

A Comparative Study of Microtubules of Disk-Shaped Blood Cells

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Experiments were performed on the disk-shaped nucleated erythrocytes of the frog and the chick, chick thrombocytes, and blood platelets of rat and man to test (a) whether the microtubules of the marginal bundles showed a similar response to different treatments and (b) whether there was any correlation between the integrity of the marginal bundle and cell shape.

After brief storage at 0°C, microtubules disappeared from chick erythrocytes and platelets while some microtubules were still present in chick thrombocytes; frog microtubules looked normal. After cooling for 24 hours, frog microtubules were still present; chick thrombocyte microtubules had disappeared. Some microtubules had reformed in the cold in chick erythrocytes, but no reformation had taken place in chick thrombocytes or in platelets.

Microtubules disappeared from platelets after treatment with Colcemid, while they looked normal in frog and chick cells. Blocking of sulfhydryl groups with *N*-ethylmaleimide caused the disappearance of microtubules from platelets and thrombocytes, but not from chick erythrocytes. *Vinca* alkaloids caused the disappearance of microtubules from chick cells and platelets (frog cells not tested). It is suggested that the differences in response may be due to differences in chemical composition of the microtubules from the different cell types.

When marginal bundles were lost, platelets and chick thrombocytes lost their normal disk shape. Chick erythrocytes remained disk-shaped despite the disappearance of microtubules. It is concluded that a marginal bundle of microtubules cannot be considered a main factor responsible for maintenance of cell shape in all cell types where such a structure is found.

At the beginning of this century, Meves (29) described a structure in the periphery of nucleated, disk-shaped red blood cells which he called "der Randreifen." Meves concluded that "es ist erhebt über jeden Zweifel dass wir in dem Randreifen ein festes und elastisches Gebilde vor uns haben, und das der Randreifen es ist, welcher die Form der roten Blutkörperchen bedingt." Fawcett (15, 16) showed that *der Randreifen* consists of a bundle of microtubules, encircling the cell immediately beneath the plasma membrane, and since then marginal bundles of microtubules

have been observed in nucleated erythrocytes of fishes, amphibians, reptiles, and birds (see, for example, 1, 3, 17, 26, 27, 44). Mammalian blood platelets are also disk-shaped and also possess a marginal bundle of microtubules (4, 21, 35, 38, 39, 40).

Since all these various cell types are nonspherical, most authors have agreed with Meves and have assumed that the marginal bundles maintained the disk shape of the cells.

If indeed the bundle of microtubules is responsible for shape maintenance, then these cells ought to alter their shape when subjected to treatments that cause disassembly of microtubules or disorganization of the marginal bundle. Also, such changed cells should exhibit a marginal bundle of microtubules when they regain their disk shape.

The present paper gives comparative observations on microtubules of frog erythrocytes, chick erythrocytes, chick thrombocytes, and blood platelets of rat and man. Evidence will be presented that the microtubules of these cells respond differently to various physical and chemical treatments, and that chick erythrocytes remain disk-shaped even though microtubules are not present.

MATERIALS AND METHODS

Siliconed glassware was used throughout.

Rat blood was obtained from anesthetized animals by puncture of the inferior caval vein; human blood was obtained from the antecubital vein; chick blood was obtained from the wing vein (*vena cutanea ulnaris*).

Either 3.8% sodium citrate or ACD solution, USP formula A, were used as anticoagulants; 1 ml of anticoagulants was added to 9 ml of blood. Anticoagulants were warmed to 37°C before being mixed with blood. Rat and human blood was centrifuged at 300 g for 10–15 minutes, and the supernatant was platelet-rich plasma (PRP). The centrifuge was warmed at 37°C to keep the blood at body temperature.

One-milliliter samples of PRP were used in the experiments.

Citrated chick blood was centrifuged for 15 minutes at 150 g, and the supernatant was a mixture of red cells and thrombocytes; 1-ml samples of this supernatant were used in the experiments, though in a few experiments whole citrated chick blood was used.

Frog red blood cells, obtained by cardiac puncture of decapitated frogs, were suspended in modified frog-Ringer solution (11), and this suspension was used in the experiments.

