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Recent studies of the mineral phase in bone and its possible linkage to the organic matrix by protein-bound phosphate bonds

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[Plates 1 and 2]

The most widely accepted hypothesis to account for maturational changes in the X-ray diffraction characteristics of bone mineral has been the 'amorphous calcium phosphate theory', which postulates that an initial amorphous calcium phosphate solid phase is deposited that gradually converts to poorly crystalline hydroxyapatite. Our studies of bone mineral of different ages by X-ray radial distribution function analysis and ^{31}P n.m.r. have conclusively demonstrated that a solid phase of amorphous calcium phosphate does not exist in bone in any significant amount. ^{31}P n.m.r. studies have detected the presence of acid phosphate groups in a brushite-like configuration. Phosphoproteins containing *O*-phosphoserine and *O*-phosphothreonine have been isolated from bone matrix and characterized. Tissue and cell culture have established that they are synthesized in bone, most likely by the osteoblasts. Physicochemical and pathophysiological studies support the thesis that the mineral and organic phases of bone and other vertebrate mineralized tissues are linked by the phosphomonester bonds of *O*-phosphoserine and *O*-phosphothreonine, which are constituents of both the structural organic matrix and the inorganic calcium phosphate crystals.

1. The nature of the mineral phase in bone

(*a*) *Introduction*

The solid mineral phase of bone has two major biological functions. First, the impregnation of the soft, extracellular organic matrix by the rock-like hydroxyapatite crystals converts the otherwise soft tissue and organ to a rigid structural material and member having the requisite mechanical properties to resist the external and internal forces to which it is subjected during gait, prehension, etc. This firm, hard, and relatively inflexible structural material is now able to preserve the shape of the organism as a whole and to protect vital organs such as the bone and lungs. Secondly, the inorganic, solid mineral phase acts as an ion reservoir, and as such is the body's major storage site for calcium, inorganic orthophosphate, magnesium, sodium, carbonate and other ions. As a result of this property, it plays a critical role in maintaining the concentrations of these ions in the extracellular fluid, which in turn is vital for a number of important biochemical reactions and physiological activities.

Because both of these functions ultimately depend in great part on a knowledge of the intricate structure and chemistry of the mineral phase, studies of the exact crystallographic and chemical characteristics of bone mineral have been made for many years by a large number

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of investigators. However, despite the fact that it has been known for over 75 years that the solid, mineral phase of bone and other calcified tissues of vertebrates consists principally of calcium and inorganic phosphorus with a small but significant amount of carbonate (Levy 1894), and for over 50 years that it has the X-ray structural characteristics of poorly crystalline hydroxyapatite (p.c.HA) (De Jong 1926; Roseberry *et al.* 1931) no general agreement has been reached either about the details of its exact molecular structure or its chemical composition (Posner 1969; Termine 1972; Wadkins *et al.* 1974; Brown & Chow 1976; Glimcher *et al.* 1981*a*; Bonar *et al.* 1983). In great part this stems from the fact that the quantitative characteristics of the X-ray diffraction patterns generated by samples of bone tissue differ significantly from those generated by standard preparations of highly crystalline hydroxyapatite (HA). Indeed, even mineral from the most mature bone generates only a few discernible reflections of hydroxyapatite and even these are quite broadened (figure 1).

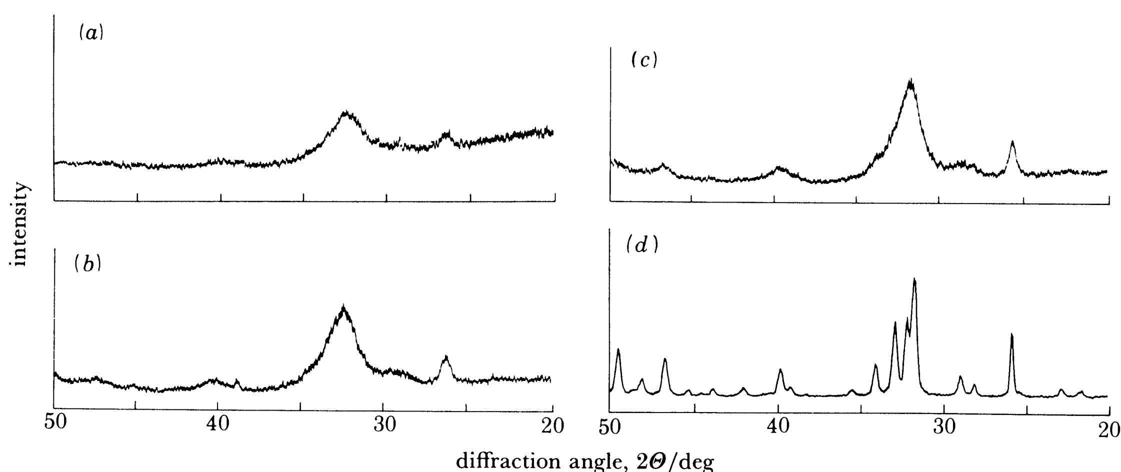

FIGURE 1. X-ray diffraction patterns generated by (*a*) 11 d old embryonic chick tibiae; (*b*) 5 week old post-natal chick tibiae; (*c*) 1 year old post-natal chick tibiae; (*d*) synthetic crystalline hydroxyapatite. (From Bonar *et al.* 1983.)

Electron micrographs of bone, which have revealed the very small size of the bone crystals (about (15 – 35 Å) × (50 – 100 Å) × (400 – 500 Å†)), help to explain this aspect of the X-ray diffraction patterns. However, other characteristics of bone mineral such as crystal strain, vacancies, addition to (for example carbonate) and adsorption into the lattice of other ions (Na, Mg, etc), also result in significant differences between bone mineral and crystalline hydroxyapatite; most importantly, progressive changes occur in the X-ray diffraction patterns of bone mineral as a function of the age of the tissue, of the animal, and of the age of the mineral itself. These X-ray diffraction changes are also accompanied by significant changes in the chemical composition of the mineral phase, namely, in the Ca/P ratio, and in the contents of carbonate, HPO_4^{2-} and H_2O (Woodward 1964; Pellegrino & Biltz 1972). Recognition that the mineral phase undergoes extensive structural and chemical changes after its initial formation has led investigators to explore the nature of the first solid phase of Ca–P deposited and the detailed changes that it undergoes during aging and maturation, and the search for the explanation of these X-ray and compositional changes in terms of the structure and chemistry of the mineral phase of bone has been the focus of much investigation (figure 1).

† $1 Å = 10^{-10}$ m.

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(*b*) *Amorphous calcium phosphate theory*

The most widely accepted hypothesis emerging from recent studies is the one proposed by Posner and his colleagues and referred to as the 'amorphous calcium phosphate' (a.CP) theory (Posner 1969). In brief, these investigators (Harper & Posner 1966; Termine & Posner 1967) observed that the X-ray diffraction intensities generated by samples of bone were lower than would be expected if all of the solid, Ca–P mineral phase present in the bone were poorly crystalline hydroxyapatite, assuming that the poorly crystalline hydroxyapatite in bone was identical to that prepared *in vitro* (that is, had the identical chemical and X-ray diffraction properties of poorly crystalline hydroxyapatite synthesized *in vitro*). From these data they

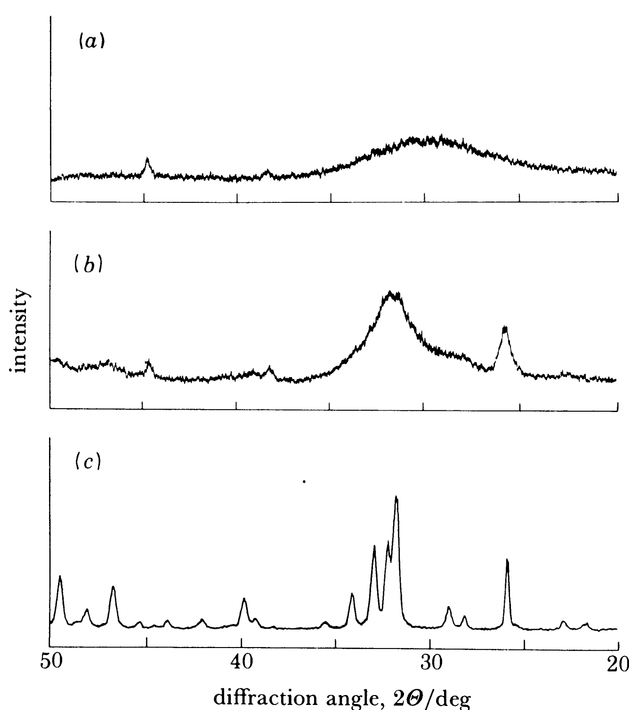

FIGURE 2. X-ray diffraction patterns generated by (*a*) *in vitro* prepared amorphous calcium phosphate solid phase (a.CP); (*b*) *in vitro* prepared poorly crystalline hydroxyapatite (p.c.HA); (*c*) highly crystalline hydroxyapatite.

concluded that bone mineral was a two phase system, one of which was poorly crystalline hydroxyapatite (p.c.HA) and the other an a.CP, which did not have a sufficient long-range order to generate an X-ray diffraction pattern of its own, or to contribute to the X-ray diffraction reflections generated by the p.c.HA. Indeed, just such an a.CP was observed as an integral, separate and intermediate phase in the formation of hydroxyapatite crystals from highly supersaturated solutions of calcium and inorganic orthophosphate ions (Eanes *et al.* 1965; Termine & Posner 1966; Termine *et al.* 1970) (figure 2).

Using X-ray diffraction intensities and infrared spectroscopy absorption data, Harper & Posner (1966) and Termine & Posner (1967) devised techniques to evaluate the proportions of a.CP and p.c.HA in mixtures of a.CP and p.c.HA prepared *in vitro*. These data were then used by these investigators and others to study the relative proportions of a.CP and p.c.PHA in bone samples from animals of widely differing ages. As they expected, immature bone from very young animals, which consisted only of recently deposited bone mineral, contained 75% (by

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mass) or more of a.CP, whereas older, mature bone, which contained a mineral phase that had presumably been in the tissue for a longer period of time and therefore had aged over a long period of time, contained 35–40% (by mass) a.CP (Posner & Betts 1975). However, on the basis of further work by this group, which took into account the presence of carbonate and other tissue electrolytes, the content of a.CP was revised downwards to approximately 50% (by mass) a.CP in young bone mineral and approximately 25–30% (by mass) a.CP in mature bone mineral (Russell *et al.* 1973; Tannenbaum *et al.* 1974; Posner & Betts 1975). In more recent work, this group could experimentally find *no* a.CP at all in mature bone, but because of instrumental considerations they revised their estimate only down to a figure of no more than 10% (by mass) (Betts & Posner 1974).

The essence of the a.CP theory is therefore twofold.

(*a*) It proposes that the initial, solid mineral phase of Ca–P deposited in bone is so structurally amorphous that it neither generates an X-ray diffraction pattern of its own nor contributes to the X-ray diffraction pattern of the poorly crystalline apatitic phase in bone. It must be emphasized that the theory does not postulate that the amorphous solid phase is merely a very rapid or transient phase, but rather that it *makes up the bulk* of the early solid Ca–P mineral phase. Indeed, it is the single, solid mineral phase of Ca–P in the earliest or first deposited bone mineral and is therefore by far the major solid phase of Ca–P present in young, newly synthesized bone.

(*b*) It proposes that the changes observed in the X-ray diffraction patterns generated by bone and in the chemical composition of bone mineral from animals of increasing age (and therefore of increasing age of the bone mineral *per se*) are due to the gradual and progressive transformation of a.CP to p.c.HA. That is, they postulate that it is the phase change of a.CP to p.c.HA, resulting in a progressive decrease in the proportion of a.CP relative to p.c.HA, that is responsible for the progressive changes observed in the bone mineral by X-ray diffraction and by chemical analysis: an increasing Ca/P ratio approaching that of hydroxyapatite, decreasing content of HPO_4^{2-} (or H^+), and increasing contents of carbonate and tightly bound water (Woodward 1964).

It is important to note that the a.CP theory is deduced from *indirect* data; it is based on the fact that the X-ray diffraction intensities generated by a sample of bone are less than those *theoretically expected* if the mineral phase consisted completely of p.c.HA. The validity of such calculations, however, is a bit worrying. As already indicated, the theory assumes that the p.c.HA in bone is identical in every respect to the p.c.HA made *in vitro*. For example, the calculations for the expected intensities depended significantly on the chemical compositions of the *in vitro* p.c.HA, especially its carbonate content. The calculations also took into account crystallite size, habit, and the presence of impurities incorporated in the crystallites or adsorbed on their surface (Harper & Posner 1966; Termine *et al.* 1973; Posner & Betts 1975).

(*c*) *Present radial distribution function analysis and nuclear magnetic resonance studies.*

Because of these and other factors, which made this indirect method open to considerable question (Elliott 1973), we chose to investigate the nature of the bone mineral and specifically whether a.CP was the initial mineral phase in bone, by a direct method, namely, radial distribution function analysis (r.d.f.). Radial distribution function analyses are plots of atomic density against atomic separation and are widely used in X-ray diffraction studies of non-crystalline materials (Warren 1969). Since all constituents in a sample will contribute to

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the r.d.f. in proportion to their X-ray scattering power, regardless of whether they are crystalline or amorphous, definitive and direct positive evidence of an amorphous solid phase in bone mineral should be readily apparent with this technique.

In addition to using a direct physical method (r.d.f.) to detect the presence of an a.CP phase in bone mineral, we made a very concentrated effort to obtain samples of bone mineral as homogeneous as possible with respect to the age of the mineral phase. While the biological complexities of growth and internal remodelling of bone as an organ and tissue have in the past frustrated most attempts to obtain homogeneous samples of bone mineral of various ages,

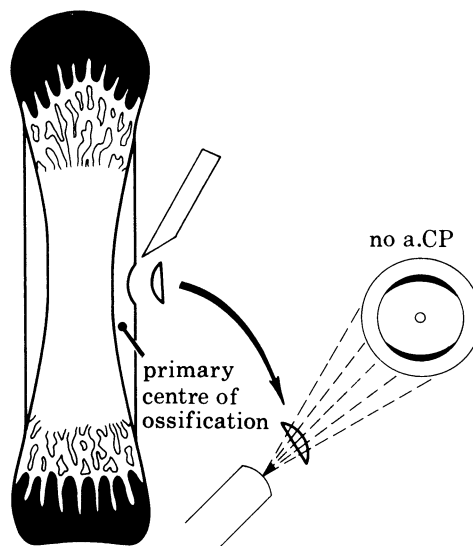

FIGURE 3. Diagrammatic representation of how newly formed bone containing very young embryonic chick bone mineral was obtained for X-ray diffraction and ^{31}P n.m.r. studies.

we were able to turn these biological complexities to our advantage. This was accomplished by three general techniques. First, we took advantage of the fact that the long bones of very young embryonic chicks are completely turned over (the tissue is synthesized and completely resorbed) within 24–48 h. Since the bone mineral in such samples can be no older than 48 h, it is necessarily young and relatively homogeneous with respect to age. Secondly, by recognizing that cylindrical growth (increase in diameter and thickness) of embryonic long bones occurs principally by outer periosteal new bone formation and endosteal resorption, it was possible to obtain an even younger and more homogeneous sample of bone mineral by using only the bone tissue scraped from the outer surface of the periosteal bone (figure 3).

Our third method for obtaining homogeneous and very young samples of bone mineral used the density centrifugation technique of Herman & Richelle (1961) (figures 4 (*a*), (*b*), (*c*)). This method fractionates and separates particles of finely ground bone powder on the basis of their physical density, which reflects their mineral content, and thus separates them as a function of the age of the tissue and of the mineral phase *per se*. Since the samples of bone powder fractionated by density centrifugation were themselves obtained from very young embryos, the relatively unmineralized low density fractions, which we were able to fractionate and isolate, represented the youngest samples of bone mineral ever studied by gross techniques.

