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THE EFFECTS OF MITOTIC INHIBITORS ON THE STRUCTURE OF VINBLASTINE-INDUCED TUBULIN PARACRYSTALS FROM SEA-URCHIN EGGS

D. STARLING
Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland

SUMMARY

Vinblastine sulphate (VLB) is known to induce *in vivo* formation of tubulin paracrystals in sea-urchin eggs. Corresponding paracrystals have been prepared in the presence of both vinblastine sulphate and other mitoclasic agents. Careful standardization of conditions was required to restrict the formation of alternative forms of the paracrystals induced by vinblastine alone. Comparisons were made between preparations in terms of paracrystal shape, size, proportion of eggs containing paracrystals, number per egg and their relative times of first appearance. A correlation between such properties was established. Comparison of paracrystals at the ultrastructural level showed them all to be similar regardless of the drugs present during their formation. The implications of tubulin polymerization in the presence of mitoclasic agents are discussed and mechanisms for paracrystal enhancement by combinations of such drugs are suggested. Some similarities of paracrystal and microtubule seeding are discussed together with the activation of tubulin in the pool.

INTRODUCTION

Numerous substances are known to inhibit mitosis. Among them are a class of compounds, mitoclasic agents, which are known to affect the mitotic spindle. This paper reports the effects of such drugs on the formation of tubulin paracrystals in sea-urchin eggs. Examples of such agents are vinblastine sulphate (VLB) (Malawista, Sato & Bensch, 1968; Palmer, Livengood, Warren, Simpson & Johnson, 1960), colchicine (Borisy & Taylor, 1967*b*), podophyllotoxin (Kelly & Hartwell, 1953), griseofulvin (Malawista *et al.* 1968) and isopropyl-*N*-phenyl carbamate (IPC) (Coss & Pickett-Heaps, 1974). There is evidence that colchicine (Borisy & Taylor, 1967*a*; Bryan, 1972*b*), vinblastine sulphate (Bryan, 1972*b*; Wilson, 1970) and podophyllotoxin (Bryan, 1972 *b*; Wilson, 1970) exert their effects by binding to 'tubulin' subunits and thus preventing their polymerization into the microtubules of the mitotic spindle fibres. In some instances microtubule disassembly is observed (Malawista *et al.* 1968) and is probably due to the restricted availability of subunits from the preformed tubulin pool, caused by mitoclasic agents binding to these subunits. Griseofulvin does not compete with either colchicine or podophyllotoxin for a binding site on the tubulin subunit (Flavin & Slaughter, 1974). Results at the cytological level suggest that tubulin polymerization is prevented, but (Malawista *et al.* 1968) in some cases

microtubule disruption does not occur (Grisham, Wilson & Bensch, 1973). IPC does not compete with colchicine or podophyllotoxin (Flavin & Slaughter, 1974), does not bind to tubulin (Bouck & Green, 1974; Coss, Bloodgood & Brower, 1974), and does not prevent *in vitro* polymerization (Coss *et al.* 1974). However, reassembly of microtubules *in vivo* has been shown to be inhibited by IPC (Coss & Pickett-Heaps, 1974) and subsequently, after drug withdrawal, the number of microtubule-organizing centres (MTOCs) is affected. It is probable therefore that both griseofulvin and IPC affect some element or elements closely involved in microtubule formation.

It has been known for some time that vinblastine sulphate or vincristine sulphate induces birefringent paracrystals in sea-urchin eggs (Bryan, 1971), starfish oocytes (Malawista & Sato, 1969), mammalian cells (Bensch & Malawista, 1969) and Hemipteran ovarioles (Stebbings, 1971). In the case of sea-urchin eggs these paracrystals are known to be composed of 2 closely similar tubulins (Bryan, 1972*a*). If it were possible to produce similar paracrystals in the presence of other mitotic inhibitors, which either bind to tubulin or affect seeding, differences in the shape and ultrastructure of the paracrystals might be expected. A study of the structures formed under such conditions might give information about the binding sites for these inhibitors on the tubulin subunit and as a consequence shed some light on the number and nature of the actual binding sites involved in the polymerization of the tubulin subunits into microtubules.