Experiments

1. Cold: (A) 1-ml samples were kept at 0°C for 1 hour and then fixed; (B) 1 ml samples were maintained at 4°C for 24 hours, and then fixed. Experiments with temperatures below 0° have not been done.
2. Rewarming: the samples were cooled as above, rewarmed to body temperature for 1 hour, and then fixed.

TABLE I
EFFECTS OF COLD TREATMENT AND REWARMING

Cell	Effect of 1 Hour Cold Treatment		Rewarming	
	Microtubules	Cell Shape	Microtubules	Cell Shape
Frog erythrocytes	Present in marginal bundle	Disks	—	—
Chick erythrocytes	Not present	Disks	Present	Disks
Chick thrombocytes	Present. Some cells had bundles, some showed random arrangement of microtubules	Irregular	Present; some cells had bundles	Irregular and disks
Rat blood platelets	Not present	Irregular and spheres	Present; arranged in bundles in majority of cells	Disks and spindle shaped (see text)
Human blood platelets	Not present	Irregular and spheres	Present; arranged in bundles in majority of cells	Disks and spindle-shaped (see text)

3. Chemicals: Chemicals were dissolved in 0.9% saline, and 0.1 ml of the solutions were added to 1 ml of the blood samples to obtain the desired final concentrations. Human, rat, and chick cells were incubated at 37°C, frog cells at room temperature. Immediately after treatment the samples were fixed. (A) Colcemid was at a final concentration of 1 mM, and cells were incubated for 1 hour. (B) *N*-Ethylmaleimide (NEM) (Sigma) was at a final concentration of 3 mM, and cells were incubated for 1 hour. (C) Vincristine sulfate (Oncovin, Lilly) or Vinblastine sulfate (Velbe, Lilly) were at final concentrations of 4×10^{-4} M, and cells were incubated for 1–4 hours.

Fixation

Fixatives were used at room temperature. Four milliliters of a 4% glutaraldehyde solution in 0.1 M cacodylate buffer at pH 6.5 was added to each 1-ml sample; after 10 minutes the cells were sedimented, resuspended in fixative, fixed therein for an additional half hour, resedimented, and then postfixed for 1 hour in 1% OsO₄ in 0.1 M cacodylate buffer at pH 6.5–7. After dehydration cells were embedded in Epon. Sections were stained with uranyl acetate and lead citrate.

OBSERVATIONS

General remarks

The cells used in this study normally are disk-shaped and have smooth contours (see Figs. 5 and 8). In the following, the term irregular denotes cells that have an irregular outline due to extension of cytoplasmic processes.

TABLE II
EFFECTS OF COLD TREATMENT FOR 24 HOURS

Cell	Microtubules	Cell Shape
Frog erythrocytes	Present	Majority disks, but some cells had bizarre shapes (see text)
Chick erythrocytes	Present	Disks
Chick thrombocytes	Not present	Spheres and irregular
Human blood platelets	Not present	Spheres and irregular

All cells possess microtubules arranged in a marginal bundle encircling the cells at their greatest circumference immediately beneath the plasma membrane. The approximate number of microtubules in the marginal bundle of the various types is as follows: frog erythrocytes, 20–30; chick erythrocytes, 10–25; chick thrombocytes, 20–35; rat and human blood platelets 5–20.

Temperature experiments

The observations on temperature-treated cells are summarized in Tables I and II.

Short cold treatment

Microtubules. After 1 hour at 0°C, the microtubules of frog erythrocytes looked normal (Fig. 1); microtubules were not found either in chick erythrocytes or in mammalian blood platelets. In chick thrombocytes microtubules were still present. In some sections of thrombocytes, bundles of microtubules were seen in transverse as well as in longitudinal or oblique section in one and the same cell; a likely explanation of this observation is that the marginal bundle of these cells was distorted but otherwise intact. In some thrombocytes only a few microtubules were present, arranged randomly in the cytoplasm.

Cell shape. Frog erythrocytes were disk-shaped, and so were chick erythrocytes, despite the absence of marginal bundle microtubules in the latter. Chick thrombocytes were irregular, as were most mammalian platelets, but some platelets were spheres with a smooth outline.

