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TWO ULTRASTRUCTURALLY DISTINCT TUBULIN PARACRYSTALS INDUCED IN SEA-URCHIN EGGS BY VINBLASTINE SULPHATE

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SUMMARY

Two types of ultrastructurally distinct tubulin paracrystals have been induced in sea-urchin eggs with vinblastine sulphate (VLB) under different sets of conditions. One type of paracrystal appears to consist of hexagonally-close-packed microtubules and closely resembles paracrystals present in mammalian cells treated with vinblastine or vincristine sulphate, but not previously reported in sea-urchin eggs. The other type is also made up of tubulin subunits, but these do not seem to have polymerized into microtubules.

Both types of paracrystal are induced in sea-urchin eggs in the presence of VLB at a time when tubulin subunits would not normally polymerize. Possible mechanisms for tubulin activation and the induction of paracrystal formation are discussed in respect to the available information on the binding sites of the tubulin subunits.

INTRODUCTION

Vinblastine sulphate (VLB) induces the formation, *in vivo*, of tubulin paracrystals in a wide variety of mammalian cells and tissues including L cells (Bensch & Malawista, 1968, 1969; Nagayama & Dales, 1970), Earle cells (Krishan & Hsu, 1969), lymphoblasts (Krishan, 1970), brain (Schochet, Lampert & Earle, 1968) and kidney (Tyson & Bulger, 1972). Similar paracrystals have also been reported from invertebrate sources such as starfish oocytes (Malawista & Sato, 1969), hemipteran ovarioles (Stebbings, 1971) and sea-urchin eggs (Bryan, 1971, 1972*a*; Fujiwara & Sato, 1972; Starling & Burns, 1975; Starling, 1976; Strahs & Sato, 1973).

The ultrastructure of such paracrystals has usually been interpreted as an array of hexagonally-close-packed microtubules (Bensch & Malawista, 1969). One recent report (Starling & Burns, 1975), however, shows clearly that the paracrystalline ultrastructure in sea-urchin eggs can take a different form. There is ample evidence that such paracrystals are composed of tubulin (Bryan, 1972*a*).

VLB has been variously reported to bind to tubulin dimers *in vitro* in equimolar amounts (Bryan, 1972*b*; Wilson, 1970) or at a ratio of 1 mole of VLB to 2 moles of tubulin (Owellen, Owens & Donigan, 1972). The binding is not reversible (Bryan, 1972*b*); neither do colchicine (Bryan, 1972*b*; Wilson, 1970) or podophyllotoxin (Bryan, 1972*b*; Wilson, 1970) compete with VLB for the same binding site on the

tubulin dimer. It has been reported, however, that VLB does compete for one of the GTP-binding sites (Bryan, 1972*b*). VLB also prevents the polymerization of tubulin into microtubules both *in vivo* (Bensch & Malawista, 1969) and *in vitro* (Bensch, Marantz, Wisniewski & Shelanski, 1969) and induces the formation of various helical spiral forms instead.

It will be demonstrated that 2 forms of paracrystal can be induced in the eggs of the sea urchin, *Echinus esculentus*, though under different conditions, and the detailed ultrastructure of both forms is discussed. Mechanisms are proposed for the induction of the paracrystals in the light of available information on the binding sites of the tubulin subunit.

MATERIALS AND METHODS

Eggs of the sea urchin *Echinus esculentus* were shed into seawater by injection of 0·56 M KC into the body cavity. After washing in filtered seawater buffered to pH 8·0 with 5 mM Tris-HCl, the unfertilized eggs were incubated with 100 μM VLB in seawater-Tris-HCl at 12 °C for 18 h. Eggs were kept in 10 ml of incubation medium at 2 different densities: high density (over 250 eggs/ml) or low density (less than 100 eggs/ml).

