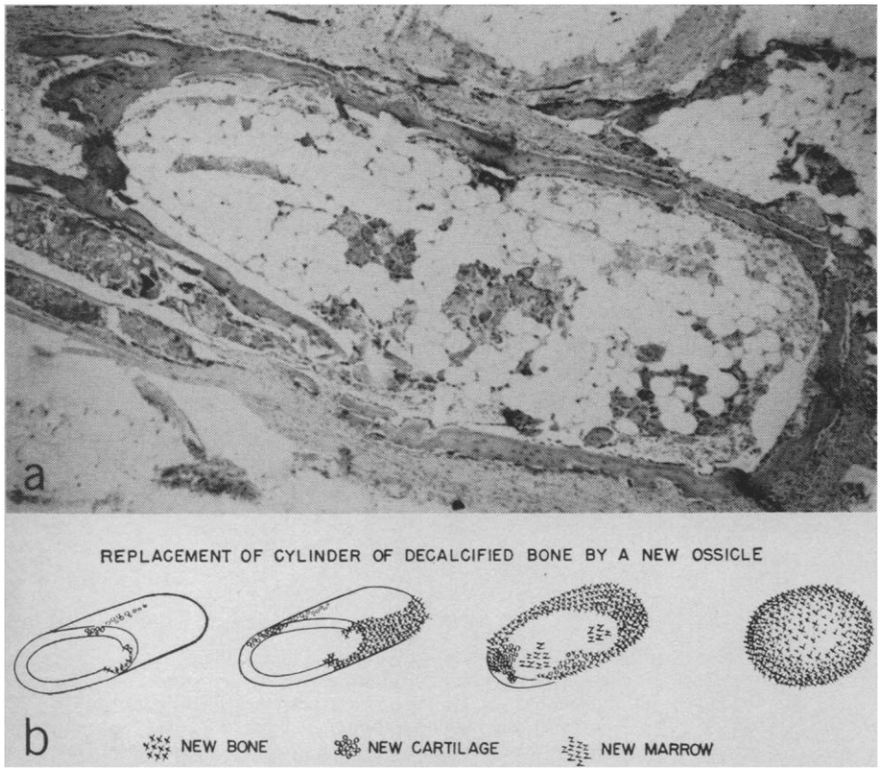


Fig. 3. (*a*) Low-power photomicrograph of ossicle shown in Fig. 2*c*. (*b*) Diagrammatic representation of the process of transformation of cylinders of decalcified bone matrix into a new ossicle. The new bone always forms inside well-vascularized excavation chambers in the interior of the matrix. New cartilage forms, not in excavation chambers, but in the closed end of old vascular channels. The volume of the living ossicle represents approximately 75 percent of the volume of the original implant of dead, decalcified matrix.

together and transmitting inductive stimuli. If the migratory stimulus is tissue-specific, it is conceivable that histiocytes with special predisposition to osteogenesis are attracted into the decalcified implant. There is considerable evidence, reviewed by Saxen and Toivonen (*8*), that there are heterogenous inductors which are (i) cross-tissue as well as cross-species specific, (ii) obtainable from adult as well as from embryonic material, (iii) active in heterotopic sites, and (iv) resistant even to chemical fixatives. This process is referred to as *heterogenous induction* and includes a hypothetical mesodermalizing principle. The sum and substance of current literature on heterogenous induction defies all present understanding of the chemistry of life processes.