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- FIG. 1. Marginal bundle of microtubules in frog erythrocyte kept at 0°C for 1 hour. $\times 100,000$.
 FIG. 2. Marginal bundle of microtubules in frog erythrocyte treated with Colcemid for 1 hour. $\times 100,000$.
 FIG. 3. Marginal bundle of microtubules in chick erythrocyte maintained at 4°C for 24 hours. $\times 80,000$.
 FIG. 4. Marginal bundle of microtubules in chick erythrocyte treated with *N*-ethylmaleimide. $\times 80,000$.

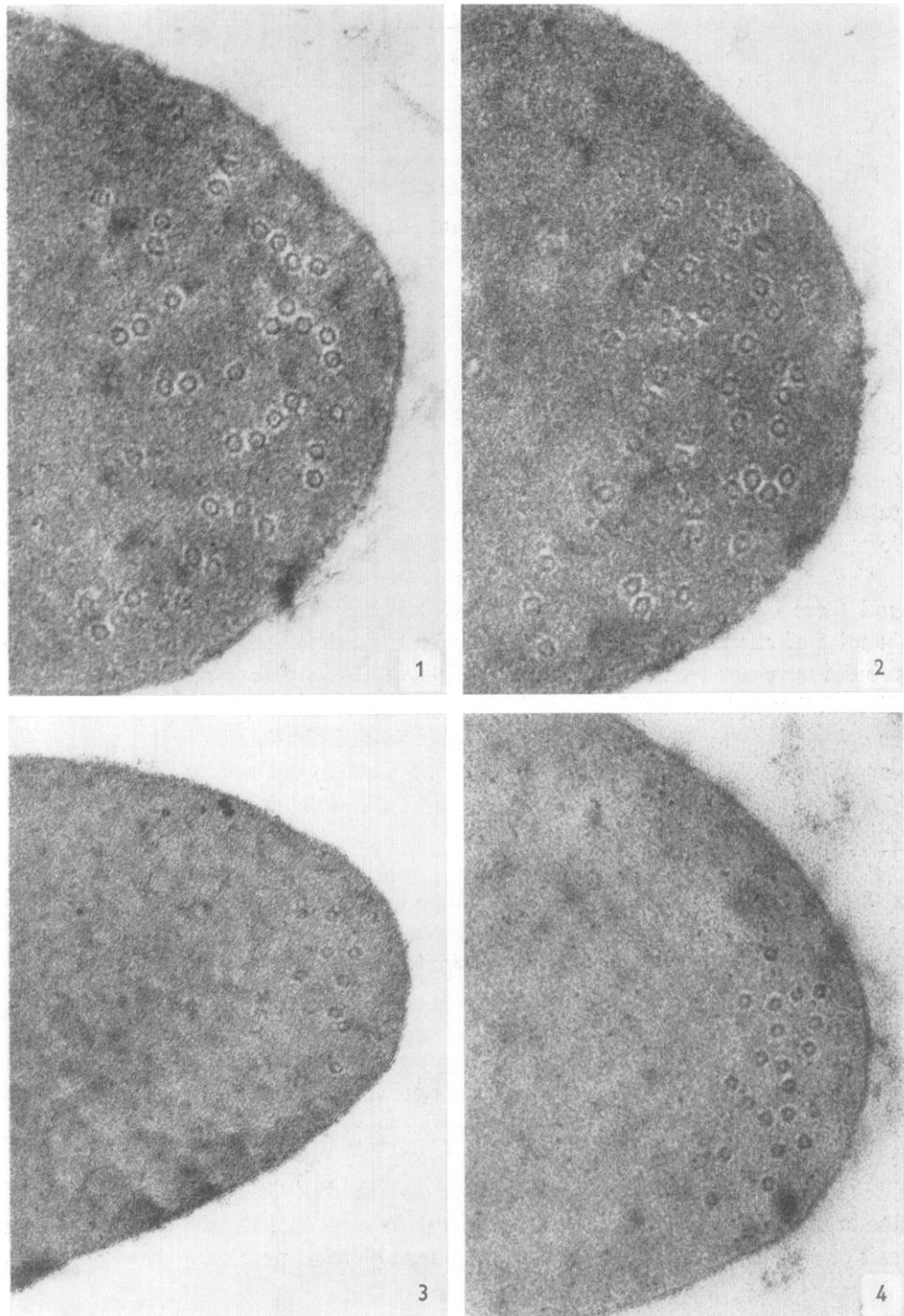


TABLE III
EFFECTS OF COLCEMID

Cell	Microtubules	Cell Shape
Frog erythrocytes	Present in marginal bundle	Disks
Chick erythrocytes	Present in marginal bundle	Disks
Chick thrombocytes	Present in marginal bundle	Disks
Rat blood platelets	Not present	Spheres
Human blood platelets	Not present	Spheres