Direct analyses by r.d.f. of samples of early bone mineral obtained by these methods including

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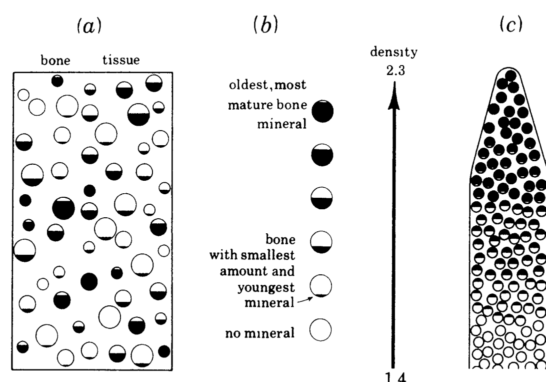

FIGURE 4. Schema for separating bone powder on the basis of mineral content. The younger mineral phase is in the lower density particles and the oldest mineral phase is in the highest density particles. (*a*) Bone tissue is never homogeneous with respect to the age of its mineral particles. (*b*) Bone tissue; the amount of bone mineral increases with increasing age and maturation. (*c*) To obtain specimens of bone containing mineral particles of different ages bone is first ground to fine powder and then separated according to its density by centrifugation.

a density centrifugation fraction representing the youngest 20% of young embryonic chick bone, revealed *no* evidence for the presence of an a.CP phase (Glimcher *et al.* 1981*a*; Grynpas *et al.* 1983) (figure 5(*a*), (*b*)). We have now obtained a sample of even younger embryonic bone mineral, which one would predict on the basis of the a.PC theory would consist almost entirely of a.PC. However, even in this sample we could find no evidence for the presence of a.CP by r.d.f. analysis (Bonar *et al.* 1984, in preparation). To demonstrate that our technique could detect small quantities of a.CP, small amounts of synthetic a.CP were added to embryonic bone and these were readily detected by r.d.f. analysis (Grynpas *et al.* 1983).

So, although the a.CP theory is an attractive and plausible explanation for the X-ray and chemical changes found as a function of age of the bone mineral, we must nevertheless conclude on the basis of our r.d.f. analyses of bone mineral that the a.CP theory is incorrect: it does not account for the structural or the chemical composition of the initial Ca–P phase deposited in bone or for the X-ray diffraction or chemical changes that occur with age. Furthermore, on the basis of additional X-ray diffraction studies as a function of age, which we conducted on chicken bone of ages from embryonic to two years old (Bonar *et al.* 1983), we conclude that bone mineral can best be characterized as a single phase of poorly crystalline non-stoichiometric hydroxyapatite, which becomes more crystalline and approaches ideal stoichiometry with time after its initial deposition.

Recent studies done in collaboration with Dr Robert Griffin and his colleagues at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, have also confirmed the absence of an amorphous Ca–P solid phase, but interestingly have detected the presence of HPO_4^{2-} groups (Aue *et al.* 1984, submitted; Roufosse *et al.* 1984, submitted). These acid phosphate groups are not present as crystalline brushite ($CaHPO_4 . 2H_2O$) but are in a brushite configuration. There is more of this HPO_4^{2-} phase in the youngest bone and it decreases with the age of the bone mineral. These results are consistent with chemical analyses for HPO_4^{2-} (Woodward 1964). As we will discuss later, it is tempting to speculate that this HPO_4^{2-} moiety may in part represent the bridge between the inorganic and organic phase of bone and tooth.

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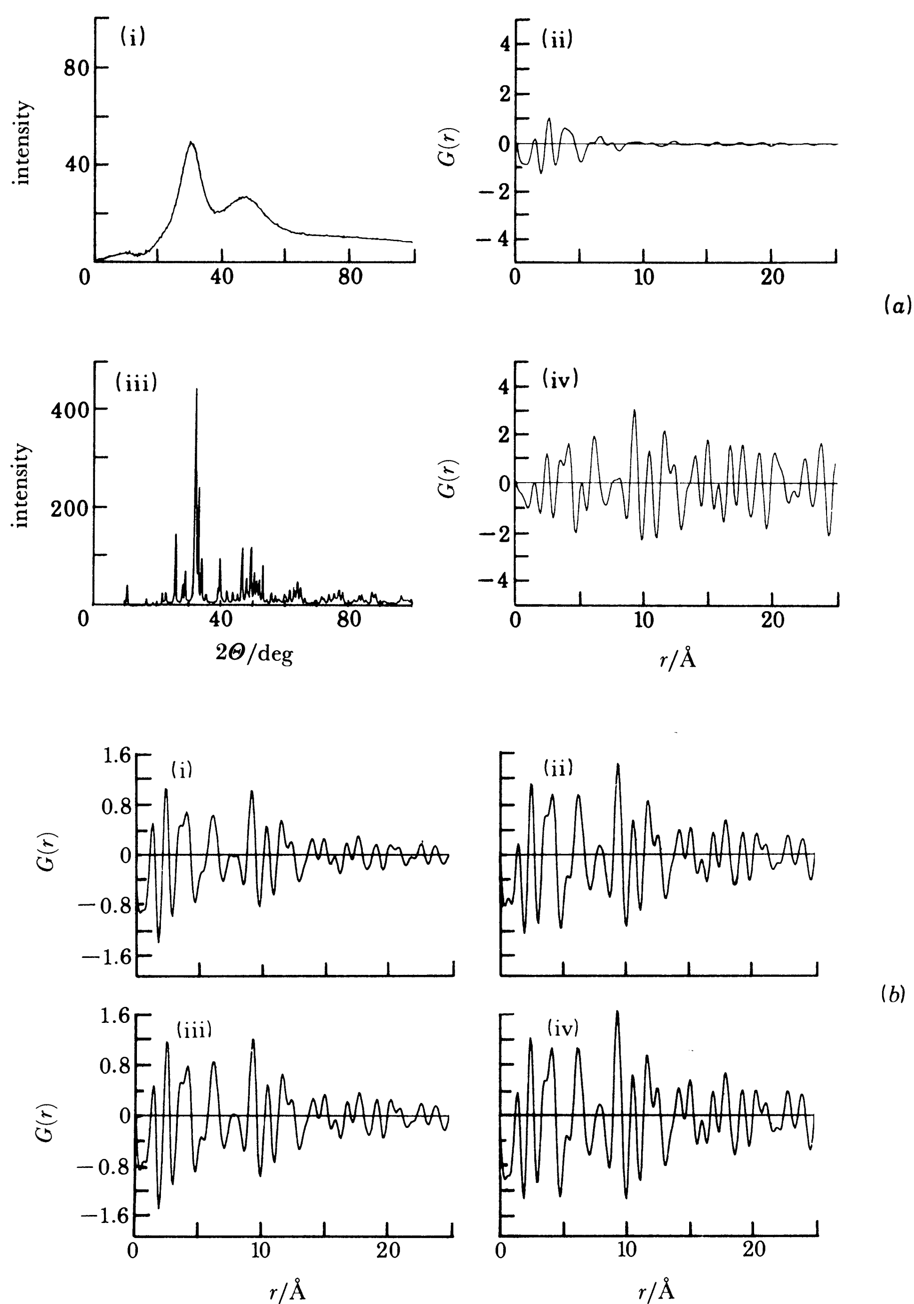

FIGURE 5. (*a*) X-ray diffraction ((i) and (iii)) and radial distribution function (r.d.f.) analyses ((ii) and (iv)); (i) and (ii) for amorphous calcium phosphate, (iii) and (iv) for synthetic, crystalline hydroxyapatite.

(*b*) R.d.f. analyses of bone from (i) 1.7–1.8 density centrifugation fraction of 17 d chick embryo bone; (ii) whole bone, 16 d chick; (iii) whole bone, 18 weeks old chicken; (iv) whole bone, 1 year old chicken. (From Grynpas *et al.* 1984).

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2. Requirements for theories of tissue calcification

(a) *Review of matrix vesicle theory; general physical and biological boundary conditions*

Before beginning our discussion of the potential role of collagen and the phosphoproteins in the calcification of bone tissue, there are several important physical chemical principles and morphological observations that must be reviewed so that the role of collagen and phosphoproteins can be seen in its proper context. First, by definition, *tissue* mineralization includes the sum total of all of the individual intra- and extracellular tissue components that eventually

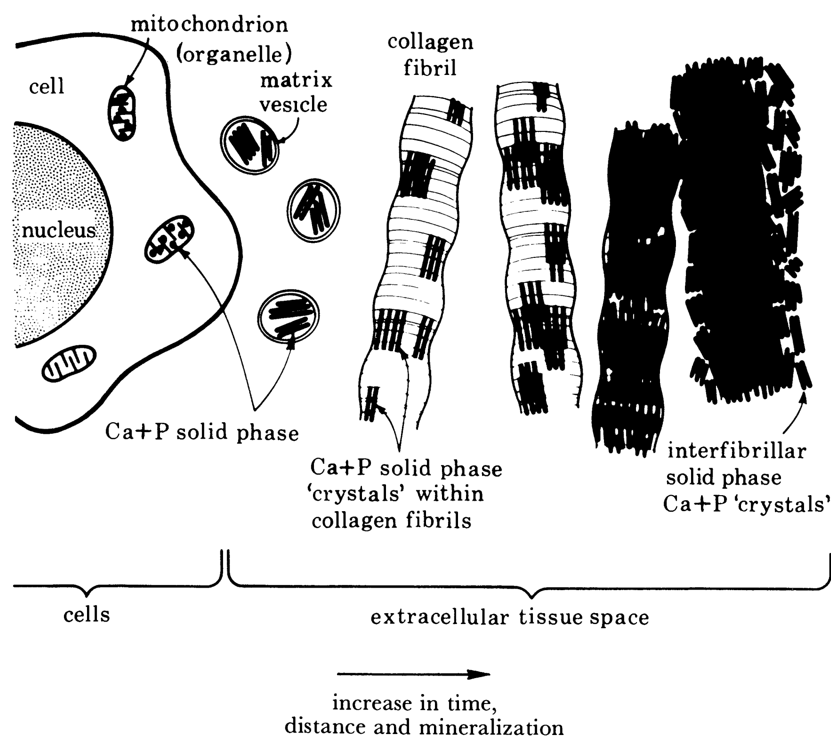

Figure 6. Diagrammatic representation of bone tissue calcification and of the putative calcification of several of its intra- and extracellular compartments and components. Serious doubts remain on the role, if any, of mitochondria and matrix vesicles in tissue calcification. (From Glimcher 1981.)

become mineralized. In bone, for example, these include the mitochondria, the endoplasmic reticulum, the extracellular matrix vesicles and the collagen fibrils (Lehninger 1970; Anderson 1973, 1983; Glimcher 1981; Wuthier 1982) (figure 6). Secondly, it is necessary to recognize that each of these cellular and extracellular components are *spatially distinct* entities, physically separated from one another. Such a morphological arrangement of spatially distinct and separated anatomical entities, each of which mineralizes, imposes a number of boundary conditions on any theory that attempts to describe the interrelations, if any, between the calcification of the individual compartments. These boundary conditions include the following: mineralization of each of the tissue components occurs *physically* independent of the others, that is, the initiation of calcification in each of the structures (nucleation of the first mineral phase particles) occurs *de novo* from a metastable solution phase containing the requisite mineral ions (Glimcher 1976). For reasons detailed elsewhere (Glimcher 1959, 1960, 1976, 1983; Glimcher & Krane 1968) this initiation of mineralization is accomplished as a physical change in state

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(a phase transformation and not a chemical reaction) initiated by heterogeneous nucleation. The nucleation catalyst in all of the compartments is considered to be a well defined and organized aggregate of macromolecules, which possess particular, reactive side-chain groups arranged in a specific stereochemical and electrochemical array, such an array constituting a nucleation site (Glimcher 1981) (figure 7).

Recognition that the calcification of each of the tissue compartments is an independent physicochemical event in which the initial crystals are formed *de novo* from a metastable

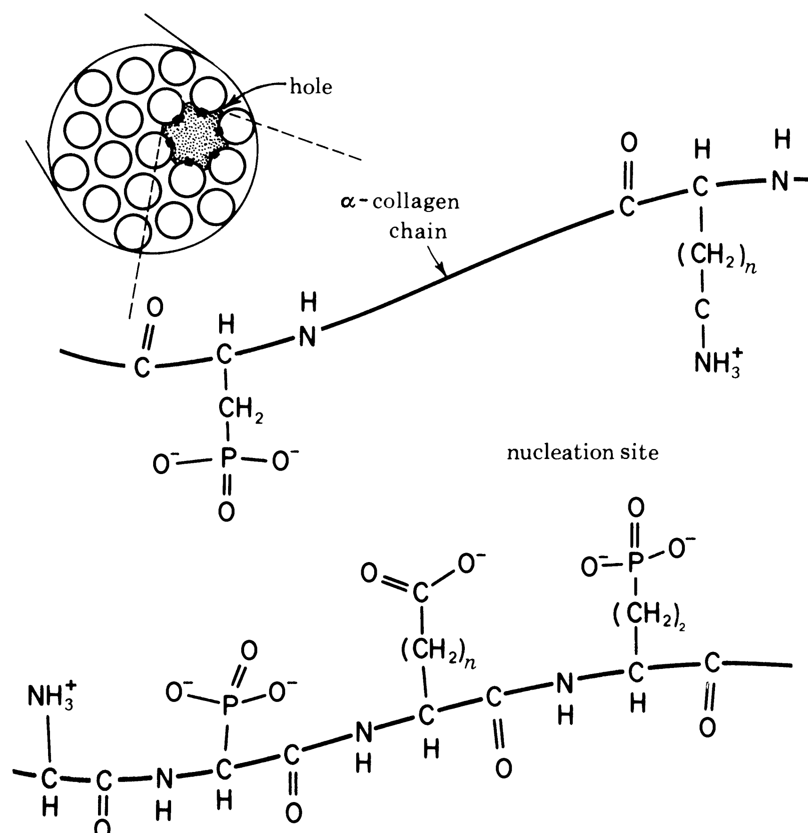

FIGURE 7. Diagrammatic representation of a nucleation site in collagen fibril. (From Glimcher 1981.)

solution phase eliminates the highly improbable hypotheses that solid phase particles of one anatomical compartment are in some way extruded and, without inciting secondary nucleation of additional crystals within the intervening space, manage to find their way to other compartments where they proceed to initiate further calcification by secondary nucleation and multiplication. For example, it has been suggested that the solid phase particles of Ca–P in the mitochondria of bone cells are extruded into the extracellular space, which they then traverse, eventually becoming lodged in the hole zone regions of the collagen fibrils (Lehinger 1970). Similarly, Wuthier (1982) has postulated that the Ca–P granules in the mitochondria of bone and cartilage cells are transported to the extracellular matrix vesicles where they constitute the first solid phase of Ca–P in these structures. Apart from the improbability of such gross particles traversing the intracellular and extracellular spaces and being 'captured', or otherwise entering matrix vesicles or collagen fibrils, extensive electron micrograph (Anderson 1969, 1973; Bonucci 1971) and electron microprobe (Ali 1976; Ali *et al.* 1977) studies of the

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temporal sequence of matrix vesicle calcification have demonstrated the presence of numerous matrix vesicles free of Ca–P particles and containing no increase in their Ca and P_i concentrations compared with the adjacent extracellular fluid, just before the time when the first crystals appear within them, and at a time when no Ca–P particles are found between the vesicles.

A now prevalent theory of tissue calcification, which purports to describe specifically how collagen fibrils are calcified, has been presented by Anderson (1983), Bonucci (1971) and Wuthier (1982). In this hypothesis, in contrast to those mentioned in the preceding paragraph, the extruded mineral phase particles *are* considered to initiate calcification within the intervening space between the two mineralizing components. The sequence suggested is as follows. Ca–P particles in the matrix vesicles pierce the membrane of the matrix vesicles and are extruded into the extracellular space. The extruded crystals then act as nucleation catalysts for the formation of additional crystals of Ca–P by secondary nucleation and multiplication, thereby progressively filling the extracellular tissue spaces between the collagen fibrils with inorganic crystals. When the crystals reach the collagen fibrils, new crystals enter or form within the hole zone region of the fibrils. Continued secondary nucleation causes new additional crystals to form from the solution phase within the hole zone region and possibly within the pore spaces as well (Glimcher 1981). In this schema, collagen fibrils are simply a passive repository for the deposition and subsequent multiplication of Ca–P crystals, which eventually results in the almost complete impregnation of the fibrils with a solid mineral phase of Ca–P. Neither the collagen fibrils nor any of the non-collagenous macromolecules associated with them are considered to play any role in the formation of the crystals of Ca–P within the fibrils.

(*b*) *Difficulties of correlating matrix vesicle theory and actual electron micrograph observations*

In addition to both the physical chemical and biological criticisms already discusssed and presented in detail elsewhere (Glimcher 1976, 1981, 1983), this proposal, which involves crystals extruded from the matrix vesicles then propagating a solid mineral phase in the extracellular tissue space, suffers from the fact that the suggested sequence of events does not in any way correspond to what is actually observed during the calcification of bone, dentin or cementum by electron microscopy. In our extensive studies of embryonic chick bone by non-aqueous as well as aqueous techniques for the preparation of the tissue samples (Glimcher & Krane 1968; Landis *et al.* 1977*a*, 1977*b*, 1980; Landis & Glimcher 1978) and in countless published and unpublished electron micrographs of others, we have never observed a stage of calcification in which the extracellular spaces were filled with bone mineral and the collagen fibrils were unmineralized. Indeed, even in the earliest stages of embryonic bone or dentin calcification, at a time when the collagen fibrils are just beginning to mineralize, the most common picture observed is of collagen fibrils separated from one another and in various stages of mineralization with little or no mineral phase between the fibrils (figure 8, plate 1).