Bone induction in decalcified matrix raises questions that are common to the whole unsolved problem of cellular differentiation. Does matrix produce a specific diffusible chemical agent that induces the cells of the host to differentiate into osteoblasts? The answer is no. The system is more complex than a simple chemical stimulus and direct cell response; its complexity suggests that we should reconsider Spemann's theory of induction. Spemann's concept (*9*), derived chiefly from experiments upon embryonic tissue, means the influence of one living cell (an inductor) upon another living one (the induced cell) to cause it to differentiate into a more specialized form. In oversimplified but realistic terms, the idea of induction comes from the observation that the cells of the "eye" cup derived from the neural plate induce cells of skin-forming ectoderm to bring about the formation of the lens. Similarly, a group of proliferating cells inside an excavation chamber in decalcified matrix induces a group of young connective-tissue cells associated with capillary sprouts to differentiate first into osteoprogenitor cells, then into osteoblasts. A group of proliferating cells in the closed end of an old vascular channel induces a group of their own offspring to differentiate into chondroprogenitor cells (without any preliminary sprouting of capillaries or absorption of dead matrix). Table 2 illustrates the sequence of appearance of various cell types in decalcified matrix induction systems for cartilage and bone.

Experiments with specific diffusible chemical agents from spinal cord and

Table 1. Bone formation in implants of decalcified bone matrix.

| Donor | Preparation | Site | Host | Positive results (%) |
|---|---|---|---|---|
| Mouse | HCl, 0.6*N* | Rectus abdominus | Mouse | 95 |
| | | | Rat | 60 |
| Rat | HCl, 0.6*N* | Rectus abdominus | Rat | 90 |
| | | | Rabbit | 70 |
| Guinea pig | HCl, 0.6*N* | Rectus abdominus | Guinea pig | 95 |
| | | | Rat | 50 |
| Rabbit | HCl, 0.6*N* | Rectus abdominus and quadriceps | Rabbit | 98 |
| | | | Rat | 60 |
| Calf | HCl, 0.6*N* | Rectus abdominus | Rabbit | 50 |
| | | | Rat | 60 |
| Rabbit | EDTA, 1*N* | Rectus abdominus | Rabbit | 80 |
| Rabbit | Formic-citric, 1*N* | Rectus abdominus | Rabbit | 85 |
| Rabbit | Lactic, 0.6*N* | Rectus abdominus | Rabbit | 10 |
| Rabbit | Acetic, 0.6*N* | Rectus abdominus | Rabbit | 50 |
| Rabbit | Nitric, 0.6*N* | Rectus abdominus | Rabbit | 0 |
| Rabbit | Nitrous, 0.6*N* | Rectus abdominus | Rabbit | 0 |
| Rabbit | β-Proprio-lactone, 10% | Rectus abdominus | Rabbit | 0 |
| Rabbit | HCl, 0.6*N*, + 0.1% toluidine blue | Rectus abdominus | Rabbit | 80 |
| Rabbit | HCl, 0.6*N*, + 70°C heat | Rectus abdominus | Rabbit | 20 |
| Rabbit | HCl, 0.6*N*, + 70% alcohol | Rectus abdominus | Rabbit | 85 |
| Rabbit | HCl, 0.6*N*, + lyophilization | Rectus abdominus | Rabbit | 80 |
| Rabbit | HCl, 0.6*N*, +0.1% 1,2-fluorodi-nitrobenzene | Rectus abdominus | Rabbit | 0 |
| Rabbit | HCl, 0.6*N*, +0.1% iodoacetamide | Rectus abdominus | Rabbit | 10 |
| Rabbit | $CaCl_2$, 25 mmole/liter | Rectus abdominus | Rabbit | 50 |
| Rabbit | HCl, 0.6*N* | Ulna | Rabbit | 95 |
| Human | HCl, 0.6*N* | Various bone defects | Human | 90 |
| Calf | HCl, 0.6*N* | Fibrous dysplasia | Human | |
| Calf | HCl, 0.6*N* | Lumbar spinal fusion | Dog | 50 |

Table 2. Cell induction sequences.