Rewarming after short cold treatment

Microtubules. After cells were rewarmed for 1 hour, microtubules reappeared in chick erythrocytes *in situ*, that is, at the margins of the disk-shaped cells, and microtubules were not seen in other parts of the cytoplasm. In mammalian platelets microtubules initially reappeared randomly in the cytoplasm (5), but after 1 hour the reformed microtubules were arranged in marginal bundles in most platelets.

Cell shape. After rewarming for 1 hour, some chick thrombocytes were discoid, and these cells had marginal bundles. Others were still irregular with distorted bundles of microtubules. Thrombocytes with but a few randomly arranged microtubules were not seen. Mammalian platelets with reformed marginal bundles were discoid. Some platelets did not regain their natural disk shape, however, but appeared as spindles, up to 8 μ in length. The spindle-shaped platelets had microtubules arranged parallel to the long axis of the cell. Spindles were seen more frequently in the experiments with rat platelets than in those with human material.

Cold treatment for 24 hours

Microtubules. After 24 hours at 4°C, microtubules of frog erythrocytes were still present and were arranged in bundles. In some cells the marginal bundles showed loops and torsions, like the figure of 8, similar to the figures described after supravital staining in nucleated erythrocytes by Meves (29) and Fawcett and Witebsky (17).

In chick erythrocytes microtubules reappeared at the margins of the cells during prolonged storage in the cold (Fig. 3); some cells had only a few microtubules while others showed marginal bundles which looked normal. Microtubules were not present in chick thrombocytes and human platelets.

Cell shape. Most frog erythrocytes were disk shaped. Cells in which torsions of the bundle had occurred presented a variety of shapes as if twisted around the long axis of the cell. Chick thrombocytes and human platelets were spherical or irregular.

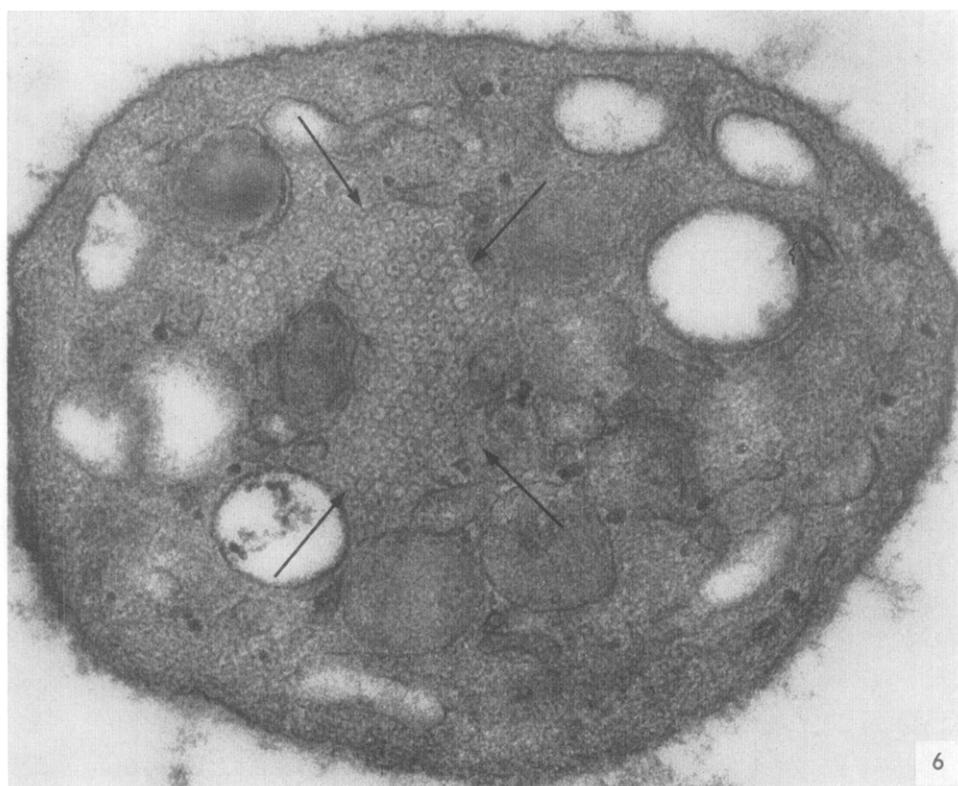
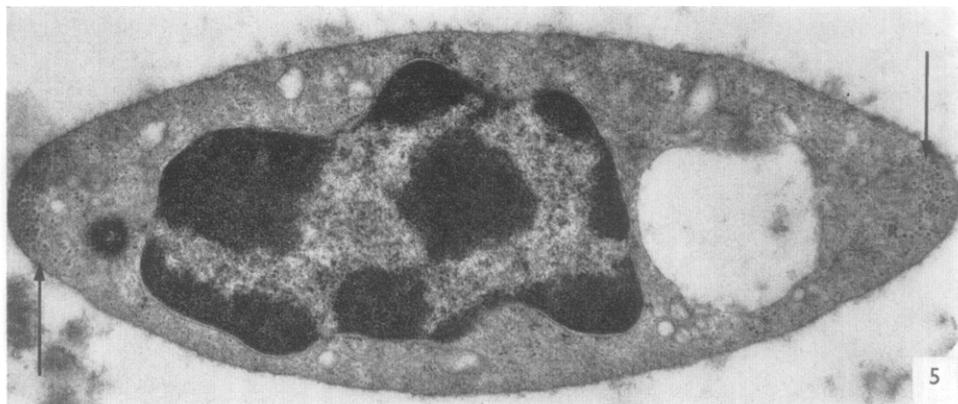