The resulting paracrystals were isolated by a procedure based on the methods of Bryan (1971). Eggs were lysed by vigorous mixing into 100 volumes of 0·2 % Nonidet P-40 (Shell Chemical Company) in medium containing 100 mM KCl, 1 mM MgCl, 100 μM EDTA, 10 mM Tris-HCl (pH 7·0). Lysed cells were centrifuged at 1000 **g** for 1 min to remove whole cells, nuclei and membrane fragments and at 2500 **g** for 15 min at 12 °C to pellet the paracrystals.

Isolated paracrystals were either resuspended in a small volume of the above medium and observed with a Zeiss photomicroscope II fitted with polarizing optics, or were prepared for electron microscopy according to the method of Starling & Burns (1975). Isolated paracrystal pellets were resuspended in the above medium to which 1 % uranyl acetate had been added for 1 h, washed for several hours and fixed in 1 % OsO_4 in the same medium. After an overnight wash, pellets were embedded in agar, dehydrated and embedded in Araldite. Sections of 50–90 nm thickness, as judged by interference colour, were cut using an LKB Ultratome III and further stained with lead citrate. Sections were examined in a Philips EM 300 fitted with a goniometer stage.

RESULTS

Induction of two types of paracrystal

Incubation of eggs at low density produces paracrystals of the general form shown in Fig. 2A, B (L.D. paracrystals), previously described at both the light-microscope level (Bryan, 1971) and the electron-microscope level (Starling & Burns, 1975). These paracrystals have been shown to be composed of equimolar amounts of 2 proteins characterized as tubulin (Bryan, 1972*a*).

Paracrystals formed in eggs incubated at high density (H.D. paracrystals) are longer and narrower than L.D. paracrystals. Their overall appearance is more 'needlelike' (Fig. 2C, D). There are usually more paracrystals per egg in eggs incubated at high density than occur in eggs incubated at low density in VLB alone. The number of L.D. paracrystals per egg can be greatly enhanced, however, by the inclusion of other mitotic inhibitors in addition to VLB in the incubation medium (Starling, 1976).

Attempts to produce the 2 forms of paracrystal by varying the VLB concentration were unsuccessful. Lower VLB concentrations often failed to produce paracrystals and when the VLB concentration was increased paracrystals of the low density type invariably resulted. Neither has it been possible in this organism to produce paracrystals with vincristine sulphate, either alone or in combination with other mitotic inhibitors.

Paracrystal ultrastructure

L.D. paracrystals are characterized by 2 very distinctive patterns at the ultrastructural level. The first 2-dimensional projection of the structure is observed in a plane at right angles to the crystal long axis and consists of a network of 6 pointed stars formed

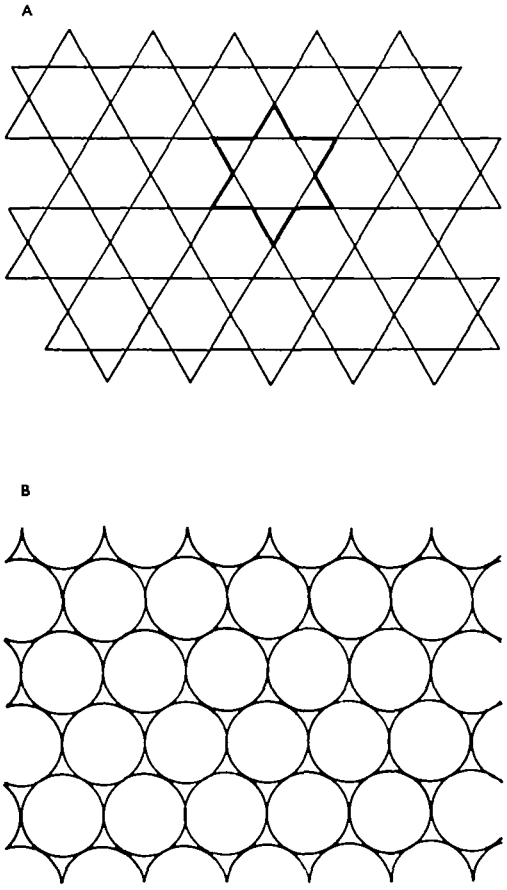

Fig. 1. A, schematic diagram of transverse section of L.D. paracrystal. B, schematic diagram of transverse section of H.D. paracrystal.