| Before induction | | | After induction | |
|---|---|---|---|---|
| Inductor cell + Responding cell | Microenvironmental variables | | Responding cell | Differentiated cell |
| A + B | + ($CO_2$ tension, $O_2$ saturation, cell metabolites) | ⟶ | B | + D |
| Wandering histiocyte + Fixed histiocyte | + (Open excavation chamber, high $O_2$ saturation, low $CO_2$, other cell metabolic products) | ⟶ | Fixed histiocyte | + Osteoprog-enitor cell and osteoblast |
| A + B | + ($CO_2$ and $O_2$ saturation, cell metabolites) | ⟶ | B | + C |
| Wandering histiocyte + Fixed histiocyte | + (Compaction of cells in closed vascular channel, high $CO_2$, low $O_2$ satura-tion, other cell metabolic products) | ⟶ | Fixed histiocyte | + Chondro-progenitor cell and chondroblast |
| C + B | + (Vascularized excavation chamber) | ⟶ | B | + D |
| Chondrocyte + Perivascular connective-tissue cell | + (Absorption cavities, high $O_2$, low $O_2$ saturation) | ⟶ | Perivascular connective-tissue cell | + Osteo-progenitor cells and osteoblast |

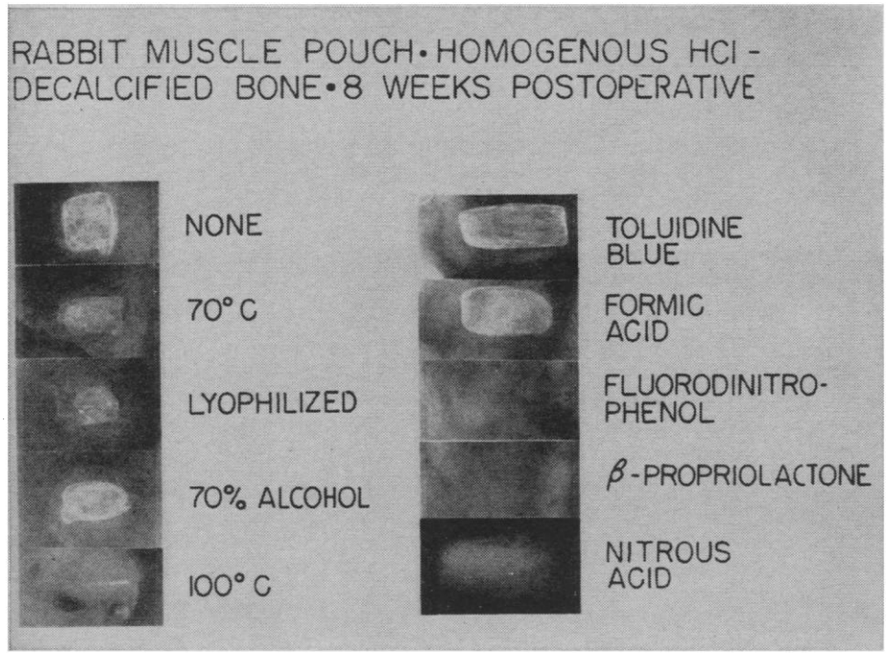


Fig. 4. Radiographs showing new ossicles formed from implants of homogenous decalcified bone in rabbits, treated in various ways and implanted in the anterior abdominal wall. Fluorodinitrobenzene, β-propriolactone, or nitrous acid prevented formation of ossicles in implants of decalcified bone matrix.

notochord which act locally to induce chondrogenesis (*10*), systems which induce osteogenesis across Millipore filters (*11*), and extracts of bone and cartilage that alter tissues to produce bone formation by induction (*12*), are not incontrovertible evidence of a diffusible inductor. The reaction of embryonic tissue can always be challenged with questions about the difficulty of assessing the stage of differentiation of cells in the preinduction phase. Experiments with extracts of tissue are often rejected as examples of the unspecific effects of chemical injury (*13*). All of these objections are avoided, however, by the hypothesis of autoinduction and the simple assumption that nonspecific substances or degradation products of dead tissue stimulate or attract the wandering histiocytes to migrate into the interior of the implant. Shipley and Macklin (*14*) describe ingestion of ultramicrons of trypan blue by histiocytes preceding osteogenesis and encourage one to speculate that these cells induce other primitive connective-tissue cells to differentiate into osteoblasts. Inasmuch as decalcified bone produces new bone even after heat shrinkage, fixation in formalin and in alcohol, lyophilization, and toluidine blue staining, and other treatments, only nonspecific acid or neutral salt–insoluble substances, which resist the most harsh treatment, and only nonspecific histiocyte-attracting chemical substances could possibly emanate from dead matrix. Furthermore, intramuscular implants of HCl-treated muscle, tendon, cartilage, and many other tissues unrelated to bone matrix may