Even without the theoretical objections that we have already voiced here and elsewhere (Glimcher 1976, 1981, 1983), these data make it clear that the proposals by Anderson (1983), Bonucci (1971), Wuthier (1982) and others to explain how collagen fibrils become calcified can be largely discounted; the events and sequences depicted in their proposals are not observed in the tissue. Moreover, the proposal that crystals of Ca–P, formed randomly by secondary nucleation and multiplication in the extracellular spaces, are somehow able to find their way selectively to only the hole zone regions of the collagen fibrils is thoroughly improbable from

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both the physicochemical and biological standpoints. If this theory were true, it would follow that at the early stages the collagen fibrils would become encrusted in a random fashion by the self-propagating and multiplying mineral phase particles in the extracellular tissue spaces, and collagen fibril calcification would proceed without the localization of the crystals to the hole zone regions of the fibrils that is actually observed by electron microscopy (Glimcher *et al.* 1966*a*; Glimcher & Krane 1968), X-ray and neutron diffraction (Engström 1966; White *et al.* 1977; Berthet-Colominas *et al.* 1979).

So it would appear that if the calcification of one compartment of a tissue in some way influences the calcification of another compartment, it does so not as a direct result of any transportation of mineral particles to the second compartment, nor as a result of any extrusion of particles into the intra- or extracellular spaces, thus inducing a self-perpetuating and propagating mass of crystals by secondary nucleation. Instead, the crystalline, solid phase particles in the first compartment may be dissolved and the Ca^{2+}, phosphate, and other ion constituents pumped out, locally increasing the concentrations of these and other ions and in other ways increasing the metastability of the solution phase perfusing the second compartment. In this fashion, calcification of one compartment could conceivably *facilitate* the heterogeneous nucleation of *de novo* crystals by organic constituents in another compartment.

Another very important point is almost always overlooked in a discussion of the possible role of extracellular matrix vesicles in the overall calcification of bone tissue as a whole. Extracellular matrix vesicles are present only in bone during the earliest stages of embryonic development. In later stages of embryogenesis and in post-natal animals, matrix vesicle are either absent or very few in number. Those who theorize that matrix vesicles are crucially important in mineralization seem to overlook the fact that during embryonic growth and development *all* of the bone synthesized during the early stages of embryogenesis is totally and completely resorbed and replaced by new bone (Glimcher 1981). In the chick, for example, depending upon the age of the embryo and the particular long bone, this complete resorption can occur in as short a period as 18–48 h. So new bone tissue is routinely synthesized during embryogenesis and post-natally at a time *when matrix vesicles are absent or near absent from the tissue*, and in this situation the collagen fibrils are calcified *in the absence of matrix vesicles*. So, even if one were to concede some role for matrix vesicles in the calcification of collagen and the tissue as a whole during the short period of embryonic development when they are present in the tissue (Glimcher 1981), it is still clear that matrix vesicle calcification is *not obligatory* for collagen or tissue calcification since almost all of the bone synthesized in the lifetime of an animal calcifies in the absence of matrix vesicles (Glimcher 1981). In terms of evolutionary biology, matrix vesicle calcification may be an expression of what Lowenstam (1981) has termed 'primitive calcification'.

3. Potential role of collagen in calcification

(*a*) *Ultrastructural observations.*

In searching for a theory of bone calcification more in keeping with physical and chemical possibilities and actual observations, we can start with the ultrastructural data demonstrating that the Ca–P crystals are not deposited randomly in the extracellular tissue spaces and do not randomly encrust the collagen fibrils. Instead, we shall see that the initial crystals formed within the collagen fibrils are located in specific regions along the axial period of the fibrils. With these data alone, one might correctly surmise that this highly selective and intimate ultrastructural

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relation between collagen fibrils and the initial deposits of Ca–P crystals is a reflection of an equally specific *physicochemical* interaction between the two components and, moreover, that there must exist in these selective regions of the collagen fibrils some combination of space, charge, and electrochemical configuration, which in some way *causes* the crystals of Ca–P to form from the solution phase in the first place.

Electron micrographs of bone tissue that is well mineralized clearly demonstrates that the mineral crystallites of Ca–P are not randomly distributed in the tissue but are organized in a highly ordered fashion (figure 9), accentuating the 700 Å (approximate) axial repeating period of the collagen fibrils. In addition, the long axes of the crystals are aligned relatively parallel to the long axes of the collagen fibrils with which they are associated. Electron diffraction confirms these observations and furthermore establishes that it is the crystallographic *c*-axis, corresponding to the physical long axis of the crystals, that is aligned roughly parallel to the long axis of the collagen fibril. In no area of the bone tissue are crystals found, either by electron microscopy or by electron diffraction, to be randomly oriented. Similar electron optical studies of fish bone, a tissue in which collagen fibrils are more widely separated than in bones of other vertebrates, revealed that the crystals of Ca–P are located *within* the collagen fibrils (figure 10), (Glimcher 1959). Electron microscope observations of the very early stages of tissue and collagen calcification also clearly demonstrated that the crystals initially deposited in the collagen fibrils are likewise located from the start in the precise positions observed in the more heavily calcified tissue and have a similar axial repeating period. Moreover, the individual, widely spaced crystal particles initially deposited within the fibrils are also, like the more mature crystals, oriented with their long, physical axis (crystallographic *c*-axis) parallel to the long axis of the collagen fibrils with which they were associated (Glimcher 1959; Glimcher *et al.* 1966*a*; Glimcher & Krane 1968) (figure 11).

This eliminates the possibility that the highly ordered orientation of the crystals is somehow due, for example, to mechanical pressure generated by dense packing of large numbers of crystals. Even at this early stage of tissue and collagen calcification, one commonly observes relatively large numbers of collagen fibrils cut in cross section, some of which are completely free of mineral, others in varying stages of being mineralized and impregnated with a solid phase of Ca–P. Most importantly, little or no mineral crystals are observed in the intervening space

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Description of plate 1

Figure 8. Electron micrographs of early stages of calcification of (*a*) embryonic chick and (*b*) 6 d mouse bone. Collagen fibrils are seen in cross section. Note mineral-free collagen fibrils (c.f.), and fibrils in varying stages of being mineralized and impregnated with a solid phase of calcium phosphate. The spaces between the fibrils are essentially free of mineral particles. The calcification of each of the fibrils is a biologically and physicochemically independent event, which starts with the nucleation of the first crystals within the fibrils. (From Landis *et al.* 1977*b*.) An osteoblast (o.b.) and two osteoblast processes (o.p.) are indicated.

Figure 9. Electron micrograph of an unstained, longitudinal section of embryonic chick bone, which has not been decalcified. The ordered disposition of the dense mineral phase along the axial direction of the collagen fibrils is evident. Note also that the mineral phase is in lateral register as well. (From Glimcher & Krane 1968.)

Figure 10. Electron micrograph of unstained fish bone, which was not decalcified. The collagen fibrils, seen primarily in cross sectional profile, are packed with the inorganic crystals. (From Glimcher 1959.)

Figure 11. Electron micrograph of an unstained, longitudinal section of young embryonic chick bone, which is not decalcified. The dense mineral phase appears to 'stain' the collagen fibril at regular intervals along its axial length. In some areas, the inorganic crystals can be seen on edge as dark lines. Most of the mineral phase is not resolvable into individual crystals. (From Glimcher & Krane 1968.)

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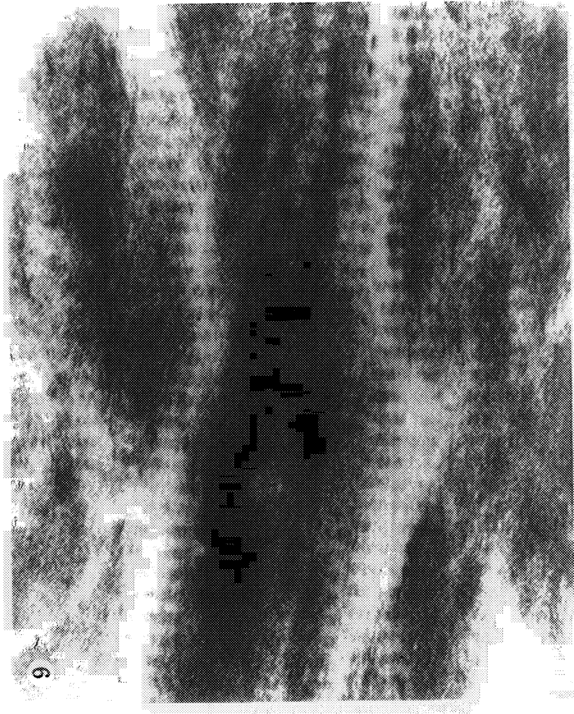

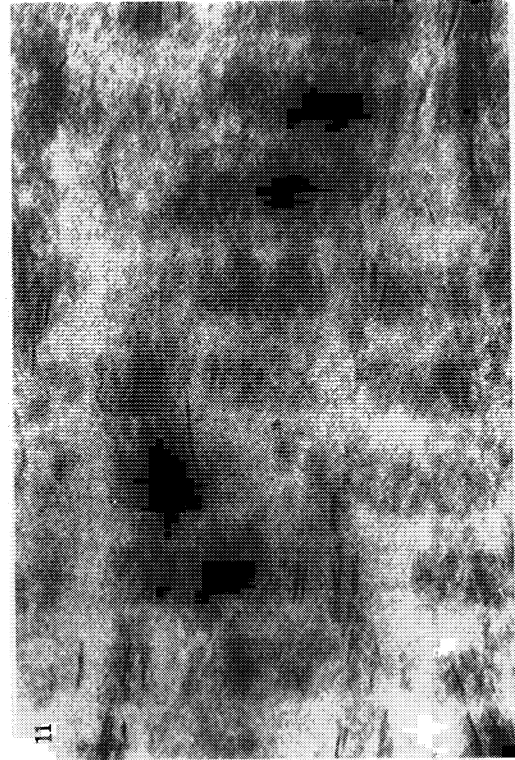

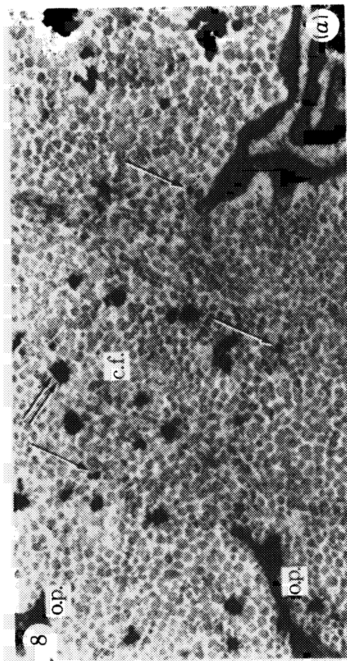

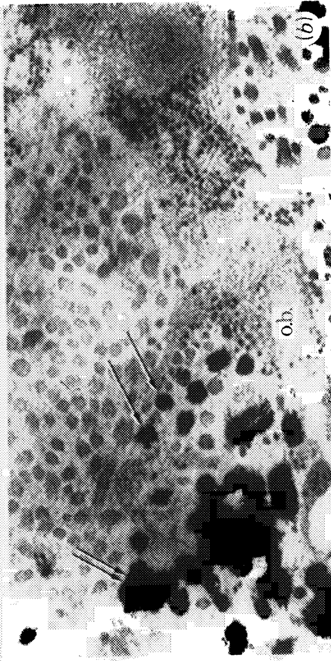

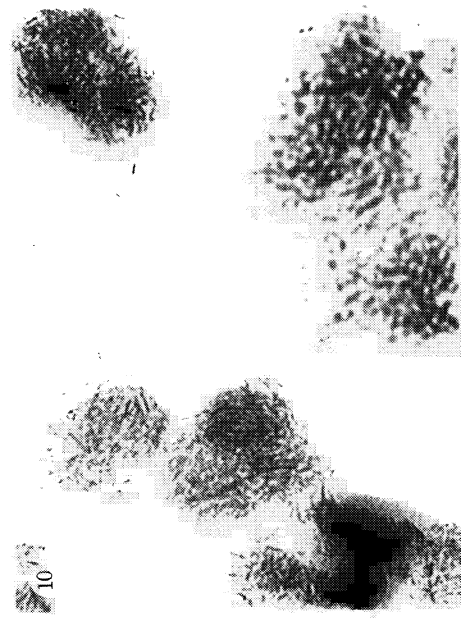

FIGURES 8–11. For description see opposite.

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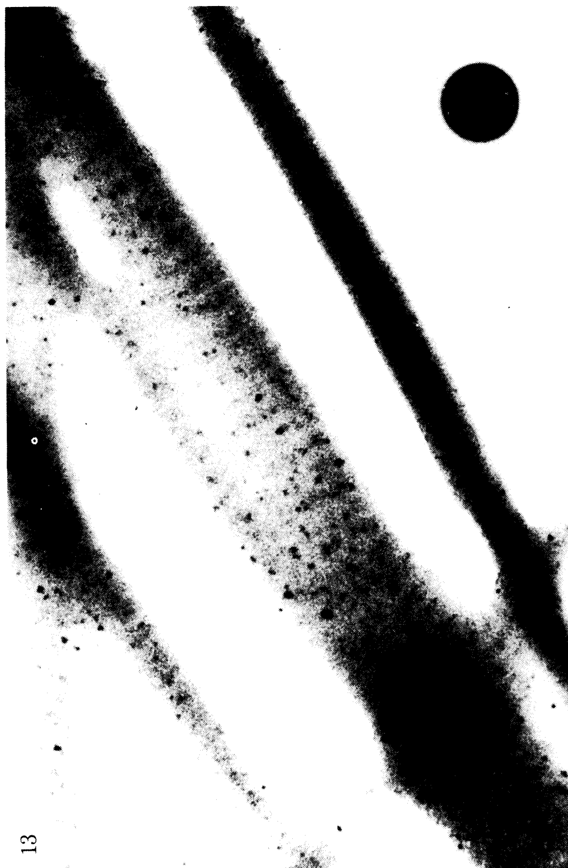

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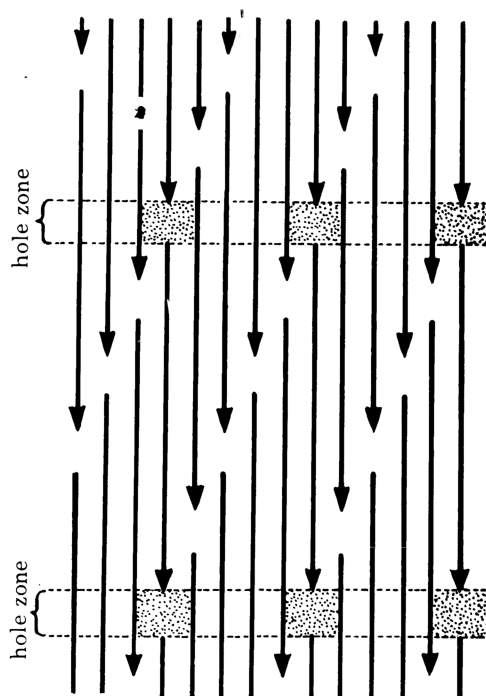

Figures 12, 13 and 15. For description see opposite.

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between the collagen fibrils. The sum total of all these observations makes it patently clear that mineralization is *initiated* within specific locations within each individual fibril, and that the process is independent of any *direct* physical contact with a solid phase of Ca–P located between the fibrils. So the suggestion that crystals, propagated by secondary nucleation and randomly oriented in the interfibrillar extracellular space after their emergence from the matrix vesicles, eventually reach the collagen fibrils and indeed enter the hole zone region of the fibrils (Anderson 1983) is clearly not supported by direct, ultrastructural observation of the tissue.

Additional electron microscope studies of the location of the mineral phase with respect to the axial period of the collagen fibrils and of the location of the holes within the collagen fibrils, also revealed that the Ca–P crystals are at least initially limited to the hole zone region of the collagen fibril (Glimcher *et al.* 1966*a*; Glimcher & Krane 1968) (figure 12, plate 2). These data have been confirmed with non-aqueous methods of tissue preparation for electron microscope study. More recently, the position of the mineral particles in the hole zone region of the collagen fibrils of whole, intact unfixed tissue has been confirmed indirectly by Engstrom (1966) using optical transforms of low angle X-ray diffraction data, and directly by very elegant studies with low angle X-ray and neutron diffraction (White *et al.* 1977; Berthet-Colominas *et al.* 1979).