However, the *in vivo* formation of paracrystals in sea-urchin eggs, under the influence of vinblastine sulphate alone, can be shown to be variable in terms of paracrystal size, shape and ultrastructural arrangement (Starling, 1976). Factors affecting paracrystal growth include vinblastine sulphate concentration in relation to the density of the egg suspension (Starling, 1976), the nature and concentration of additional agents (Strahs & Sato, 1973) and presumably the precise physiological state of the eggs. This paper reports the effects of mitotic inhibitors added to suspensions of sea-urchin eggs in the presence of a fixed concentration of vinblastine sulphate. Since it has been reported previously that colcemid enhances the rate of formation of paracrystals (Strahs & Sato, 1973) a systematic study was made of the formation of vinblastine sulphate-induced paracrystals in the eggs of the sea urchin *Echinus esculentus* in the presence of several mitoclasic agents. Particular attention has been paid to paracrystal shape, both at the gross and the ultrastructural levels, to the number of paracrystals per egg and the proportion of eggs containing paracrystals and to the ultimate size and relative time of first appearance of such paracrystals.

MATERIALS AND METHODS

Eggs from the sea urchin *Echinus esculentus* were shed into filtered seawater following injection of 0·56 M KCl. The eggs were washed in fresh seawater and resuspended to a final concentration of about 100 eggs/ml in filtered seawater buffered to pH 8 with 5 mM Tris-HCl, to which 100 μM VLB had been added. In each experiment a further mitotic inhibitor, dissolved in a suitable solvent, was added to each of four 10-ml samples as shown in Table 1. A fifth sample was maintained in the presence of VLB alone. The eggs were then incubated at 14 °C for various periods of time up to 30 h.

At intervals eggs were removed and photographed with a Zeiss photomicroscope II using polarizing optics with polarizer and analyser crossed. The remaining eggs were harvested by low-speed centrifugation and resuspended in crystal isolation buffer (Bryan, 1971; Starling & Burns, 1975), where the eggs were lysed by vigorous mixing. Small samples of these crystals were photographed with the polarizing microscope, whilst the remainder were fixed and embedded for electron microscopy as previously described (Starling & Burns, 1975; Starling, 1976).

Table 1. *Details of additions of mitotic inhibitors*

Mitoclasic agent	Colchicine	Podophyllin	Griseofulvin	Isopropyl-*N*-phenyl carbamate
Final concentration	100 μM	1 % of soluble part of sat. soln. (6·45 mg/ml)	100 μM	100 μM
Solvent	Water	Water	Acetone	Ethanol
Final solvent concentration	—	—	1 % (v/v)	1 % (v/v)

RESULTS

Light microscopy

It has been shown (Starling, 1976) that vinblastine sulphate causes the formation of 2 types of paracrystalline inclusions in unfertilized sea-urchin eggs. Standardized conditions of vinblastine sulphate concentration and egg density allow the formation of paracrystals of the type described (Starling & Burns, 1975). The inclusion of known mitotic inhibitors in addition to vinblastine sulphate did not prevent the formation of paracrystals though the size, shape, number per egg, frequency and rate of formation were all systematically affected by the presence of the other drugs. The results are summarized in Table 2.

As can be seen from Table 2 there is often a logical correlation between observations on several properties of paracrystal formation. For example, podophyllin causes a marked increase in the number of paracrystals per egg and increases the number of eggs with paracrystals, but they are usually smaller than those produced by VLB alone and considerably smaller than paracrystals produced in the presence of the other drugs tested. Conversely griseofulvin delays the appearance of paracrystals, when formed they are fewer in number than those grown in the presence of other drugs and attain a larger size. Colchicine and IPC also accelerate the appearance of paracrystals, which are increased in final size. Additionally, IPC greatly increases the numbers of paracrystals per egg. These 2 drugs often bring about the appearance of bilobed paracrystals.