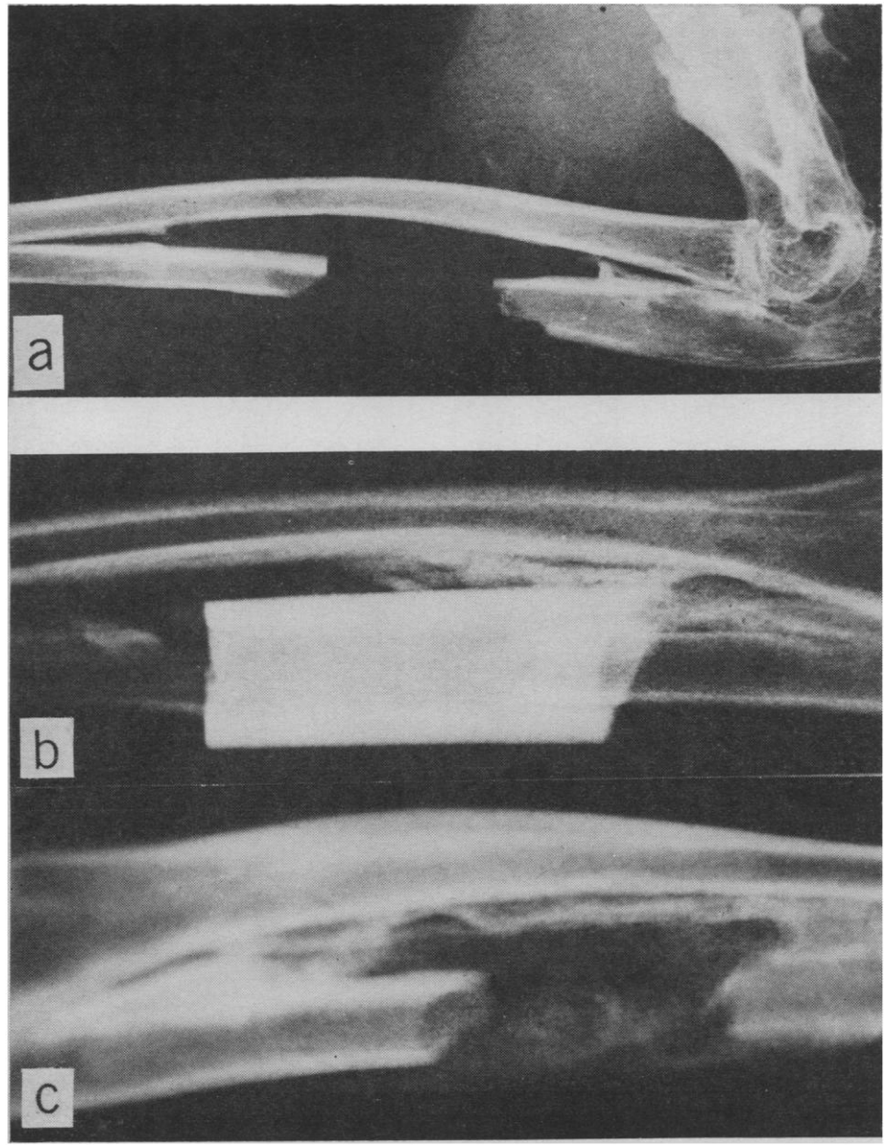


Fig. 5. Radiographs of ulna of rabbits showing (*a*) resection of middle third of the shaft; (*b*) replacement with *undecalcified* homogenous segment of diaphysis; (*c*) replacement with an HCl-decalcified segment of diaphysis. Twelve weeks after the operation, the decalcified matrix has been completely replaced, and the defect is filled with new bone which is so abundant that it produced radioulnar synostosis. The undecalcified implant (*b*) is largely unabsorbed and unreplaced; it is united to only one bone end of the host.