The intimate and ordered organization of the Ca–P crystals within the collagen fibrils must be explained by any theory of collagen calcification or of calcification of the tissue as a whole. Specifically, all theories must account for the following facts: (*a*) the solid Ca–P mineral phase is located within the collagen fibrils; (*b*) it is deposited within the fibrils in specific locations; and (*c*) the inception of crystallization within the collagen fibrils must be considered an independent physical chemical event in which *de novo* crystals are formed from a solution phase within the fibrils at a time when mineralization does not occur from this same fluid between the fibrils.

The *de novo* formation of solid phase particles of Ca–P within the collagen fibrils, from a solution phase free of mineral particles, must clearly begin with the nucleation of the crystals. This could arise either from the spontaneous precipitation of crystals, caused simply by an increase in the metastability of the solution phase perfusing the collagen fibrils to the point of thermodynamic instability – in which case the collagen fibrils would serve simply as a *passive* reservoir and provide the necessary space and volume within which the crystals could be deposited – or alternatively, the collagen fibrils could play an *active* role in initiating crystal formation from otherwise stable (metastable) solutions of Ca–P (heterogeneous nucleation) (Glimcher 1959, 1976; Glimcher *et al.* 1957; Glimcher & Krane 1968). The notion that it is possible to increase the metastability of Ca–P solution perfusing the collagen fibrils to the point of spontaneous precipitation (homogeneous nucleation) can be discounted on operational

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Description of plate 2

Figure 12. The identification of the location of the mineral phase in bone collagen between the a^3 and c^2 bands places the crystals in the hole zone. (From Glimcher & Krane 1968.)

Figure 13. Electron micrograph of a very early state in the *in vitro* nucleation of apatite crystals by reconstituted collagen fibrils. The electron diffraction pattern identifies the mineral phase as apatite. (Magn. × 60000.) (From Glimcher 1959.)

Figure 15. Electron micrograph of *in vivo* calcified reconstitued skin collagen implanted into peritoneum. (By courtesy of Dr Marie Nylen, NIDR.)

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physicochemical grounds, since it has been amply demonstrated that even under the most ideal conditions it is rarely if ever possible to reach this point of unstable equilibrium with solutions of inorganic ions. Instead, with solutions of inorganic ions, heterogeneous nucleation occurs on the surface of the containers or on extraneous particles within the solution phase before the point of unstable equilibrium is reached. It is therefore highly improbable that the point of instability of the extracellular fluids with regard to calcium and inorganic orthophosphate could be reached and homogeneous nucleation occur in tissues, in as much as biological tissues offer so many discrete components, the surface of any one of which could easily serve as a nucleation catalyst.

Moreover, as already discussed, spontaneous nucleation of crystals in the extracellular tissue spaces would result in a random precipitation of particles, thereby filling the extracellular space between the fibrils before calcification within the fibrils could occur. This is likewise contrary to what has been observed in the native tissue both by electron microscopy and X-ray diffraction. No observer has ever reported that all of the spaces between the collagen fibrils are first filled with a solid mineral phase before calcification of collagen fibrils begins, an observation that would be dictated by such a proposal (Wuthier 1982; Anderson 1984). Indeed, the striking feature of early mineralization in young embryonic bone is the deposition of crystals within the collagen fibrils selectively within the holes of the fibrils at a time when there is little or no mineral between the collagen fibrils.

We conclude that the putative solid phases of Ca–P present in matrix vesicles and other intra- and extracellular components cannot themselves play a *direct* physical role in the calcification of another relatively remote component such as the collagen fibrils (Glimcher 1976, 1981). Even if matrix vesicles and other extra- and intracellular components do produce an increase in the metastability of the extracellular fluids permeating the collagen fibrils by whatever means, their role in the calcification of the collagen fibrils must necessarily be one of *facilitating* the nucleation of mineral particles within the collagen fibrils.

To explain the ultrastructural observations and to satisfy the physical chemical boundary conditions, we postulated that collagen fibrils in their native state of aggregation were themselves responsible for the *de novo* formation of the initial Ca–P crystals in the hole zone regions of the fibrils (Glimcher *et al.* 1957, 1966; Glimcher 1959, 1976; Glimcher & Krane 1968).

(*b*) *In vitro and in vivo observations of the nucleation of calcium phosphate crystals by collagen fibrils*

This thesis was first tested experimentally *in vitro* by extracting, purifying, and reconstituting native type collagen fibrils, and by extensively decalcifying and extracting bone collagen fibrils. Both preparations were then exposed *in vitro* to operationally metastable solutions of Ca–P (Glimcher *et al.* 1957; Strates *et al.* 1957). As might have been predicted from the intimate ultrastructural relation between the collagen fibrils and the mineral phase of bone, as observed by electron microscopy as well as from theoretical physicochemical considerations (Glimcher 1959), native type reconstituted collagen fibrils and extensively demineralized and extracted particles of bone consisting almost wholly of collagen fibrils, were found to be potent nucleation catalysts for the heterogeneous formation of apatite crystallites (Glimcher *et al.* 1957; Glimcher 1959). Failure of other structural proteins, including, tropomyosin, myosin, fibrin, and a host of other proteins, macromolecules and intact cartilage to do the same established the specificity of collagen fibrils as nucleation catalysts of apatite crystals from metastable solutions of Ca–P. The additional discovery that aggregates of collagen macromolecules into fibrils different from

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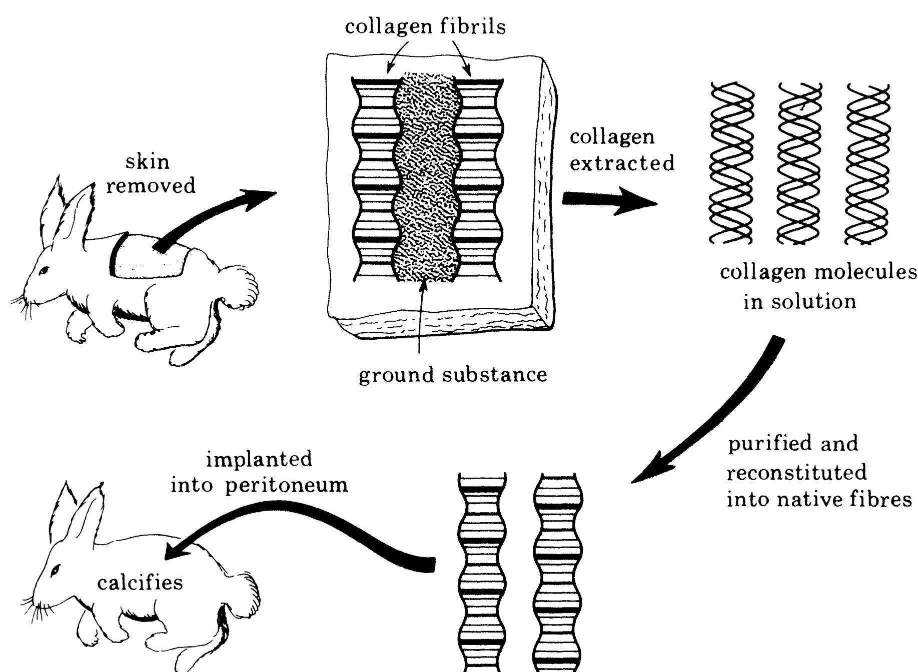

FIGURE 14. Schema of *in vitro–in vivo* experiments to show ability of native type reconstituted collagen fibrils to calcify *in vivo*. (From Glimcher 1981.)

the native form failed to nucleate Ca–P crystals from identical metastable solutions of Ca–P, aptly demonstrated that the exquisite and specific property of heterogeneous nucleation catalysis resided in the very special way that collagen macromolecules are aggregated in native type fibrils. Electron microscopy of the *in vitro* nucleated crystals showed that these mineral particles were not randomly disposed within the collagen but were instead located within the hole zone regions of the fibrils and were distributed at regular intervals along the axial period (Glimcher 1959) (figure 13).

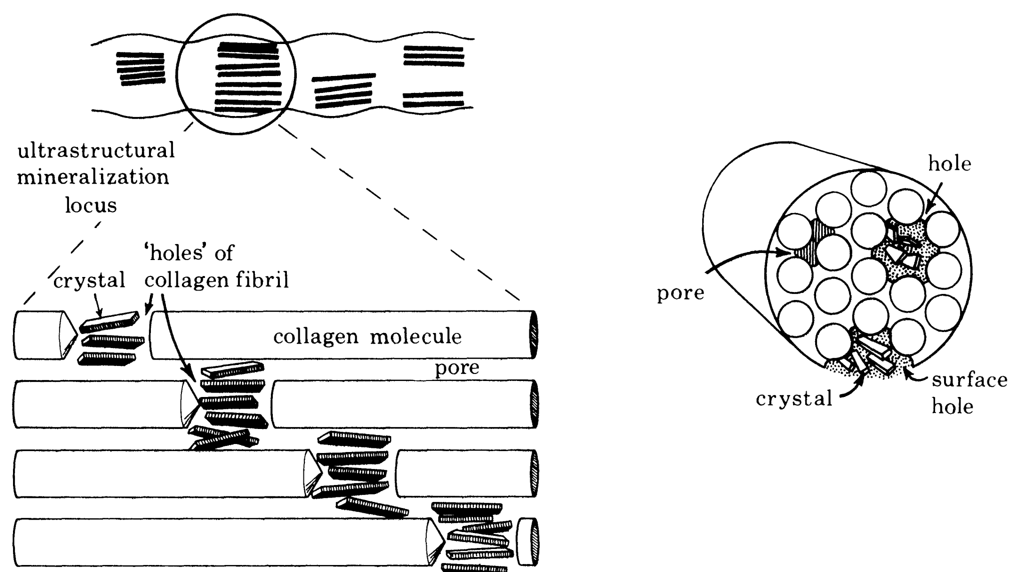

FIGURE 16. Schematic diagram of nucleation within holes of collagen fibril. A cross section of fibril is shown on the right. (From Glimcher 1981.)

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Even more convincing evidence that native type collagen fibrils have the inherent capacity to induce the formation of Ca–P crystals from metastable solutions came from experiments in which collagen was obtained from the skin of rats, rabbits, and guinea pigs, and was purified and reconstituted into native type fibrils, unstructured fibrils, and fibrous long spacing fibrils, which then were all placed back in the animals, intraperitoneally and subcutaneously (Mergenhagen *et al.* 1960; Glimcher *et al.*, unpublished work). After a moderate period of time, deposition of apatite crystals occurred with the hold zone regions of *only the native type collagen fibrils* (figures 14 and 15).

So not only has the native type aggregation of collagen fibrils been found to be necessary for the heterogeneous catalysis of Ca–P crystals within the fibrils *in vitro* and *in vivo*, but the unusually high selectivity of the site where Ca–P nucleation occurs within the collagen fibrils has been found to be identical in native tissues and in reconstituted collagen fibrils calcified *in vitro* and *in vivo*. These data provide very strong evidence that it is the specific aggregation state of native type collagen fibrils that produces the volume of space and the electrochemical and stereochemical distribution of side chain groups, which together constitute a nucleation site (figure 16) (Glimcher 1981).

It is therefore reasonable to conclude that the native aggregation state of collagen fibrils is *necessary* for their calcification but may not be *sufficient*, at least not *biologically* sufficient. This point requires further discussion.

4. Potential role of phosphoproteins of bone matrix in calcification

(*a*) *Isolation and characteristics of bone phosphoproteins and interactions with Ca^{2+} and inorganic phosphate ions*

Although the evidence is quite strong that it is the native type of aggregation of the collagen macromolecules that is necessary for the nucleation of a Ca–P solid phase within the hole zone regions of the fibrils, this property may not be sufficient, or if necessary and sufficient from the standpoint of physical chemistry, may not be sufficient biologically. That is, the native aggregation state alone may not be capable of inducing nucleation of mineral crystals in the short period of time required for this event in the functioning tissue. For example, in many instances it took at least several weeks and longer for native type reconstituted collagen fibrils or decalcified bone collagen fibrils to initiate mineralization within them when placed either in the peritoneal cavity or in subcutaneous tissues. Under these circumstances we need to redefine our boundary conditions and define 'sufficient' to mean *biologically sufficient*. In other words, for a condition to be biologically sufficient, it must enable an event to occur at a tempo consistent with biological functioning. So even if the conditions in the extracellular fluids are adequate and stable (extent of metastability), there may be other structural and chemical factors intimately related to the collagen fibrils, which, together with normal conditions in the extracellular fluid, constitute the necessary and biologically sufficient conditions for the heterogeneous nucleation of Ca–P crystals.

We have detailed elsewhere the arguments why organically bound phosphate groups are likely to play such a role (Glimcher 1960, 1976; Glimcher & Krane 1968). In addition to physicochemical considerations, the necessity of incorporating organically bound phosphate groups into potential sites in the organic matrix, in order for that region to function efficiently as a nucleation site, would permit precise cellular control and molecular localization of the

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mineralization process. This ultimate control of mineralization by tissue cells is a feature that makes this theory very attractive from the biological point of view.

Phosphoproteins, almost all of which are soluble in EDTA, were first isolated and purified from chicken and bovine bone (Spector & Glimcher 1972, 1973) in the early 1970s. The partially purified proteins from 8 species (now over 14 species) were shown to contain both Ser(P) and Thr(P), which together account for essentially 100% of their total organic phosphorus content (Cohen-Solal *et al.* 1978). Further work on chicken bone identified a homogeneous phosphoprotein of approximate molecular mass 12000 Da (Lee & Glimcher 1981) and another, not quite completely homogeneous one of 30000 Da (Lee & Glimcher 1979) both of which contained Ser(P) and Thr(P), establishing that the two phosphorylated amino acids were present in individual bone matrix proteins. ^{31}P n.m.r. studies confirmed that all of the organic phosphorus was in the form of monoesters in the intact protein (Lee & Glimcher 1981). Carboxylic acid side chain groups and organic phosphate groups of Ser(P) and Thr(P)

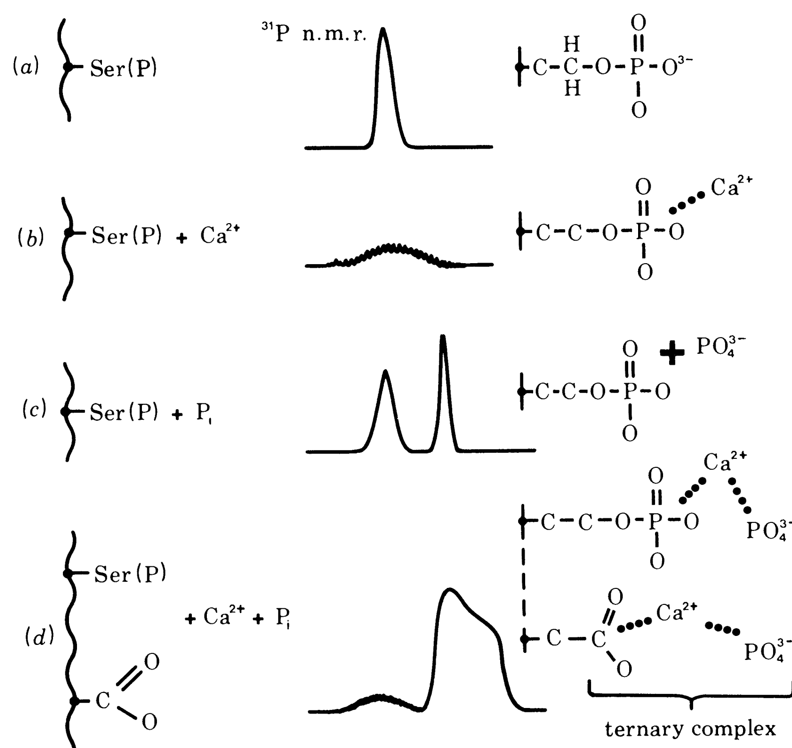

FIGURE 17. Diagrammatic representation of the effect of calcium ions, inorganic orthophosphate ions, and calcium and inorganic orthophosphate ions together, on the ^{31}P n.m.r. spectra of the protein-bound monoester bond of serine phosphate and of inorganic orthophosphate ions.

(*a*) Typical phosphomonoester spectrum of protein bound *O*-phosphoserine.

(*b*) Addition of P_i to a solution of phosphoprotein reveals two distinct peaks, identical to those obtained from the individual components (namely P_i, *O*-phosphoserine). This indicates that there is no significant interaction between inorganic orthophosphate ions and the phosphoprotein.