from 3 sets of parallel lines, 4–5 nm broad, spaced 24–28 nm apart and each set inclined at angles of between 50 and 80° to the other 2 sets such that pairs of triangles of height approximately 14 nm are formed between resulting hexagons (Fig. 4A).

In projections at right angles to the 'star pattern', the second characteristic pattern is often observable. This pattern consists of beads of 8–9·5 nm diameter, 16–18 nm apart, arranged in straight rows of some 24–28 nm spacing (Fig. 4B). Beads in adjacent rows are slightly out of register, giving a pitch of 9–15°. These patterns have been observed only in paracrystals isolated from eggs of the sea urchin *Echinus esculentus* and have been described previously in more detail (Starling & Burns, 1975). (See Fig. 1.)

At the ultrastructural level H.D. paracrystals appear similar to paracrystals previously reported in other organisms, but are different from L.D. paracrystals. H.D. paracrystals can also be represented by 2 characteristic patterns. At right angles to the long axis of H.D. paracrystals an array of circular profiles (Fig. 5*a*) of mean outside diameter 23 nm and wall thickness varying from 4·5 to 8 nm is observed. These circules are not uniformly distributed, in contrast to the very regular stars of L.D. paracrystals, but are arranged into a series of hexagonally close-packed 'domains'. Large numbers of such domains form a single cross-section of a paracrystal (Fig. 1).

In a plane at right angles to such a projection, the appearance of a paracrystal (Fig. 5B) is of groups of beads from 9·5 to 15 nm diameter, spaced at intervals of between 17 and 21 nm along straight, or slightly curved rows, which have a separation of 19–24 nm. The beads in any row are displaced with respect to those in adjacent rows, but the displacements are often insufficiently regular to determine a good line joining nearest neighbour dots and hence assess the angle of pitch. Regions where beads in adjacent rows are in register give a pitch angle of about 12°, but such regions are limited in extent.

The spacings of the beaded views of both H.D. and L.D. paracrystals may not be significantly different from each other, especially when the difficulties of making good average measurements on H.D. paracrystals are taken into account. The close-packed hexagonal pattern of H.D. paracrystals is very different from the star pattern of L.D. paracrystals. Occasionally, however, an intermediate appearance is presented in transverse section (Fig. 3), confirming that only slightly different conditions are required to produce either of the two forms.

DISCUSSION

Formation of the two types of paracrystal

H.D. paracrystals are distinctive even at the light-microscope level. Their induction in sea-urchin eggs has not previously been reported. The literature on paracrystals from sea-urchin eggs is, however, more extensive than that on related structures from any other organism (Bryan, 1971, 1972*a*; Fujiwara & Sato, 1972; Starling & Burns, 1975; Starling, 1976; Strahs & Sato, 1973). Thus it would seem that H.D. paracrystals are more difficult to induce in sea-urchin eggs in spite of their apparently close similarities to those induced by VLB in other organisms (Bensch & Malawista, 1969; Krishan & Hsu, 1969; Krishan, 1970; Malawista & Sato, 1969; Nagayama & Dales, 1970; Schochet *et al.* 1968; Stebbings, 1971; Tyson & Bulger, 1972). Since only L.D. paracrystals are produced if the VLB concentration is raised or if another

mitotic inhibitor is added (Starling, 1976) conditions for the production of H.D. paracrystals in sea-urchin eggs appear to be fairly critical.