similarly produce new bone by autoinduction. Thus, RNA and degradation products of various intracellular proteinaceous materials, previously claimed to act as inductors, would enter only into the preinduction process. In the bone-induction process involving, as noted in Table 2, the interaction of living cells under normal conditions in the living animal, the inductor would not need to diffuse beyond subultramicroscopic distances. Cell interaction could take place immediately before mitosis, and the inductor could flow along membranes from one cell to another.

The evidence for osteogenesis by autoinduction is entirely morphological but, nevertheless, substantial. Bone formation occurs in extraskeletal implants of decalcified bone matrix in the interior of excavation cavities, and the new osteoblasts are derived, not from elements of the donor tissue, but from proliferating pleuripotent, ingrowing cells of the host. Comparable implants of *undecalcified* dead bone also produce osteogenesis, but only very rarely, only in scanty amounts, and then only after a latent period of several months (*15*). Ray and his associates (*16*) and Sherrard and Collins (*17*) describe rapid replacement of decalcified bone in bone defects, but as new bone arises from surrounding bone tissue (*18*), the orthotopic system does not offer convincing evidence of induction. In an extraskeletal or heterotopic implant of decalcified dead matrix, cell-induction sequences produce an entirely new ossicle with a marrow cavity as the end-product, and not merely histiotypic osteogenesis. Because differentiation of new-bone marrow occurs concomitantly with the process of new-bone formation by induction, the possibility of implanting decalcified bone matrix in subcutaneous soft parts in individuals with severe anemia, to produce a new site of active hematopoiesis, is important and interesting to contemplate. Uncertainty exists, however, about whether the period of survival of an ossicle in soft parts would be determined by the physiological demands for bone marrow, or mechanical stimuli for bone tissue. In a normal animal, extraskeletal ossicles decrease in size and may be entirely resorbed after a few years in the anterior abdominal wall.

MARSHALL R. URIST
*Department of Surgery, University of California Center for Health Sciences, Los Angeles 90024*