(*c*) Addition of $CaCl_2$ to the phosphoprotein results in a remarkable broadening of the phosphomonester resonance, almost obliterating it. This is evidence for the strong interaction and binding of calcium to the phosphomonester side chain groups of Ser(P).

(*d*) Addition of P_i and $CaCl_2$ to the solution of the phosphoprotein broadens both the phosphomonoester resonance peak and the P_i resonance peak. This demonstrates that in contrast to (*b*), in which inorganic orthophosphate ions in the absence of calcium ions are *not* bound to the protein, inorganic orthophosphate ions are almost completely bound, presumably to the protein bound calcium ions, in the presence of the phosphoprotein and Ca^{2+}. (From Lee *et al.* 1983.)

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together account for approximately one half of all the amino acid residues of the bone phosphoproteins, thus providing an abundant number of sites for the binding of calcium ions. The potential ability to bind calcium ions is, of course, one of the requirements necessary for a component to participate in the nucleation of Ca–P crystals from solution. Calcium binding studies of purified bone matrix proteins have not yet been completed but results using the phosphoproteins of dentin have shown that there are extensive calcium binding sites corresponding to the phosphate and carboxyl side chain groups in these phosphoproteins (Lee *et al.* 1977; Zanetti *et al.* 1981). However, as we have pointed out in the past, the ability of a protein to strongly bind large numbers of calcium ions might just as easily serve to *decrease* mineralization as to increase it, since the strong binding of calcium ions may inhibit any or all of the physicochemical steps involved in the deposition of a solid phase of Ca–P, namely, nucleation, crystal growth, or crystal multiplication (Glimcher 1960, 1976; Glimcher & Krane 1968). For example, the calcium may be bound either electrochemically or stereochemically in such a way as to be unreactive and unavailable for further interaction with inorganic phosphate ions and other ions that participate in the formation of the Ca–P solid phase, for example, the chelation of Ca^{2+} by EDTA *prevents* nucleation and crystal growth. Unfortunately, this important consideration is often overlooked in formulating specific roles for certain macromolecular components or compartments in biological mineralization in general.

To determine whether calcium ions bound to the Ser(P) groups of the phosphoproteins of dentine were capable of further reaction with inorganic orthophosphate ions, additional experiments were done. The phosphoprotein was titrated with $CaCl_2$ in the presence of inorganic orthophosphate ions and examined by ^{31}P n.m.r. (Lee *et al.* 1983). In the absence of $CaCl_2$ the addition of inorganic orthophosphate ions causes neither a shift or a broadening of the phosphomonoester peak. However, when increasing amounts of $CaCl_2$ are added to a solution containing both the phosphoprotein and inorganic orthophosphate ions, the phosphomonoester peak and the inorganic orthophosphate peak are both markedly broadened or even obliterated (figure 17). This indicates that ternary complexes have been formed between the protein phosphomonoester groups, the ions and the inorganic orthophosphate ions (figure 17). These data demonstrate that the phosphoproteins possess the requisite physicochemical properties necessary for them to participate directly in the nucleation of a Ca–P solid phase from solution.

(*b*) *Synthesis of phosphoproteins by bone in organ and cell culture*

Having established that, at least from the physicochemical point of view, the phosphoproteins are capable of participating in the mineralization of bone and tooth, our attention was next directed to two other important questions: (*a*) are the phosphoproteins true bone matrix constituents, i.e., synthesized by bone cells; and (*b*) are they located in the sites in the tissue where mineralization actually occurs. The first question was a very pertinent one, since two of the major non-collagenous proteins found in bone and dentine, albumin and α_2HS-glycoproteins (Triffitt & Owen 1973; Triffitt *et al.* 1976, 1978) are synthesized in the liver and then transported to bone, where they are adsorbed and concentrated. The second question is equally critical and again often overlooked when formulating theories of calcification.

To answer the first question, we cultured fetal chick mandibles *in vitro* for 6 d. Measurements of the masses of the explants, their mineral and protein components, and the EDTA-extractable protein, established that bone tissue synthesizes Ser(P)- and Thr(P)-containing phosphoproteins, which are similar to those present in both embryonic and post-natal chicken bone matrix (Glimcher *et al.* 1982, 1984) (tables 1–3).

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Table 1. Overall composition of embryonic chick mandible (12 d old) *in ova* and after 6 d of growth in tissue culture and in ova†

sample	mineral (dry mass %)				organic (dry mass %)					
							collagen		non-collagenous proteins	
	total ash mass	Ca	P	Mg	total organic constituents	total protein		percentage of total protein content		percentage of total protein content
12 d embryo chick mandible	41.6	15.5	8.2	0.37	58.4	34.8	17.5	50.3	17.5	49.7
12 d embryo chick mandible + 6 d of tissue culture	52.9	19.9	9.7	0.39	47.1	30.6	19.9	65.1	10.7	34.9
18 d embryo chick mandible	54.3	20.1	9.8	0.40	45.7	32.0	21.3	66.6	10.7	33.4

† Mandibles were cut into two halves, each 8.1 mm in length. The Meckel cartilage was removed. Values are the average of two pooled duplicate samples, each of which contained 16 one-half mandibular pieces of bone. (Adapted from Glimcher *et al.* 1982, 1984.)

Table 2. Growth of embryo chick mandibles (12 d old) in organ culture and *in ova*: changes in overall composition

(Results expressed per one-half mandible)†

sample	total dry mass mg	increase (%)	ash mass (mineral) mg	increase (%)	total protein mass mg	increase (%)	collagen mass mg	increase (%)	non-collagenous proteins mass mg	increase (%)
12 d embryo chick mandible	2.08	—	0.86	—	0.72	—	0.36	—	0.36	—
12 d embryo chick mandible + 6 d of tissue culture	5.31	156	2.81	225	1.62	124	1.06	190	0.57	58
18 d embryo chick mandible	6.40	208	3.48	302	2.05	183	1.36	275	0.69	92

† Average value of two pooled, duplicate samples, each of which contained 16 half mandibles (8.1 mm length). (Adapted from Glimcher *et al.* 1982, 1984.)

Note how very closely growth, protein synthesis and mineralization in organ culture parallel the same parameters *in vivo* over a 6 d period (tables 1–3) (Glimcher *et al.* 1982, 1984). This underscores the likelihood that the data on protein and especially on phosphoprotein synthesis obtained from organ culture is representative of what occurs *in vivo*. Figures 17(*a*), (*b*), and (*c*) demonstrate the isolation and partial purification of the phosphoproteins from embryonic chick mandibular bone. The molecular sieve and ion exchange characteristics of the phosphoproteins are similar to those already deposited for post-natal bone (Lee & Glimcher 1981) (table 4).

The molecular sieve and ion exchange chromatographic behaviour of the EDTA extracts from 12 d old chick mandibles and from 12 d old chick mandibles after 6 days of growth *in vitro* and *in ova* were similar and also had the same characteristic behaviour described previously for the EDTA-extractable phosphoptoteins of 10–14 week old chicken bone (Spector &

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TABLE 3. GROWTH OF EMBRYO CHICK MANDIBLES (12 d OLD) IN ORGAN CULTURE AND *IN OVA*: MAJOR MINERAL CONSTITUENTS, PHOSPHOAMINO ACIDS, AND γ-CARBOXYGLUTAMIC ACID (Gla) CONTENTS

(Results expressed per one-half mandible.)†

sample	Ca mass/mg	increase (%)	P mass/mg	increase (%)	Mg mass/mg	increase (%)
12 d embryo chick mandible	0.32	—	0.17	—	0.008	—
12 d embryo chick mandible + 6 d of tissue culture	1.06	228	0.51	202	0.021	169
18 d embryo chick mandible	1.28	298	0.63	269	0.026	232
sample	Ser(P) quantity/nmol	increase (%)	Thr(P) quantity/nmol	increase (%)	Gla quantity/nmol	increase (%)
12 d embryo chick mandible	5.9	—	1.1	—	2.2	—
12 d embryo chick mandible + 6 d of tissue culture	13.6	131	2.3	109	5.2	136
18 d embryo chick mandible	15.0	154	3.1	182	8.3	277

† Average value of two pooled, duplicate samples, each of which contained 16 mandibles. (Adapted from Glimcher *et al.* 1982, 1984.)

Glimcher 1972; Lee & Glimcher 1981). The synthesis of the phosphoproteins in bone was further confirmed by the demonstration that radioactively labelled Ser(P) and Thr(P) were identified in bone and EDTA extractable phosphoproteins after pulse labelling chick mandibles *in vitro* with radioactively labelled serine and threonine (Glimcher *et al.* 1982, 1984).

Although these experiments demonstrated the synthesis of matrix phosphoprotein by chick bone as a tissue, cells specific to bone, namely osteoblasts, osteocytes, and osteoclasts, constitute only a small fraction of the cells resident in bone, especially in the young growing skeleton. Therefore, to explore whether the phosphoproteins of bone are synthesized by bone-substance cells *per se*, we isolated such cells from young mouse calvaria. By using the incorporation of [^{3}H] serine into [^{3}H] Ser(P) as an indication of phosphoprotein synthesis (Gotoh *et al.* 1983) it was found that of all the isolated fractions of bone cells, the osteoblast-rich one was the most active in synthesizing phosphoproteins that contain Ser(P). The behaviour of these phosphoproteins on molecular sieving and ion exchange chromatography was found to be similar to that of the phosphoproteins from young mouse calvaria – indeed, from the same calvaria from which the bone cells were derived (figure 18).

The elegant ^{33}P radioautographic experiments of Weinstock & Leblond (1973) show that $^{33}P_i$ injected into rats is first observed in the odontoblasts and then extruded and concentrated at the site of mineral deposition in the dentine. One presumes that the ^{33}P label is part of the phosphoproteins, although no biochemical evidence was obtained for this. We repeated these experiments with embryonic chicks, and like Weinstock & Leblond, found that the ^{33}P was first observed in the preosteoblasts and osteoblasts and then transported extracellularly to the sites where calcification was occurring (Sanzone *et al.* 1982; Landis *et al.* 1984) (table 5). In

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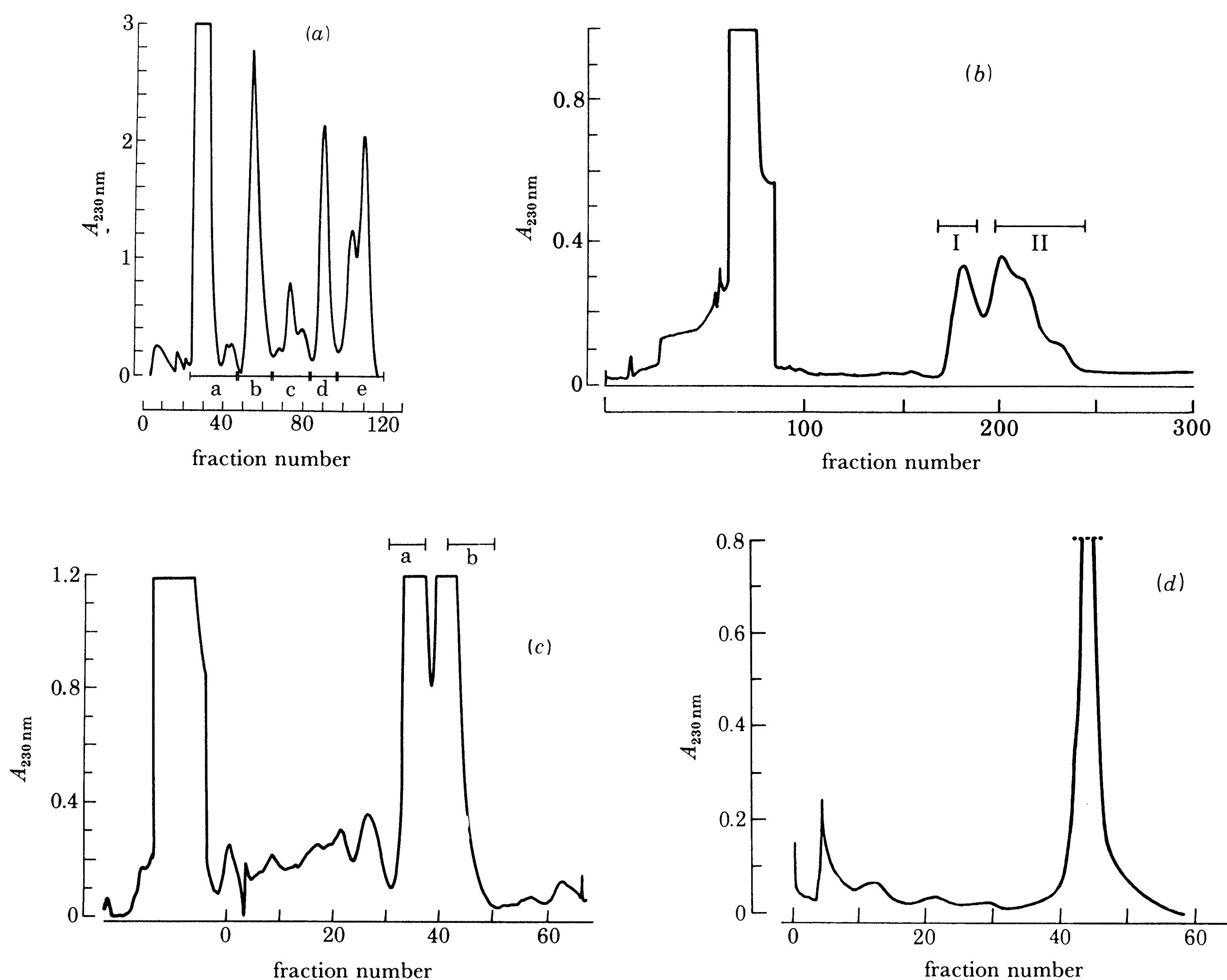

FIGURE 18. (*a*) Elution profile from Sephadex G-100 molecular sieving of an EDTA extract of tibiae from 18 d *in ova* embryonic chick tibiae. Distribution of Ser(P) in various peaks shown in table 4. Thr(P) was also identified in peaks a, b, c.

(*b*) Elution profile of peak b from Sephadex G-100 sieving shown in (*a*) chromatographed on DEAE-cellulose. Peak II contained almost all of the Ser(P) and Thr(P) applied to the column.

(*c*) Elution profile of peak II from DEAE-cellulose chromatography shown in (*b*) molecularly sieved through Sephacryl S-200 in 5 M·Gdn HCl.

(*d*) Rechromatography of peak b. Both a and b fractions contained Ser(P) and Thr(P). Fraction b was homogeneous with an apparent M_r = 12–14000 Da. Fraction a was not homogeneous. The major component had a M_r = 28–30000 Da (Glimcher *et al.* 1982, 1984).

these same experiments, however, we also isolated the phosphoproteins and found that the ^{33}P had indeed been incorporated into the phosphoproteins Ser(P) and Thr(P) (table 6).

These experiments confirm that it is the osteoblasts that synthesize the phosphoproteins and establish that the phosphoproteins are located at the sites of mineralization, a necessary requirement if they are to play the role envisaged for them in mineralization.

(*c*) *Other in vivo evidence for the role of phosphoproteins in calcification*

There is other *in vivo* evidence for the role of the phosphoproteins in calcification. When bone is powdered and separated by density centrifugation on the basis of its mineral content and therefore on the basis of the temporal sequence of calcification, one finds that the Ser(P)

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TABLE 4. DISTRIBUTION OF RECOVERED PROTEINS AND Ser(P) AFTER G-100 MOLECULAR SIEVING

(Twelve day old embryo chick mandibles were cultured *in vitro* for 6 d, after which proteins were extracted in 0.4 M EDTA, pH 7.5, at 4 °C for 1 week. Approximately 80 % of the protein and 90 % of the Ser(P) applied to the column was recovered in the 4 fractions (figure 18*a*). Fraction e, salt.)

sample	distribution: total yield (mass %)	distribution: Ser(P) (mass %)	concentration of Ser(P)† nmol/g
whole EDTA extract	—	—	1.9
G-100 sieving			
fraction a	57	19	0.73
fraction b	23	74	6.9
fraction c	5	3	1.3
fraction d	15	4	0.5

† Uncorrected for destruction during hydrolysis and for maximum yield. (Adapted from Glimcher *et al.* 1982, 1984.)

TABLE 5. RATE OF ^{33}P ACCUMULATION IN EMBRYONIC CHICK TIBIAE AFTER INJECTION OF 5mCi OF $Na_2{}^{33}PO_4$ INTO EACH EMBRYO

area analysed	increase in rate of ^{33}P accumulation (%)†
slide background (no tissue)	—
non-osseous tissue	—
outer periosteal cells	242
osteoblasts and osteoid	279
mineralized matrix	646

† From 10–30 min and 30–240 min after injection (Sanzone *et al.* 1982; Landis *et al.* 1984).