Paracrystal shape was found to depend upon the combination of drugs used and can best be judged from Fig. 1. Paracrystals prepared in the presence of VLB alone have previously been described as 'square' (Bryan, 1971) and, as can be seen from Fig. 1 A and F, they appear roughly rectangular in section, though sometimes the sides are somewhat curved and cross-sections at right angles to the long axis have been found which are almost circular. The basic rectangular shape is not much modified

Table 2. *Effects of various mitotic inhibitors on paracrystal formation*

Mitoclasic agent ...	VLB	VLB + colchicine	VLB + podophyllin	VLB + griseofulvin	VLB + IPC
Relative size	Medium	Large	Small	Very large	Large
Shape	Rectangular	Oval/lozenge	Rectangular	Barrel	Oval/lozenge
Relative No. per egg	Variable	Several	Very many	Several	Very many
Relative No. of eggs containing crystals	Variable	Most	Most	Moderate	Almost all
Relative time of first appearance	Late	Early	Early	Late	Early
No. of lobes	One	One or two	One	One or rarely two	Usually two

by podophyllin (Fig. 1 C, H), whereas in the presence of griseofulvin the 'long sides' sometimes appear more curved, tending to give some paracrystals a 'barrel-shaped' cross-section (Fig. 1 D). The shape is most radically different in those paracrystals grown in the presence of colchicine (Fig. 1 B, G) or IPC (Fig. 1 E, K), where they take on a shape intermediate between an oval and a lozenge. Frequently such paracrystals have 2 quite distinct lobes when grown in either of these 2 drugs. Since the overall shapes of the paracrystals appear so different in the presence of different mitotic inhibitors it was considered worthwhile to characterize their ultrastructure in the hope of obtaining information about the location of the various drug-binding sites on the tubulin subunits.

Electron microscopy

VLB-induced paracrystals appear very different at the ultrastructural level, depending on the plane of section etc. (Starling & Burns, 1975). Two very characteristic patterns appear (Starling, 1976; Starling & Burns, 1975) at these VLB concentrations. The first of these consists of an array of hexagons and triangles (Fig. 2 A) forming a star pattern. Perpendicular to the plane in which this pattern is observed the paracrystals appear as a regular array of electron-dense beads, arranged in straight rows, staggered with respect to their neighbours and interconnected by less electron-dense material (Fig. 2 B). A search was made in sections of each type of paracrystal for both the above patterns. The results are shown in Fig. 2. All 5 types of paracrystal exhibit both patterns. Measurements of characteristic spacings (Starling & Burns, 1975) show that at the ultrastructural level the packing arrangement of paracrystals is similar in the presence of all the mitotic inhibitors used in this study. Explanation of the variability in shape of paracrystals at the light-microscope level is therefore at least as likely to come from differential inhibition (or enhancement), of growth rates at paracrystal faces as from radical alostearic changes of the tubulin subunit, by the binding of an additional mitoclasic agent. However, it is interesting that, when VLB concentrations are standardized, even the addition of the above-mentioned mitotic inhibitors does not cause detectable alterations to the clearly defined ultrastructural arrangement, though paracrystal ultrastructure can be varied by altering the effective concentration of VLB alone (Starling, 1976).

DISCUSSION

Sea-urchin eggs are known to contain a pool of tubulin (Raff, Greenhouse, Gross & Gross, 1971), which under normal conditions does not polymerize to form microtubular structures in the unfertilized egg. It is therefore interesting that addition of the 'mitoclasic agent' vinblastine sulphate causes extensive polymerization of tubulin into structures not unlike aggregates of microtubules (Starling, 1976) and, under slightly different conditions, into structures which are unique to the aggregation of the tubulin–VLB complex (Starling & Burns, 1975). In addition it has now been shown that the formation *in vivo* of tubulin paracrystals, induced by VLB in sea-urchin eggs, is not inhibited by the presence of other mitoclasic agents. Moreover,

paracrystal formation is invariably enhanced, as judged by rate of formation or number of paracrystals per egg, or final size, when an additional mitotic inhibitor is applied. In some cases induction of more than one of the above effects takes place simultaneously in the presence of only one additional drug.

The above observations would seem to suggest that there is no direct competition for the VLB-binding site on the tubulin molecule from the other drugs. Nor would there appear to be a major degree of stearic hindrance. These observations are consistent with biochemical data that neither colchicine nor podophyllotoxin (Bryan, 1972*b*; Wilson, 1970) competes for the vinblastine-binding site on the unpolymerized tubulin subunit. This is all the more interesting in view of the observation (unpublished results) that each drug, when used alone, interferes with the *in vitro* assembly of microtubules from mammalian brain.