12 NOVEMBER 1965

**References and Notes**

1. J. Nageotte, *Compt. Rend. Soc. Biol.* **81**, 113 (1918); J. Chalmers and H. A. Sissons, *J. Bone Joint Surg.* **41-B**, 209 (1959); M. R. Urist and F. C. McLean, *ibid.* **34-A**, 443 (1952); R. W. Selle and M. R. Urist, *J. Surg. Res.* **1**, 132 (1961); P. S. Zaccalini and M. R. Urist, *J. Trauma* **4**, 344 (1964); M. R. Urist, T. H. Wallace, T. Adams, *J. Bone Joint Surg.* **47-B**, 304 (1965).
2. A series of papers giving detailed results has been prepared for publication by K. A. van de Putte and me.
3. K. A. van de Putte and M. R. Urist, *Clin. Orthoped. Rel. Res.*, in press.
4. C. A. L. Bassett, *J. Bone Joint Surg.* **44-A**, 1217 (1962).
5. C. M. Lapiere and J. Gross, in *Mechanisms of Hard Tissue Destruction*, R. F. Sognnaes, Ed. (AAAS, Washington, D.C., 1963), pp. 663–694.
6. J. T. Irving, *ibid.*, pp. 515–530.
7. A. A. Mascona, in *Developing Systems and Their Control* (Ronald, New York, 1960).
8. L. Saxen and S. Taivonen, *Primary Embryonic Induction* (Prentice-Hall, Englewood Cliffs, N.J., 1962).
9. H. Spemann, *Embryonic Development and Induction* (Yale Univ. Press, New Haven, Conn., 1938).
10. J. W. Lash, S. Holzer, H. Holzer, *Exptl. Cell Res.* **13**, 292 (1957); J. W. Lash, F. A. Holmes, F. Zilliken, *Biochim. Biophys. Acta* **56**, 313 (1962).
11. P. Goldhaber, *Science* **133**, 2065 (1961).
12. M. L. Moss, in *Calcification in Biological Systems*, R. F. Sognnaes, Ed. (AAAS, Washington, D.C., 1960), pp. 323–328.
13. A. Danis, *Acta Orthopaed. Belg.* **22**, 501 (1956).
14. P. G. Shipley and C. C. Macklin, *Am. J. Physiol.* **42**, 117 (1916).
15. R. G. Burwell, in *Modern Trends in Orthopedics*, J. M. P. Clark, Ed. (Butterworth, Washington, D.C., 1964), pp. 138–190; M. R. Urist, in *Instructional Course Lectures Am. Acad. Orthoped. Surg.*, F. Reynolds, Ed. (Mosby, St. Louis, Mo., 1960), vol. 17, pp. 184–195.
16. R. D. Ray and A. Holloway, *J. Bone Joint Surg.* **39-A**, 1119 (1957); R. Freiberg and R. D. Ray, *Arch. Surg.* **89**, 417 (1964).
17. W. J. W. Sherrard and D. H. Collins, *Proc. Roy. Soc. Med.* **54**, 1101 (1961).
18. M. H. Young, *J. Bone Joint Surg.* **46-B**, 329 (1964).
19. I am indebted to K. A. van de Putte, R. Townsley, D. Yomans, J. M. Adams, M. Hermans, and T. Adams for professional and technical assistance. Work supported by grants-in-aid from the Josiah Macy, Jr. Foundation, Squibb Institute for Medical Research, and the U.S. Army (DA-49-193 MD 2556).

9 July 1965

## Electrophysiological Studies of Chilean Squid Axons under Internal Perfusion with Sodium-Rich Media

Abstract. *Electrophysiological properties of giant axons of Chilean squid were examined by intracellular perfusion combined with various recording techniques. The amplitude of the action potential of the axons perfused with equal sodium concentrations (0.35M) on both sides of the membrane was approximately 75 millivolts (glutamate salts being used internally); the resting potential recorded with a calomel electrode was roughly 45 mv. These observations confirm and extend previous data, obtained from Chilean squid axons, which show that the amplitude of the action-potential overshoot does not agree with the Nernst equation applied to sodium ion. The discrepancy between these results and those obtained by others is attributed to differences in the procedures used to determine the resting potential.*

Giant axons in the stellate nerve of the Chilean squid, *Dosidicus gigas*, are very large (0.6 to 1.5 mm in diameter) and have no branches in the portion near the ganglion. Therefore, it is relatively easy to apply the method of intracellular perfusion with multiple cannulae to electrophysiological studies of these axons (*1*). This method gives important information concerning the physicochemical nature of the process of nerve excitation by directly controlling the chemical composition of the fluid media on both sides of the axon membrane.

Among several results obtained by this method (*1*), was the finding that *Dosidicus* axons could maintain relatively large action potentials under perfusion with a sodium-rich solution. With the same sodium concentrations across the membrane the resting potential of these axons was close to normal, and smaller than the amplitude of the action potential. The significant conclusions from these experiments are (i) that, at the inner surface of the axon membrane, a wide variation in the ratios of Na to K concentrations does not affect the resting potential significantly, and (ii) that combination of a high external with a low internal concentration of Na is not an indispensable condition for the maintenance of excitability. Furthermore, these experiments revealed a significant difference in physiological behavior between the inner and outer surfaces of the axon membrane.

Hodgkin and Chandler (*2*) have suggested that the technique of action-potential recording in the aforementioned study might have resulted in large, spurious electric responses because of the high resistance of the electrode. Therefore the electrophysiological properties of intracellularly perfused Chilean squid axons were examined with other recording techniques (*3*).

For intracellular perfusion the tech-