TABLE 6. RECOVERY OF ^{33}P AS Ser(^{33}P) IN WHOLE BONE TISSUES AFTER INJECTION OF 5 mCi $NaH_2{}^{33}PO_4$ INTO 14 d OLD CHICK EMBRYOS

(Tissue was fixed in glutaraldehyde and demineralized in EDTA-glutaraldehyde solution.)

time after injection/min	^{33}P recovered as Ser(^{33}P) per milligram of tissue
10	96
30	487
240	17941

(From Landis *et al.* 1984.)

concentration is much higher in the low density fractions corresponding to the earliest stages of tissue mineralization. In the lowest density fraction, the ratio of Ser(P)/Ca is highest and progressively and rapidly decreases as mineralization progresses (figure 19) (Lian *et al.* 1982*b*). This is exactly what one would predict if the Ser(P)- containing phosphoproteins were involved in the initial steps in calcification – once the first crystals are produced by heterogeneous nucleation, a step involving the Ser(P) groups, additional crystals are then formed next to the first crystals by secondary nucleation and multiplication. Since the Ser(P)-containing phophoproteins would not be involved in the crystal multiplication process that occurs by secondary nucleation, their concentration relative to Ca would fall as the number of Ca–P crystals and the amount of total mineral progressively increased.

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Similar results were obtained in the organ culture experiments and during growth *in ova*. For example, calcification of the chick mandibles both in tissue culture and *in ova* is accompanied by a significant drop in the ratio of Ser(P) to calcium, whereas the Gla to calcium ratio remains roughly the same (table 7). These data suggest that the fall in Ser(P)/Ca ratio results from the fact that a certain critical concentration of phosphoprotein is needed to initiate mineralization, but once deposition of a solid phase of Ca–P begins, additional amounts of the solid mineral phase are formed principally by secondary nucleation of new particles from the mineral-phase crystals *per se* (Glimcher 1981; Lian *et al.* 1982*b*) without the need for more Ser(P). Similarly,

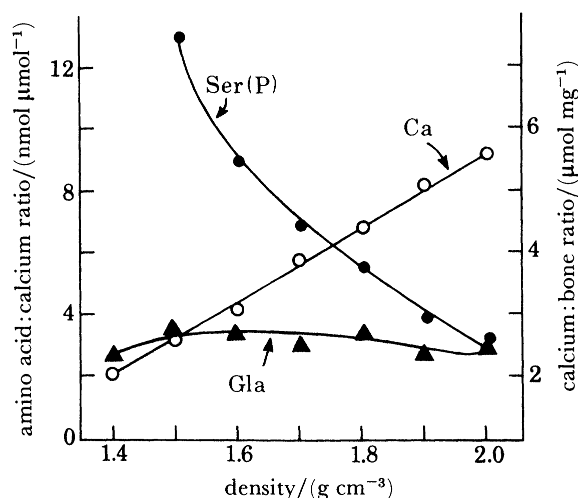

FIGURE 19. Change in Gla (▲) and Ser(P) (●) in the density fractions of bone plotted as a function of the calcium content of the tissue. Total calcium content (○) of each fraction is also shown. (From Lian *et al.* 1982*b*.)

TABLE 7. CALCIUM AND PHOSPHORUS CONCENTRATIONS/(μmol mg^{-1}) AS A FUNCTION OF PROTEIN CONCENTRATIONS IN EMBRYO CHICK MANDIBLES *IN OVA* AND AFTER TISSUE CULTURE†

	calcium		phosphorus	
sample	collagen	n.c.p.‡	collagen	n.c.p.
12 d embryo chick mandible	22.1	22.3	15.0	15.2
12 d embryo chick mandible + 6 d of tissue culture	25.0	46.6	15.6	29.3
18 d embryo chick mandible	23.5	46.8	14.8	29.5

† Average value of two pooled, duplicate samples, each of which contained 16 mandibles. (Adapted from Glimcher *et al.* 1982, 1984.) ‡ Non-collagenous protein (n.c.p.).

the relatively constant ratio of Gla to calcium during progressive mineralization of the tissue is to be expected if one postulates that osteocalcin or its precursors, or both, is synthesized by the bone cells, is excreted, does not participate directly and structurally in the nucleation and formation of the Ca–P solid mineral phase, but instead is simply bound to the calcium ions on the surface of the *already formed apatite crystals* (Glimcher *et al.* 1979; Lian *et al.* 1982*b*).

The relatively constant ratios of Ca and P_i to collagen during ossification and mineralization, at the same time that the Ca and P_i ratios to n.c.p. significantly increase (table 8), are also consistent with the suggestion that phosphorylated n.c.p. is needed principally to help facilitate

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the *onset* of mineralization and that once started, mineralization at a local site proceeds to a great extent by secondary nucleation (Glimcher 1981). The increase in mineral mass without any further increase in the phosphoproteins and other n.c.ps results in an increase in the Ca/n.c.p. and P_i/n.c.p. ratios. On the other hand, the newly formed mass of the solid phase requires a steadily and progressively increasing volume of space to house it (Glimcher 1976), which is provided for principally by the collagen (Glimcher 1976, 1981). Consequently, the collagen fibrils increase in mass at approximately the same rate as the mineral phase during

TABLE 8. Ser(P) AND Gla CONCENTRATIONS AS A FUNCTION OF CALCIUM CONTENT IN EMBRYONIC CHICK MANDIBULAR BONE *IN OVA* AND AFTER TISSUE CULTURE†

sample	Ser(P) concentration nmol/mmol calcium	Gla concentration nmol/mmol calcium
12 d embryo chick mandible	1100	292
12 d embryo chick mandible +6 d of tissue culture	562	219
18 d embryo chick mandible	605	230

† Average value of two pooled, duplicate samples, each of which contained 16 half mandibles. (Adapted from Glimcher *et al.* 1982, 1984.)

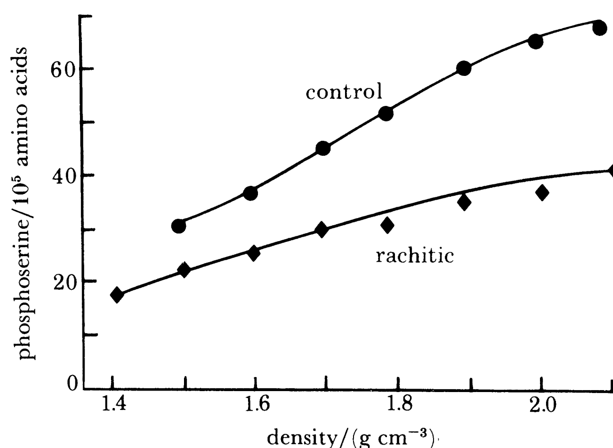

FIGURE 20. The concentrations of *O*-phosphoserine in the density fractions of normal and rachitic chicken bone. Results of a typical experiment. Values represent average of two determinations, which varied less than 5%. (From Lian *et al.* 1982*a*.)

the early stages of osteogenesis. The compositional data are also consistent with the thesis proposed much earlier (Glimcher 1959, 1960, 1976) and with more recent data (Baylink *et al.* 1972; Howell, 1976; Howell *et al.* 1969; Pita *et al.* 1979) that mineralization is inhibited by certain n.c.ps and proteoglycans, which are progressively removed as increased mineralization of the tissue proceeds.

The results from another group of *in vivo* experiments were also consistent with the hypothesis that phosphoproteins play a critical role in the formation of the mineral phase of bone and tooth. In these experiments, osteomalacia, a condition in which bone and teeth mineralize less than normally and more slowly, was induced in experimental animals by making them severely deficient in vitamin D. For rats, the reduction in the mineralization of tooth enamel was accompanied by a large reduction in the Ser(P)-containing enamel phosphoprotein (Glimcher

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et al. 1966*b*). Similarly, in chicks, the total amount of Ser(P) and Thr(P) as well as the concentrations of these phosphoamino acids in EDTA extracts and in fractions obtained by molecular sieving were markedly reduced (Lian *et al.* 1982*a*).

Recently, investigators have also reported that the concentration of phosphoproteins in dentine in human cases of dentinogenesis inperfecta is also diminished (Takagi *et al.* 1983). So in all the major mineralized tissues of vertebrates (tooth enamel, dentine, and bone), all of the *in vivo* evidence that has been gathered supports the thesis that phosphoproteins are an integral part of the calcification mechanism.

From the physiological, biochemical, structural and physicochemical points of view, an organic component, in order to play a significant and positive role in the formation of the mineral phase should ideally: (1) be synthesized by the cells of the tissue (Glimcher 1960, 1976; Glimcher & Krane 1968) and probably by those cells that synthesize the major structural components within which the mineral will be deposited; (2) be located ultrastructurally at the sites where mineralization occurs; (3) interact with the appropriate mineral ions constituting the inorganic crystals; (4) bind the ions in such a way that the bound ion remains reactive and can further interact with other inorganic ions to eventually form the inorganic crystals. It is clear from the data presented that the phosphoproteins of bone (and dentine) satisfy these conditions.

With regard to mineralized collagenous tissue, the strong interactions between collagen and phosphoproteins may reflect a specific binding of the phosphoproteins to particular sites at the hole zone regions of the collagen fibrils (Dimuzio & Veis 1978; Glimcher 1981). The specific interaction between the phosphoproteins and native type collagen fibrils, which appears to be present in all normally and pathologically calcified tissues, is not found in normally unmineralized connective tissues. Indeed, the tissue specificity of the phosphoproteins of mineralizing tissues is an important issue to stress. We have been unable to isolate phosphoproteins similar to those of bone or tooth from normally unmineralized connective tissues. On the other hand, we have shown that the characteristic phosphoproteins of bone begin to appear in progressively increasing amounts as pathological calcification of tendon (Glimcher *et al.* 1979) and skin (Glimcher *et al.* 1981) proceeds. This is in sharp contrast to the recent findings that osteonectin, originally believed to be a bone specific protein involved in mineralization (Termine 1981; Termine *et al.* 1981*a*, *b*), is in fact synthesized by cells from a wide variety of unmineralized connective tissues (Wasi *et al.* 1983). So osteonectin is not bone specific at all and may be a constituent of a large number of both mineralized and unmineralized connective tissues. It may be totally unrelated to the process of tissue mineralization (Wasi *et al.* 1983).

It may be informative at this point to further examine osteonectin not only as a specific component of the organic matrix, potentially important in calcification, but as an example of how all such postulated components need to be closely studied to see if they even possess the necessary physicochemical and biological properties to participate in mineral formation, let alone whether they actually use these properties *in vivo* in the manner proposed.

The suggested role of osteonectin in mineralization, indeed its very name, derives from the fact that supposedly, it specifically and strongly binds to collagen and to apatite crystals. If one scrutinizes the available information on osteonectin carefully, however (Termine *et al.* 1981*a*), there is nothing in the reported data to indicate a *specific* binding of osteonectin by collagen; the data as reported do not differentiate between a non-specific interaction between collagen and osteonectin and a specific binding of osteonectin by collagen fibrils. Similarly, the

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binding of osteonectin to *already formed apatite crystals* (Termine *et al.* 1981*a*) has little physicochemical meaning in terms of its being involved in the *formation* of the inorganic crystals. Literally hundreds of proteins have been shown to bind to hydroxyapatite. Indeed, hydroxyapatite crystals but have long been used as an ion exchange material for protein purification. The question is not whether a protein constituent can be bound by *already formed hydroxyapatite crystals* but whether osteonectin or any other component has the physicochemical

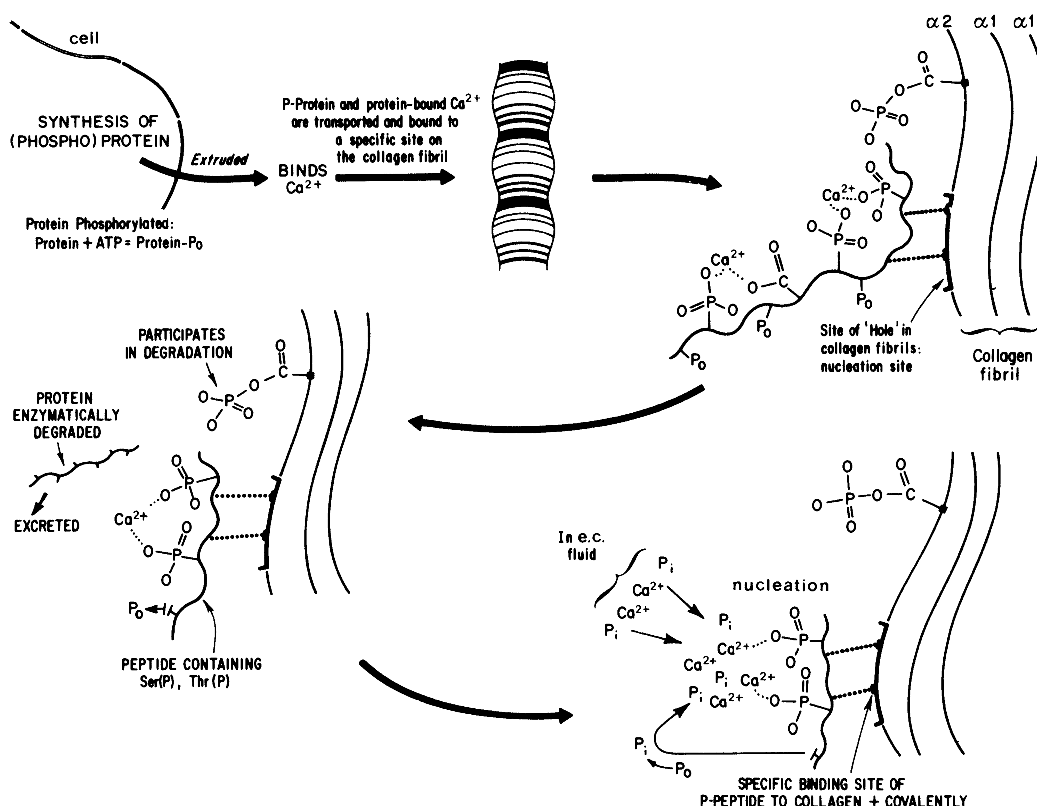

FIGURE 21. Schema for the role of phosphoproteins in mineralization of collagenous tissues. (From Glimcher 1981.)

properties to facilitate and participate in the *initiation* or the *formation* of the crystals. At least for the phosphoproteins, our own ^{31}P n.m.r. studies have shown that the phosphoproteins can form ternary complexes with calcium and phosphate ions. So they at least have the potential for doing so *in vivo*. In any event, much more basic information on the properties of osteonectin will be required before any role for this protein in mineralization can be rationally proposed.

To conclude, we believe that there is strong evidence based on the organization and location of mineral phase particles in collagenous mineralized tissues, the *in vitro* and *in vivo* properties of native type collagen fibrils and on *in vivo* studies of changes in tissue components in pathological states where mineralization is reduced, that in the presence of normal extracellular fluid, collagen macromolecules aggregated to produce native type fibrils together with phosphoproteins bound to specific regions of the fibrils, constitute a system that is both *necessary* and *biologically sufficient* for the calcification of collagen fibrils, and thus for normal calcification of the tissue as a whole (figure 21).

We would like to offer one piece of speculation to tie together the new information on the presence of HPO_4^{2-} groups arranged in a brushite configuration in the mineral phase of bone

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and the demonstration that Ser(P) and Thr(P) can form ternary complexes with Ca and inorganic orthophosphate ions. We propose that at least some of these HPO_4^{2-} groups may be contributed by Ser(P) and Thr(P). Since the number of such groups relative to the total number of inorganic orthophosphate groups in the mineral is very small, and since such organically bound phosphate groups (and possibly their next nearest phosphate ion neighbours)

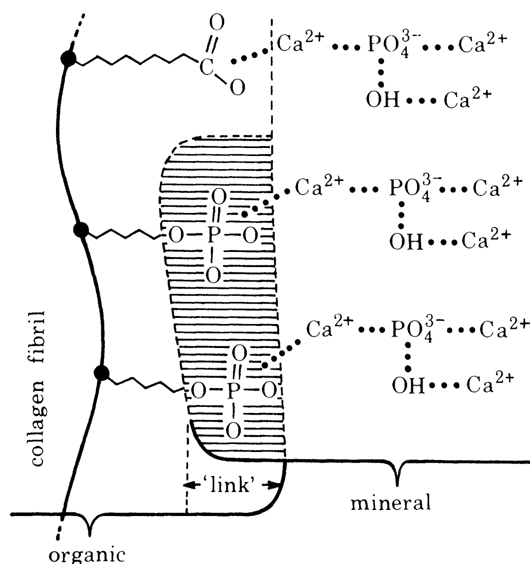

FIGURE 22. Schematic diagram illustrating how protein-bound phosphomonoester groups are constituents of both the organic and the mineral phases and thus serve as a bridge, chemical and physically linking the organic structural molecules of the organic matrix to the inorganic mineral crystals.

would extend over only a short distance, they would not generate a coherent X-ray diffraction pattern. This is consistent with our experimental findings. In any event, where the monoester groups of Ser(P) and Thr(P) are in a brushite configuration or not, we visualize them as constituents of both the inorganic and organic phases in bone and tooth and thus as chemical bridges linking the mineral crystals to the fibrous structural proteins in the tissue (figure 22).