It is not possible to make precise deductions about the mechanisms of action of powerful drugs like the ones used in this study when applied to complex organisms like the sea-urchin egg, particularly in view of the known multiple effects of some of them. However, in view of the information about tubulin-drug interactions (briefly reviewed above) available from *in vitro* experiments on mammalian brain, it is possibly useful to make some very tentative suggestions about their interactions. The possibility of indirect action of any or all of these agents cannot be excluded until such time as suitable tubulin paracrystals can be formed *in vitro*.

If the drugs do act directly on the tubulin subunits, it is possible that none of the normal tubulin polymerization sites for microtubule formation are used in the formation of paracrystals, which may be brought about, for example, by charge effects due to the binding of the vinblastine ion. Enhancement of paracrystal formation by combining VLB with another mitotic inhibitor might suggest that the tubulin molecule is 'prepared' for aggregation in some way. For example, colchicine, which has been reported to bind reversibly to the tubulin dimer (Bryan, 1972*b*; Wilson, 1970) may shift the balance sufficiently between tubulin monomers and dimers in the pool to allow enhanced aggregation. A roughly analogous argument might be advanced in the case of IPC though the failure of this drug to bind to tubulin (Bouck & Green, 1974; Coss *et al.* 1974) makes this an unlikely mechanism. Alternatively, since the number of paracrystals formed is increased by the presence of IPC, this drug may have an effect on the actual seeding process of the paracrystal. An increase in the number of poles of a newly forming mitotic apparatus after IPC treatment has recently been demonstrated (Coss & Pickett-Heaps, 1974). Podophyllin also dramatically increased the number of paracrystals formed. From the increase in the number of eggs with paracrystals in the presence of any combination of VLB and another drug, over those treated with vinblastine alone, it is clear that mitoclasic agents shift the very delicate balance (Starling, 1976) between tubulin and vinblastine sulphate which allows paracrystal formation. In addition, the number of microtubule-organizing centres may be affected.

With the possible exception of eggs treated with a combination of vinblastine sulphate and IPC, which have more large crystals per egg than any other preparation, it is unlikely that all the tubulin in the pool of any egg is 'converted' into a form

suitable for incorporation into paracrystals. It is therefore possible that tubulin in the pool of a sea-urchin egg exists in more than one form; that the forms are interconvertible to some extent and that this extent is affected by the presence of mitotic inhibitors. Thus tubulin may be activated by some simple mechanism but further conditions must be fulfilled before polymerization takes place. For example the relative concentration of tubulin in a particular state may be crucial, or a specialized seed may be required.

The bilobed appearance of paracrystals formed in the presence of colchicine and IPC may be allied to the crystallographic phenomenon of 'twinning' which is thought to arise as a result of either the seeding of the crystal or differential growth rates during its development. The fact that paracrystals formed in different drugs look different in the light microscope, but remarkably similar at the electron-microscope level can be explained on the basis that the binding of an additional mitoclasic agent to the tubulin subunit alters either the overall charge distribution of the tubulin subunits, or causes a slight alostearic change. Either of these minor changes might tend to alter differentially the acretion rate of subunits on to the various faces of the forming paracrystal without bringing about a detectable change in paracrystalline ultrastructure, which might result from a more radical change in subunit shape. Any differences detectable in Fig. 2 between hexagonal or beaded views are probably due to variations of rotation and tilt of the goniometer stage. All differences between paracrystals prepared with different drugs correspond closely to variations observed when rotating and tilting paracrystals prepared in VLB alone (Starling & Burns, 1975). It may be that with better resolution subtle differences would become apparent Ultimately, if paracrystal sizes could be increased sufficiently, X-ray diffraction might be used to determine the tubulin structure and the drug-binding sites. It is already clear, however, that the use of mitoclasic agents as chemical probes may provide useful information about the processes of tubulin polymerization.