Ultrastructure of the two types of paracrystal: transverse sections

On close examination the ultrastructure of the 2 types of paracrystal appear distinct. In transverse section the width of the electron-dense lines of the L.D. paracrystals is 4–5 nm (Starling & Burns, 1975), suggesting that tubulin monomers are arranged in single rows. Slight variations may be due either to a slightly non-vertical arrangement of subunits, as demonstrated in ciliary microtubules (Amos & Klug, 1974) or to the coincidence of lines at different levels in the depth of the section.

In contrast, the electron-dense parts of transverse sections of H.D. paracrystals vary from 4·5 nm at the edges of domains, etc., to 9 nm where, presumably, the walls of 2 adjacent microtubules or 2 helical filamentous strands are in close contact, as would be predicted for a hexagonally close-packed arrangement. The diameter of the circles seen in such sections is about 23 nm, which is well within the range reported for microtubules of other sea-urchin species (Stephens, 1967; Rebhun & Sander, 1967; Goldman & Rebhun, 1969; Fulton, Kane & Stephens, 1971). Conversely the inter-line spacing in L.D. paracrystals is of the order of 28 nm (Starling & Burns, 1975).

Longitudinal sections

In longitudinal sections, both H.D. and L.D. paracrystals exhibit a superficially similar appearance. However, L.D. paracrystals are made up of a large continuous array of beads, whereas H.D. paracrystals appear to be broken up into much smaller regions and are less regular. The spacing between beads may not be significantly different, though the beads in H.D. paracrystals appear to be larger than those of L.D. paracrystals, possibly because of the close packing of the H.D. paracrystals so that 2 8-nm beads from adjacent tubules occur close together. In L.D. paracrystals the beads may represent those regions at which protein subunit strands cross over or are adjacent to one another. The spacing of the longitudinal rows also differs, having a maximum value of 24 nm in H.D. paracrystals, consistent with the structure being formed from hexagonally packed microtubules, and 24–28 nm in L.D. paracrystals in agreement with the spacings of the transverse sections.

An alternative explanation of the appearance of longitudinal sections of one or both types of paracrystal may be provided by considering the helical filaments, 'bedsprings' or 'macrotubules' which can be induced in response to VLB treatment both *in vitro* in tubulin from mammalian brain (Bensch *et al.* 1969; Warfield & Bouck, 1974) and *in vivo* in cranefly spermatids (Behnke & Forer, 1972) and in a foraminifer (Hauser & Schwab, 1974). In the cranefly the resulting 'circular profiles' seen in transverse section are reported to have a diameter of 34–36 nm, whilst in the foraminifer they are only 18 nm in diameter. Neither value agrees with the corresponding dimensions reported here for either type of paracrystal from the sea urchin, but since the reported values vary so greatly the possibility of an intermediate value for sea urchins should not be ruled out. The mechanisms by which VLB causes tubulin to polymerize into these helical forms is not known and is more surprising in sea-urchin eggs at a stage

of development when extensive tubulin polymerization would not be expected. Nevertheless polymerization of tubulin from the pool does occur either in direct or indirect response to VLB. It is not as yet possible, however, to decide unequivocally whether both forms of paracrystal are formed from 'bedsprings'.

It seems probable that H.D. paracrystals might be made up of small regions of hexagonally packed microtubules. It has previously been suggested (Stebbings, 1971) that intact microtubules are needed to seed the formation of paracrystals. L.D. paracrystals on the other hand seem to consist of a 3-dimensional network of 'strings' of tubulin subunits, which bear little resemblance to microtubules. Optical diffraction studies to examine the ultrastructure of both types of paracrystal in more detail are in progress.

Implication of paracrystal induction

Sea-urchin eggs have a pool of tubulin subunits (Raff, Greenhouse, Gross & Gross, 1971). An important implication of paracrystal induction is to consider how the tubulin subunits can be induced to polymerize in the presence of VLB into 2 different sorts of structure, one closely resembling arrays of microtubules (H.D. paracrystals) and the other apparently completely different (L.D. paracrystals); both of which might possibly be formed from helical filaments.