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Discussion

H. Muir (*Kennedy Institute of Rheumatology, London, W6 1DW, U.K.*). Why did the constituted collagen fibre mineralize like bone when implanted in the peritoneal cavity? Is myositis ossificans an abnormality of the phosphoproteins that Dr Glimcher described?

M. J. Glimcher. I think that there are several reasons for the constituted collagen fibre mineralizing like bone when implanted in the peritoneal cavity.

(*a*) The collagen fibrils were reconstituted into the native type aggregation state (approximately 700 Å axial repeat), which does provide the proper spaces (holes) and electro- and stereochemical environment for nucleation to occur.

(*b*) As opposed to the original tissue (skin or tendon), the reconstituted fibrils were essentially free of the proteoglycans, which we believe normally function to inhibit mineralization, namely, they 'protect' the collagen fibrils from nucleating Ca–P crystals. Their removal, therefore, would permit the native type collagen fibrils to nucleate the apatite crystals from physiological solutions *in vivo*.

(*c*) There is a possibility that phosphoproteins were synthesized and bound to the collagen fibrils, which would also help facilitate the nucleation of the apatite crystals. However, even if no phosphoproteins were synthesized, the combination of native type collagen fibrils without any inhibiting proteoglycans associated with them should prove sufficient for the nucleation of apatite crystals from physiological solutions containing calcium and inorganic orthophosphate ions, although the rate might be considerably diminished.

I do not think that myositis ossificans is an abnormality of the phosphoproteins, i.e. an excess of phosphoproteins inciting or inducing abnormal calcification. I believe that myositis ossificans is probably related to changes in cell differentiation in the connective tissues of the muscle, namely, differentiation of primitive mesenchymal cells to osteoblasts, which then form ectopic bone tissue that then undergoes normal calcification.

On the other hand, there is emerging evidence that in conditions where there is deficient mineralization, for example dentinogenesis imperfecta and rickets (osteomalacia), that the decreased mineralization is accompanied by a diminution in the concentrations and content of phosphoproteins in the tissues.

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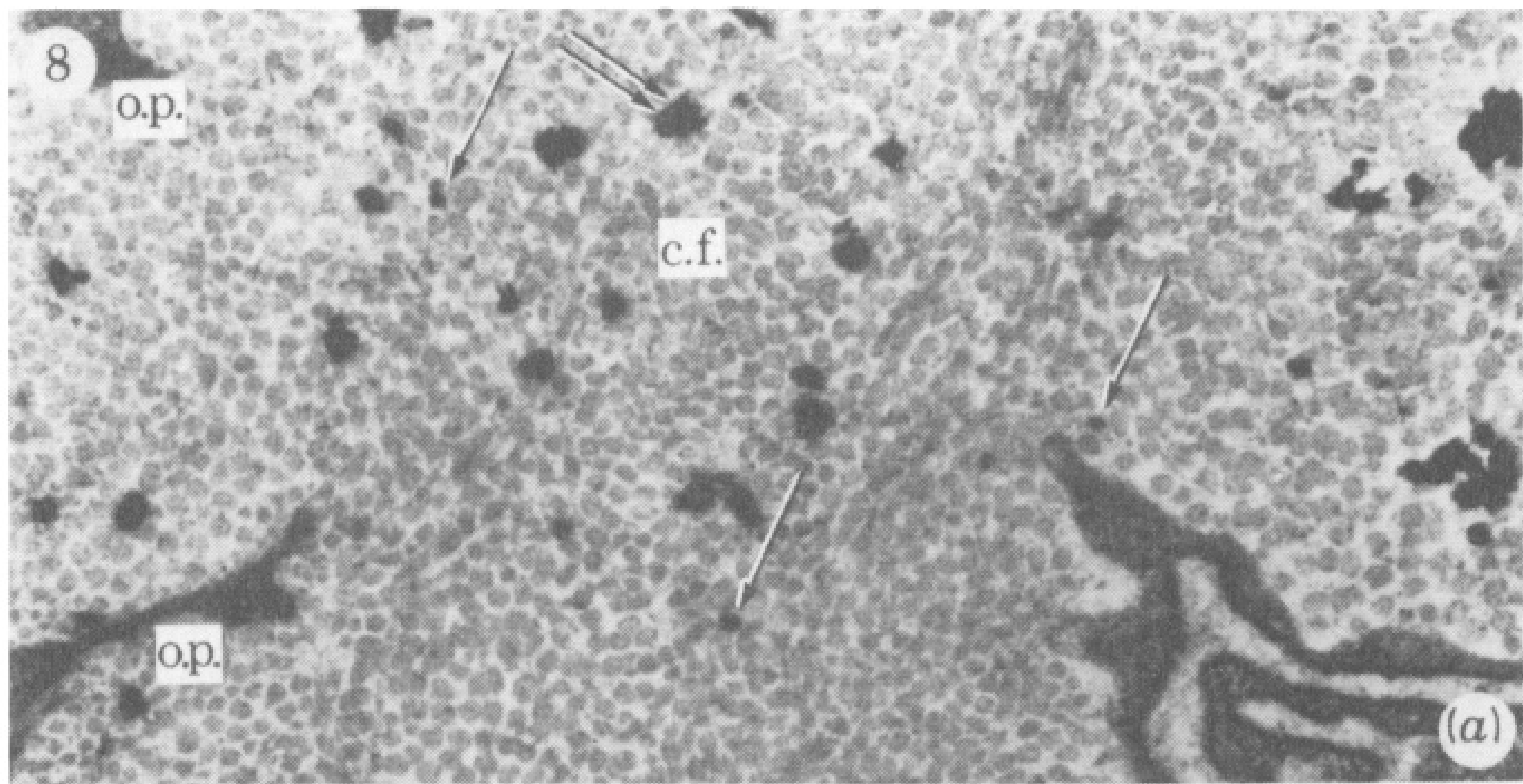

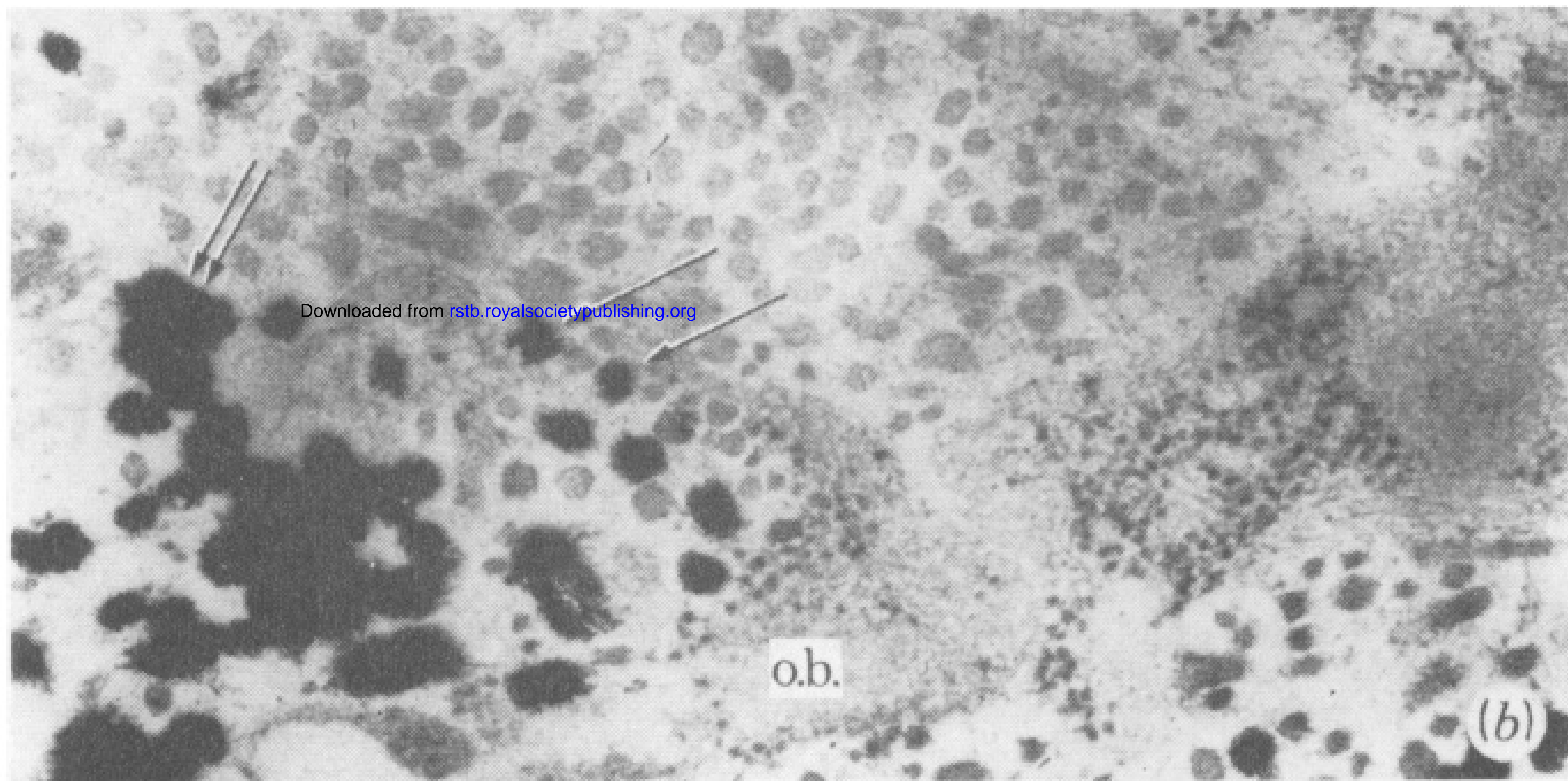

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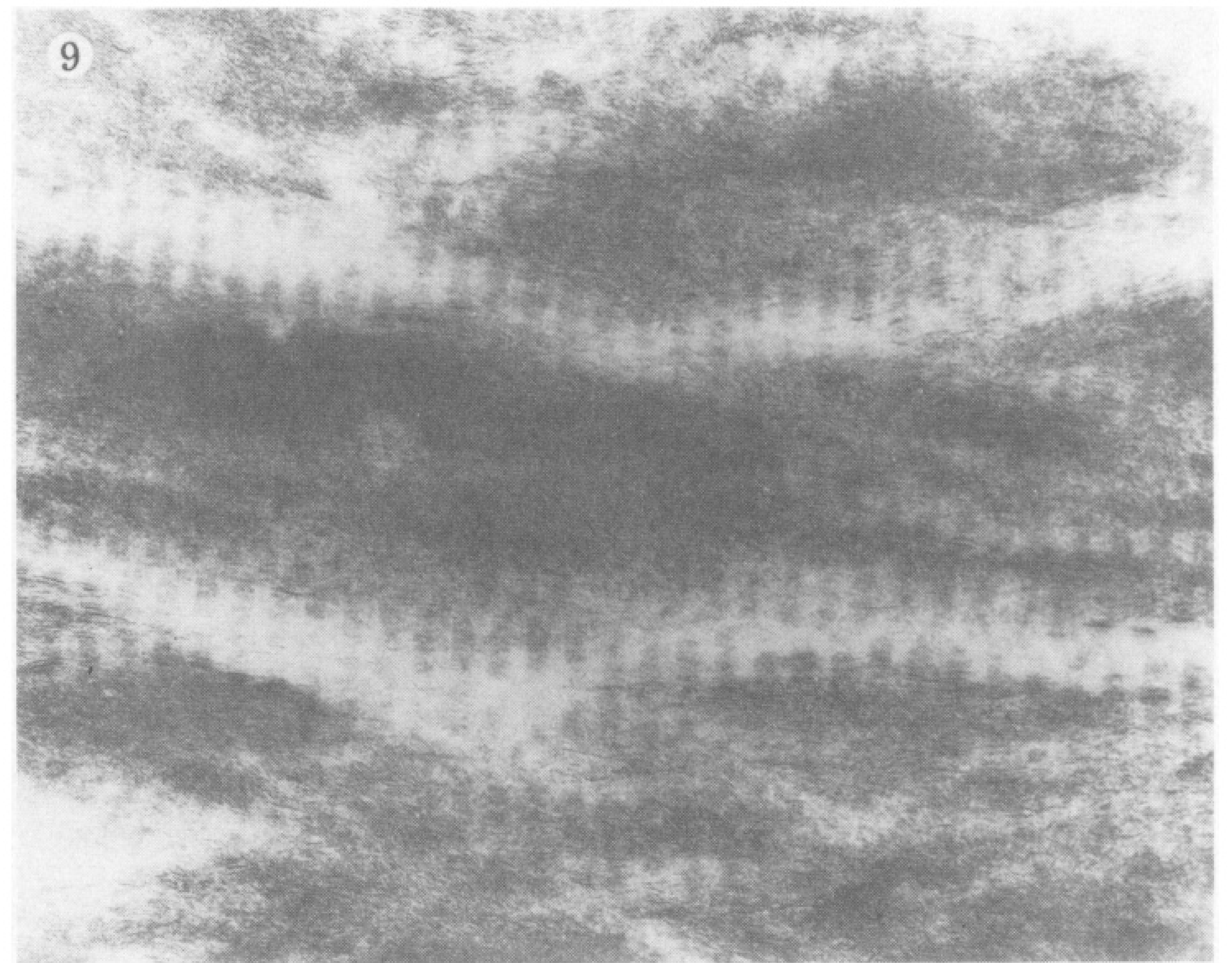

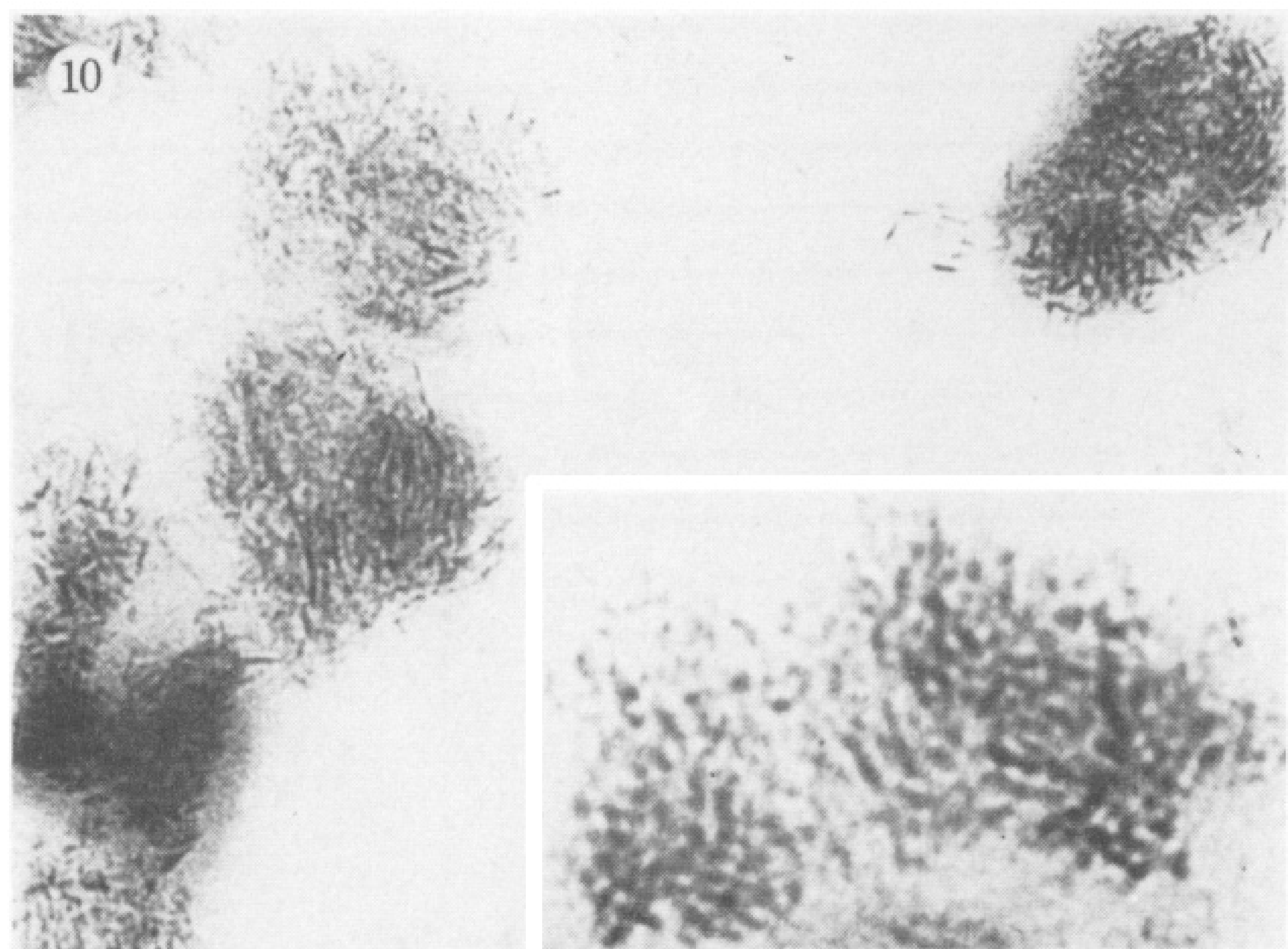

FIGURES 8–11. For description see opposite.