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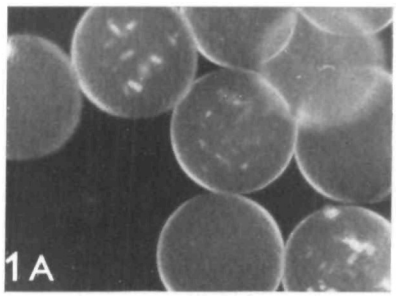
Fig. 1. Sea-urchin eggs incubated in vinblastine sulphate: A, alone; B, plus colchicine; C, plus podophyllin; D, plus griseofulvin; and E, plus IPC. × 160. Paracrystal prepared in vinblastine sulphate: F, alone; G, plus colchicine; H, plus podophyllin; J, plus griseofulvin; and K, plus IPC. × 800.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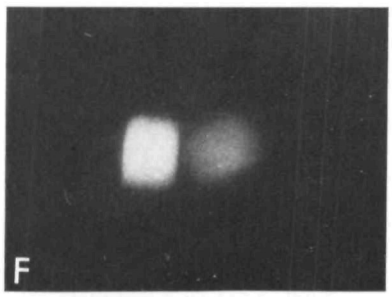
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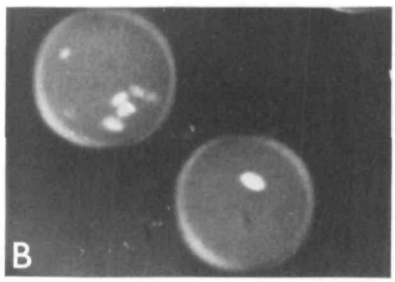
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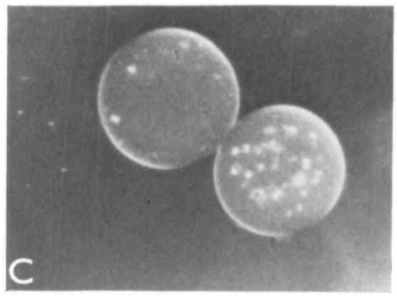
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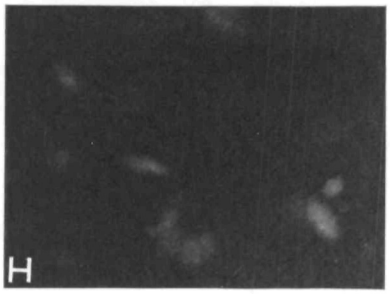