One possible explanation is that tubulin exists in an 'inactive' form in a pool of subunits in the unfertilized sea-urchin egg. As suggested by Farrell & Burns (1975) these subunits can be 'activated', for example as a result of fertilization and are then able to polymerize into microtubules. In the case of paracrystal induction, VLB must bring about activation either directly or indirectly. Polymerization into microtubules need not of necessity result from this activation. Indeed other forms of polymer are clearly possible.

Firstly, tubulin might be activated directly by the binding of a VLB molecule to a tubulin subunit. If this is the case, the ultrastructural arrangement of L.D. paracrystals might be explained on the basis that VLB binds to one of the normal polymerization sites of the tubulin subunit; probably the dimer (Bryan, 1972*b*; Wilson, 1970), and prevents the formation of microtubules by virtue of stearic hindrance, even though the subunits have been activated. These modified tubulin subunits pack into the rather open network of L.D. paracrystals. It is difficult to explain the formation of H.D. paracrystals in similar terms. H.D. paracrystals seem superficially to be composed, at least when transverse sections are viewed, of hexagonally-close-packed microtubules. Thus it would seem unlikely that the VLB occupies a polymerization site on the tubulin subunit. It may be that under the conditions of egg density required for H.D. paracrystals to be formed, insufficient VLB is present to occupy all the binding sites (this idea is supported by the intermediate form shown in Fig. 3), whilst still being available in sufficient concentration both to cause activation and to alter the overall charge distribution on the microtubules so that they form paracrystals. Perhaps this occurs when VLB is bound to tubulin in the ratio of one mole of VLB per tetramer of tubulin (Owellen *et al.* 1972). It is therefore necessary to examine mechanisms for activation other than that of direct binding of VLB to tubulin subunits.

It has been shown (Wilson, Bryan, Ruby & Mazia, 1970) that VLB causes the precipitation *in vitro* of a number of other structural proteins such as actin, membrane protein and vitelline protein. These proteins can also all be precipitated by Ca^{2+} ions, though at considerably higher ionic concentrations than are required for VLB precipitation. It has been suggested that the precipitation of such proteins is brought about by the vinblastine ion behaving as a divalent cation and binding to a divalent cation-binding site on the protein (Wilson *et al.* 1970). Thus in the formation of tubulin paracrystals it may be that VLB behaves as a divalent cation which cannot be chelated *in vivo* in the same way as smaller cations can be and in some ways triggers activation.

It is also possible that VLB brings about tubulin activation indirectly in that it acts upon some cellular component other than tubulin, since there is ample evidence (Wilson *et al.* 1970) that VLB reacts with numerous proteins and other cellular components. The binding of VLB to the subunits, whilst in itself not causing tubulin activation directly, would lead to paracrystal formation due to interference with microtubule polymerization. It is, however, difficult to account for the 2 distinct forms of paracrystal using a model of this type.

It is interesting that a single drug, under only very slightly different conditions, can produce 2 such apparently different paracrystalline structures from tubulin in an organism which otherwise would maintain most of its tubulin pool in subunit form.