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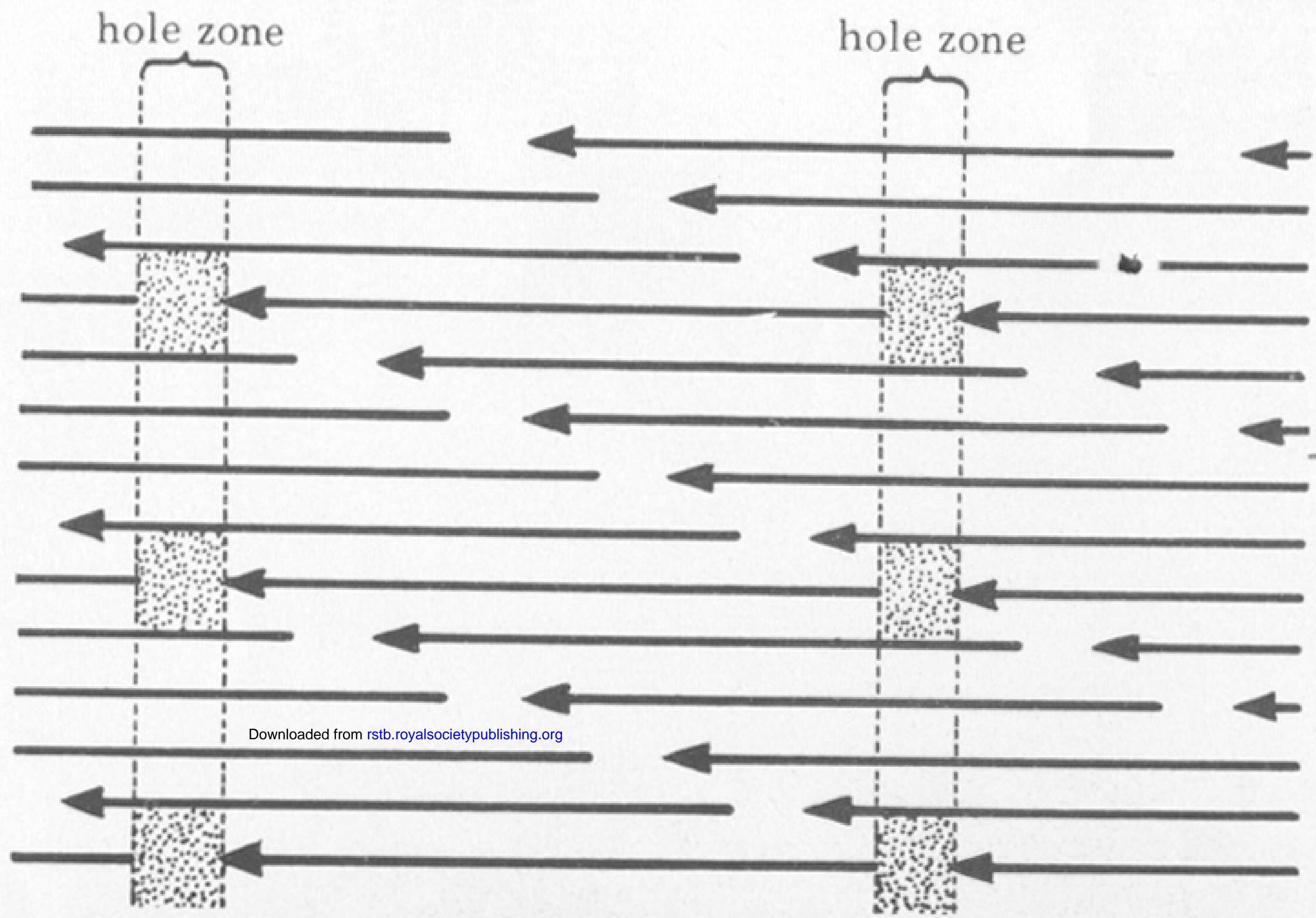

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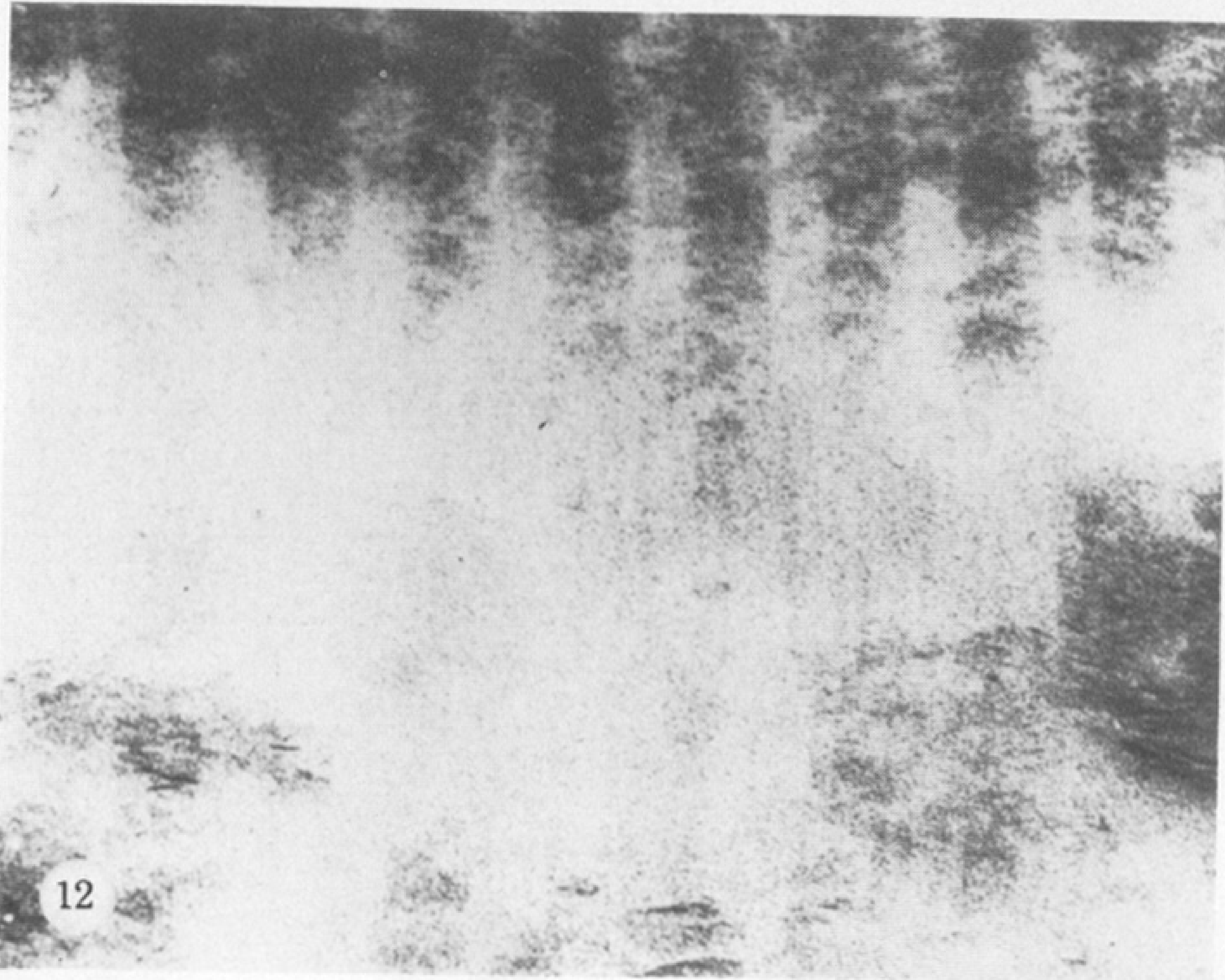

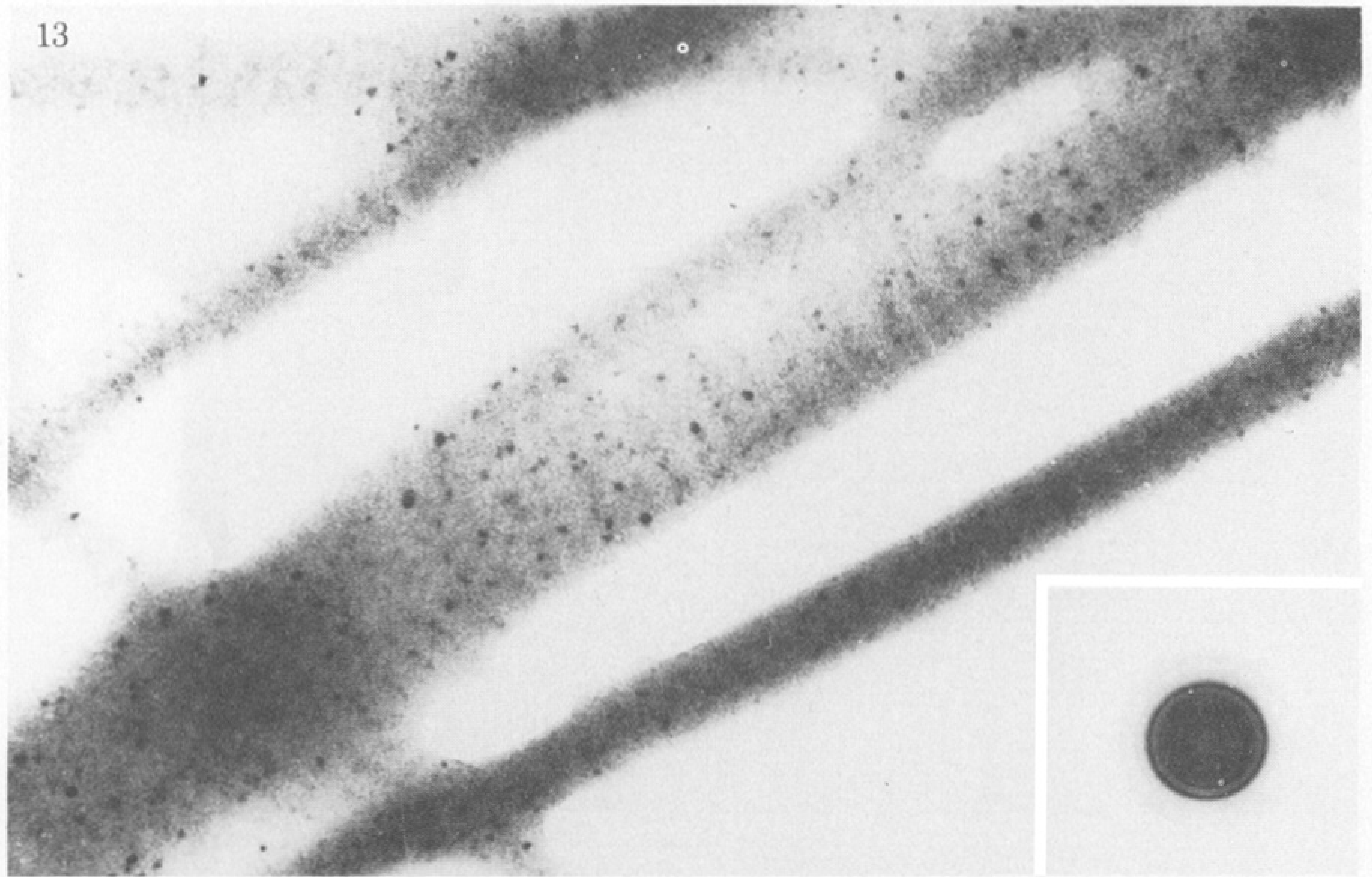

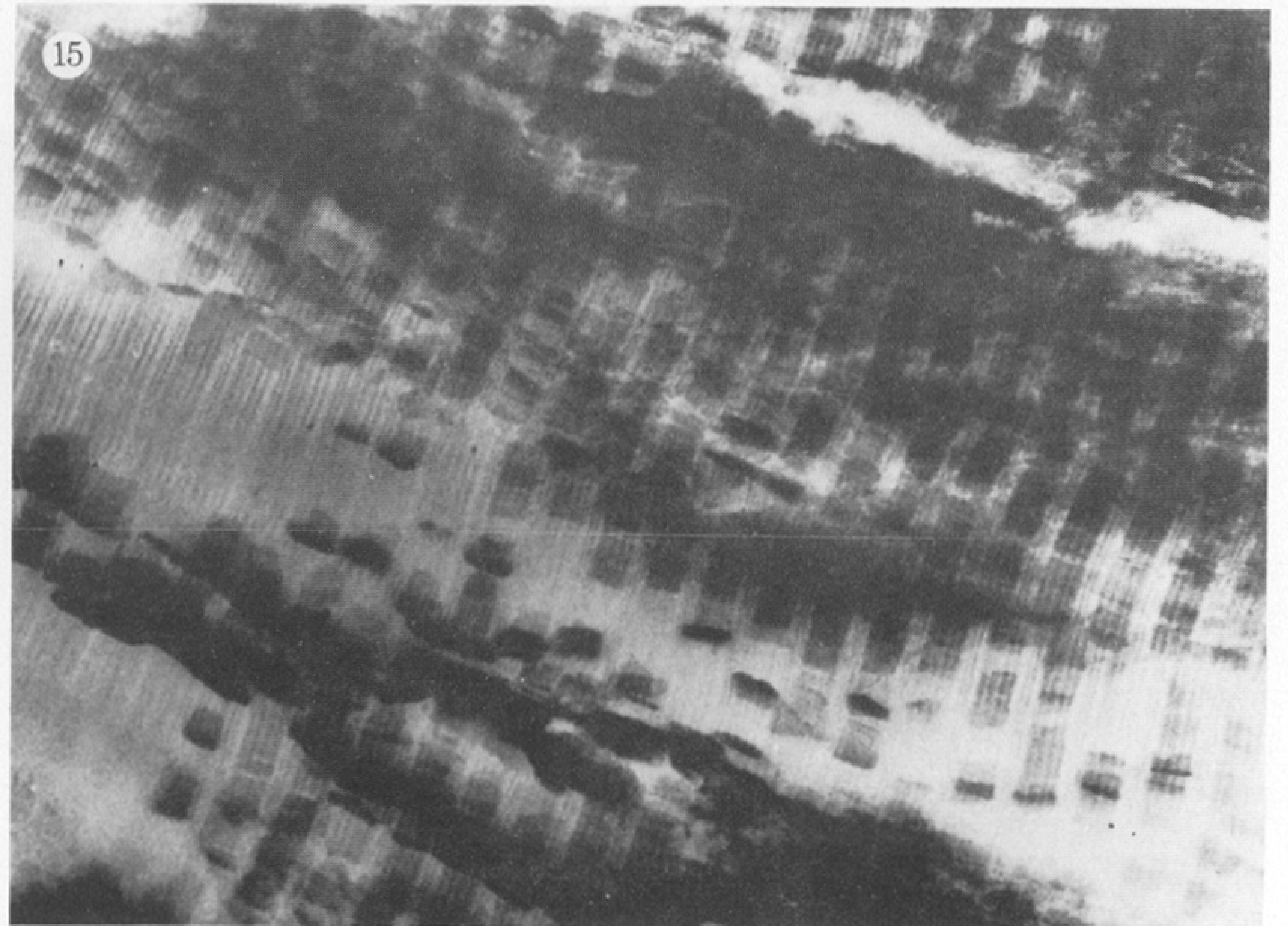

FIGURES 12, 13 AND 15. For description see opposite.

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