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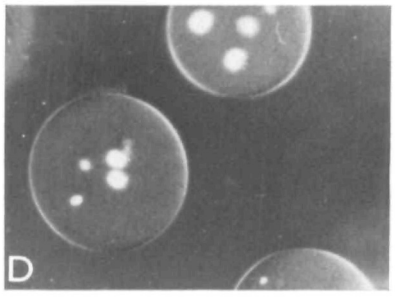
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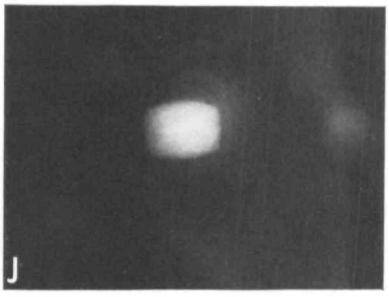
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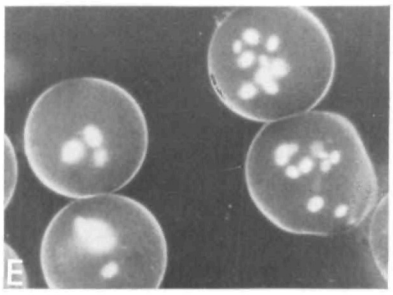
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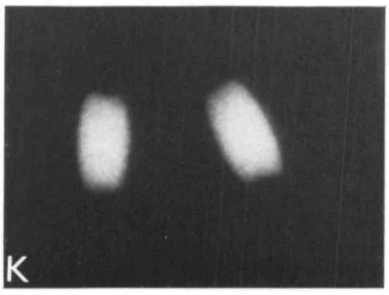
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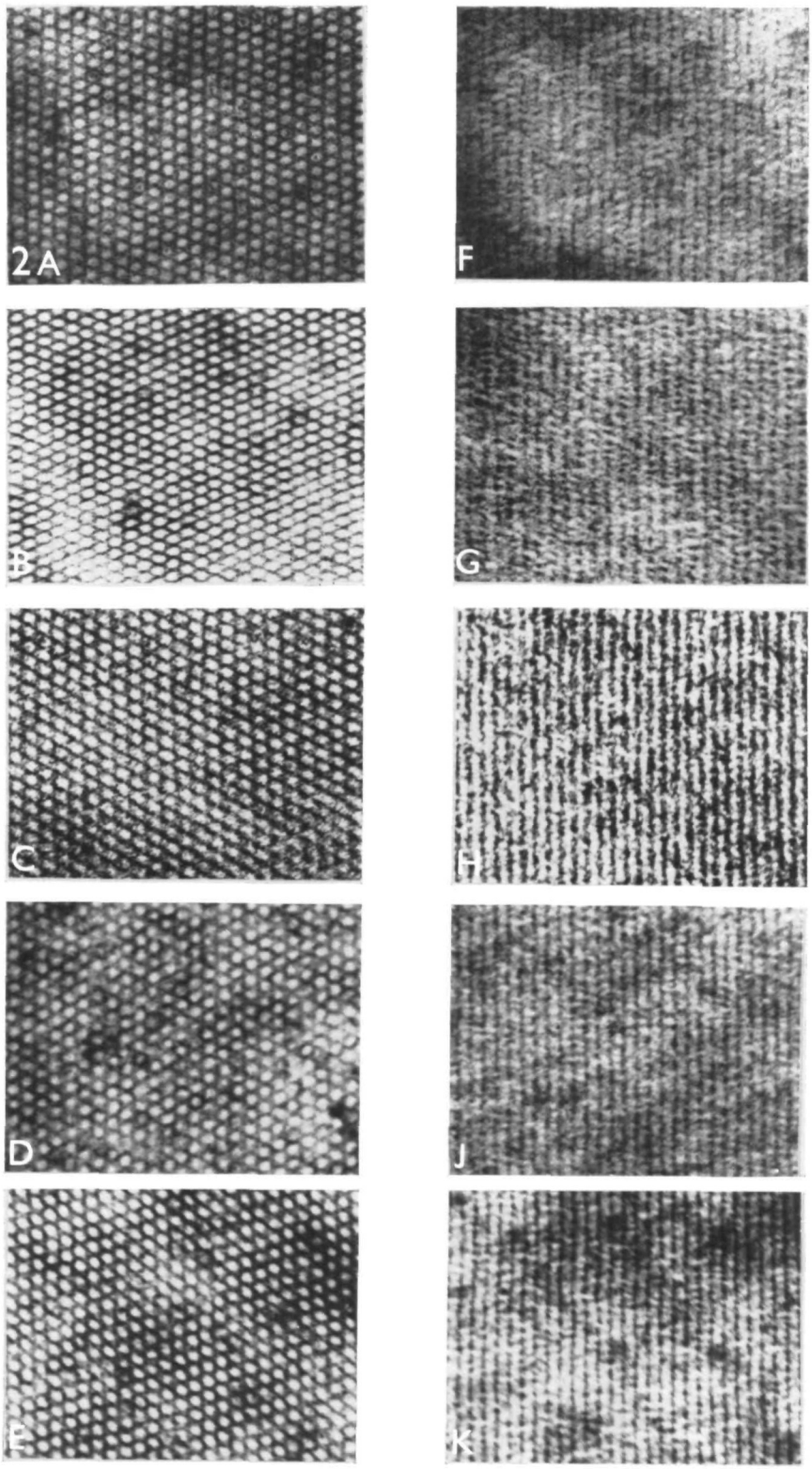

Fig. 2. Star patterns from paracrystals prepared in vinblastine sulphate: A, alone; B, plus colchicine; C, plus podophyllin; D, plus griseofulvin; and E, plus IPC. ×90000. Beaded patterns from paracrystals prepared in vinblastine sulphate: F, alone; G, plus colchicine; H, plus podophyllin; J, plus griseofulvin; and K, plus IPC. ×90000.