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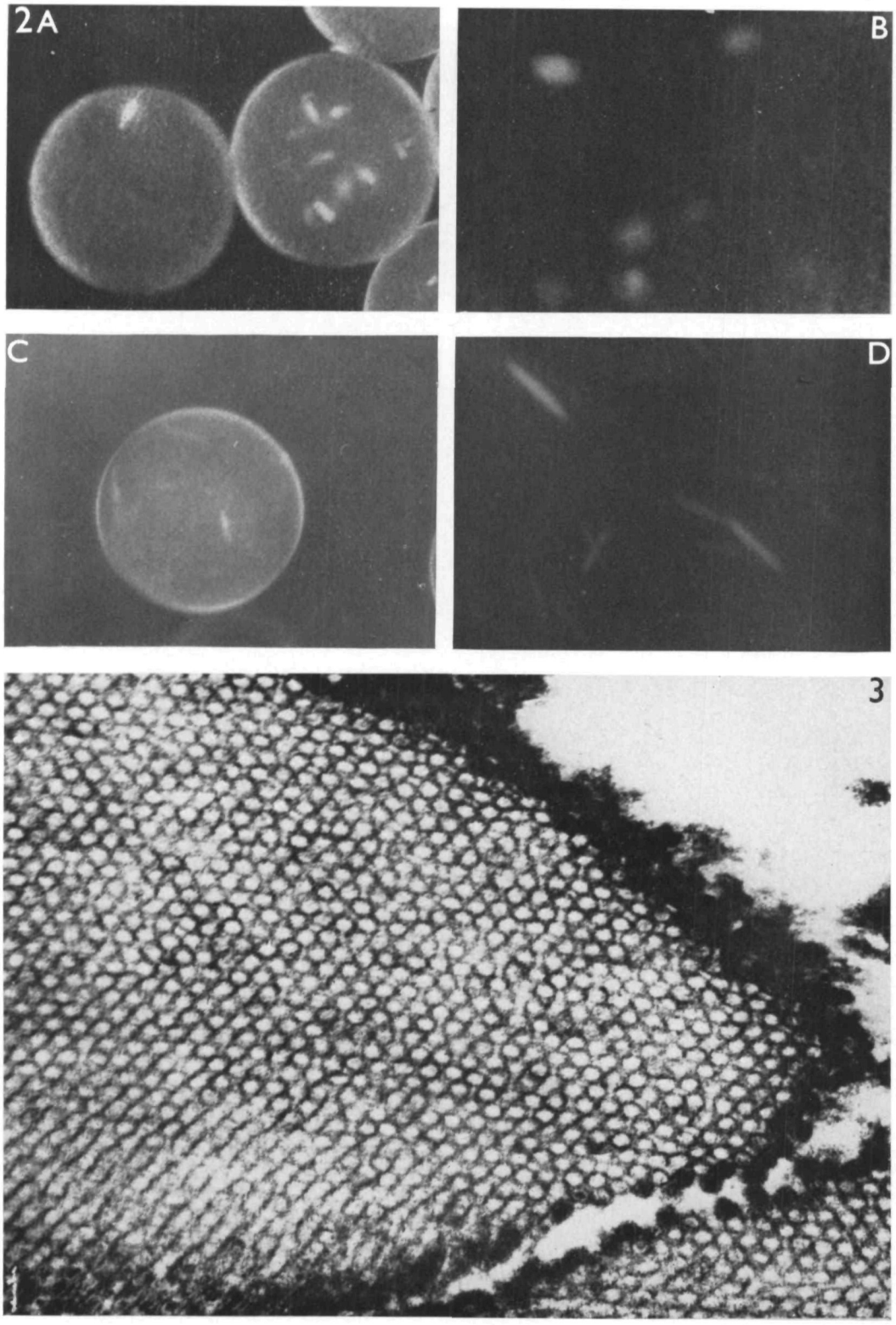
Fig. 2. A, sea-urchin egg with L.D. paracrystal. Polarizing optics; polarizer and analyser crossed; no compensator. × 320. B, L.D. paracrystal. Polarizing optics; polarizer and analyser crossed; no compensator. × 800. C, sea-urchin egg with H.D. paracrystals. × 320. D, H.D. paracrystal. × 800.

Fig. 3. Intermediate form of paracrystal. × 120000.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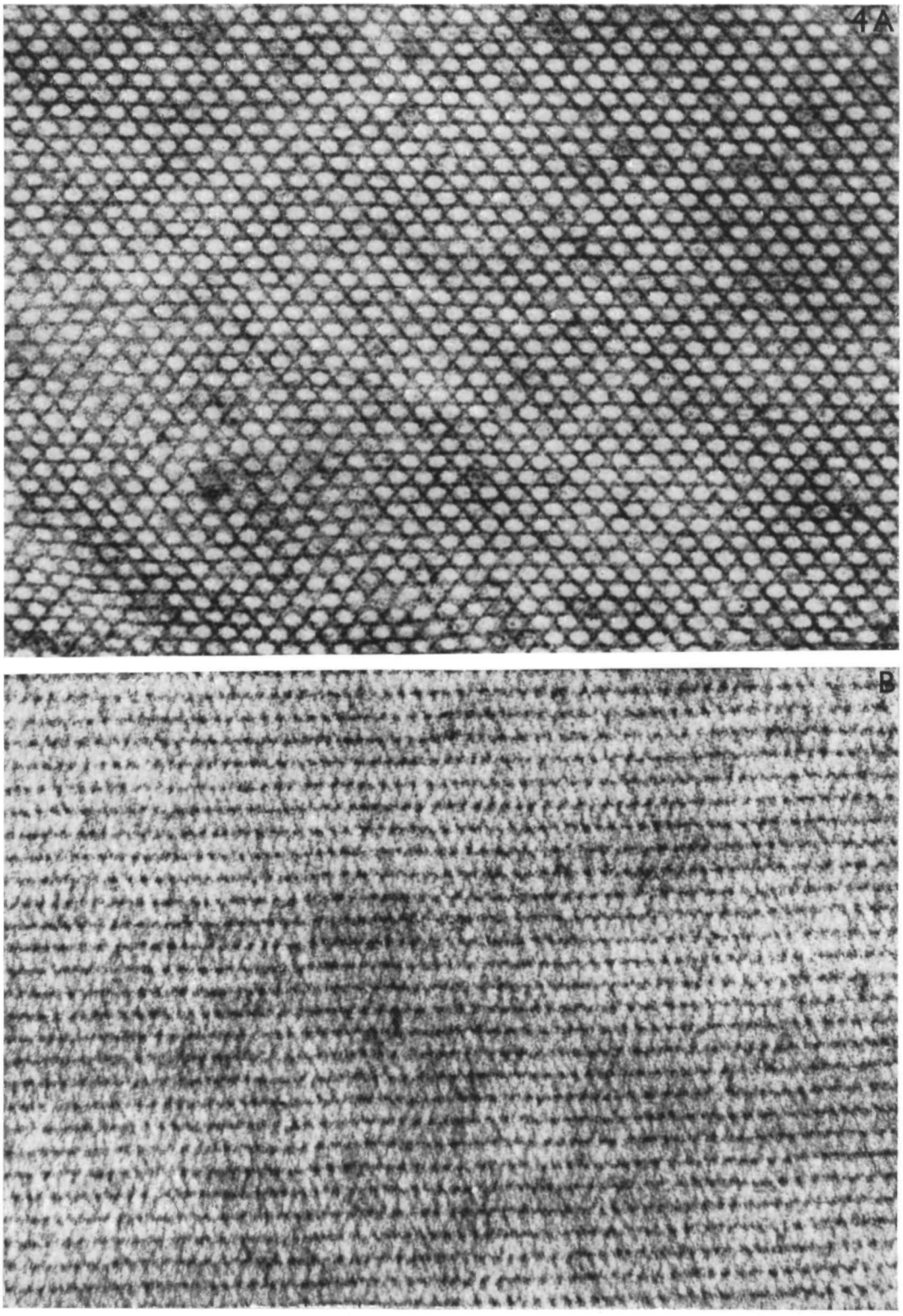

Fig. 4. A, star pattern from L.D. paracrystal. × 120000. B, beaded pattern from L.D. paracrystal. × 120000.

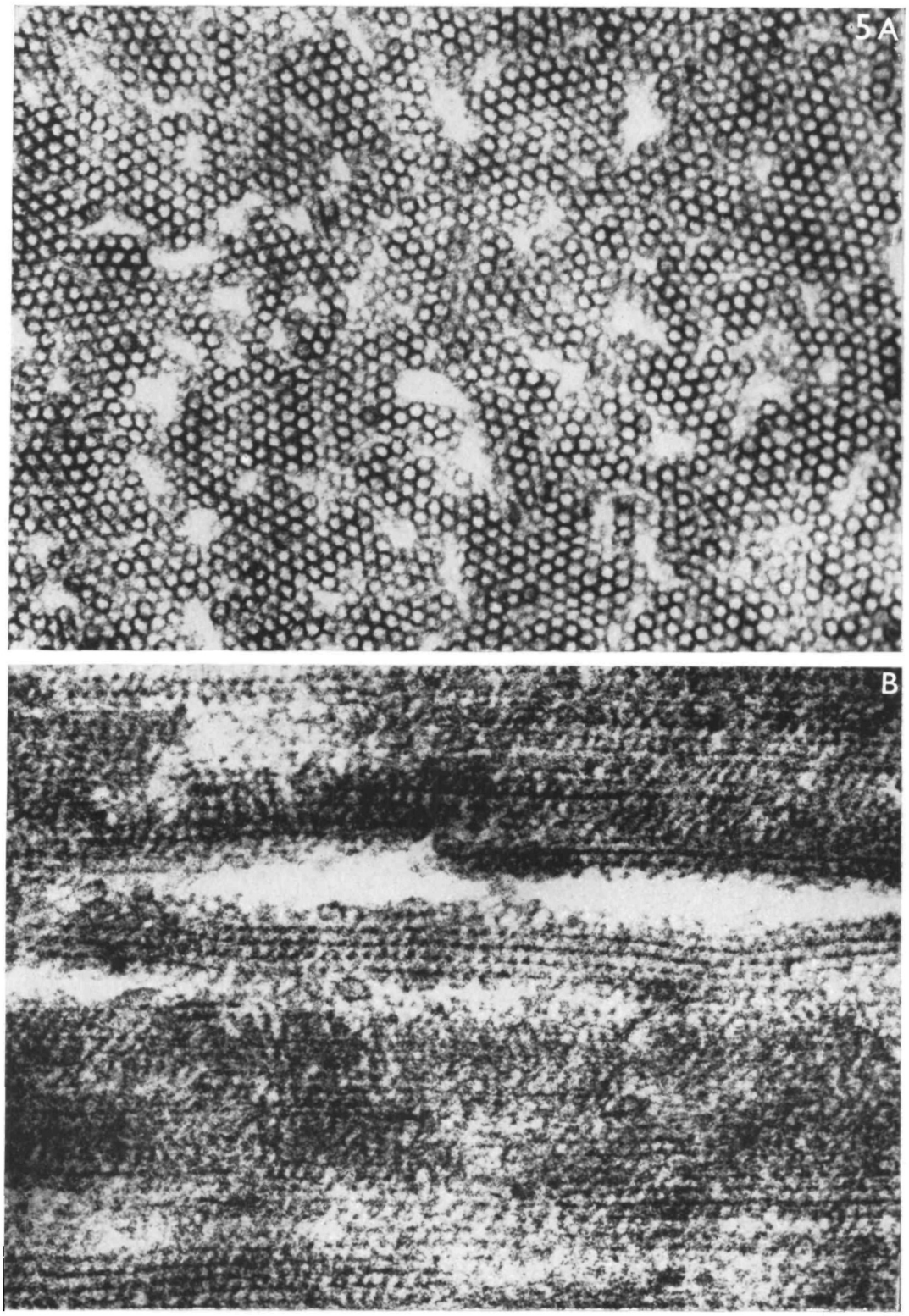

Fig. 5. A, hexagonally-close-packed pattern from H.D. paracrystal. × 120000.
B, beaded pattern from H.D. paracrystal. × 120000.