FIG. 5. Chick thrombocyte treated with Colcemid. The marginal bundle (arrows) looks normal, and the cell is disk-shaped. $\times 16,000$.

FIG. 6. Human blood platelet treated with Vincristine sulfate for 4 hours. Microtubules are not visible. A "microtubular crystal" is indicated by arrows. $\times 67,000$.

TABLE IV
N-ETHYLMALEIMIDE

Cell	Microtubules	Cell Shape
Chick erythrocytes	Looked normal; marginal bundles intact	Disks
Chick thrombocytes	Present but in reduced numbers; marginal bundles disorganized	Irregular
Human blood platelets	Not present	Irregular and spheres

Colcemid

Microtubules. After treatment with Colcemid the marginal bundle microtubules of frog (Fig. 2) and chick erythrocytes and chick thrombocytes (Fig. 5) looked normal, while microtubules were not present in platelets (Table III).

Cell shape. After treatment with Colcemid, the nucleated cells were disk shaped. The majority of platelets were spherical; in contrast to the experiments with cold, there were few irregular cells (Fig. 9).

N-Ethylmaleimide (NEM)

Experiments were performed only on chick erythrocytes, chick thrombocytes, and human platelets (Table IV).

Microtubules. After treatment with NEM, the microtubules of chick erythrocytes looked normal (Fig. 4). In chick thrombocytes marginal bundles were disorganized, but microtubules were still present, although in reduced numbers. Microtubules were not present in human platelets.

Cell shape. After treatment with NEM, chick erythrocytes were disk-shaped and the cytoplasm looked normal. Thrombocytes and platelets were swollen and irregular and the cytoplasmic matrix appeared extracted in both cell types. An abundance of crisscrossing filaments, approximately 50 Å in diameter, and patchy accumulations of a dense material, located preferentially at the plasma membranes, were predominant in the cytoplasm (Fig. 7).

Vinca alkaloids

Experiments were not performed on frog erythrocytes.

Microtubules. After treatment for 1 hour, Vincristine sulfate or Vinblastine sulfate caused the disappearance of microtubules from chick erythrocytes and thrombocytes and from rat and human platelets (Table V).

FIG. 7. Human blood platelet treated with *N*-ethylmaleimide for 1 hour. The cytoplasmic matrix is dominated by filaments. Microtubules are not present. $\times 50,000$.

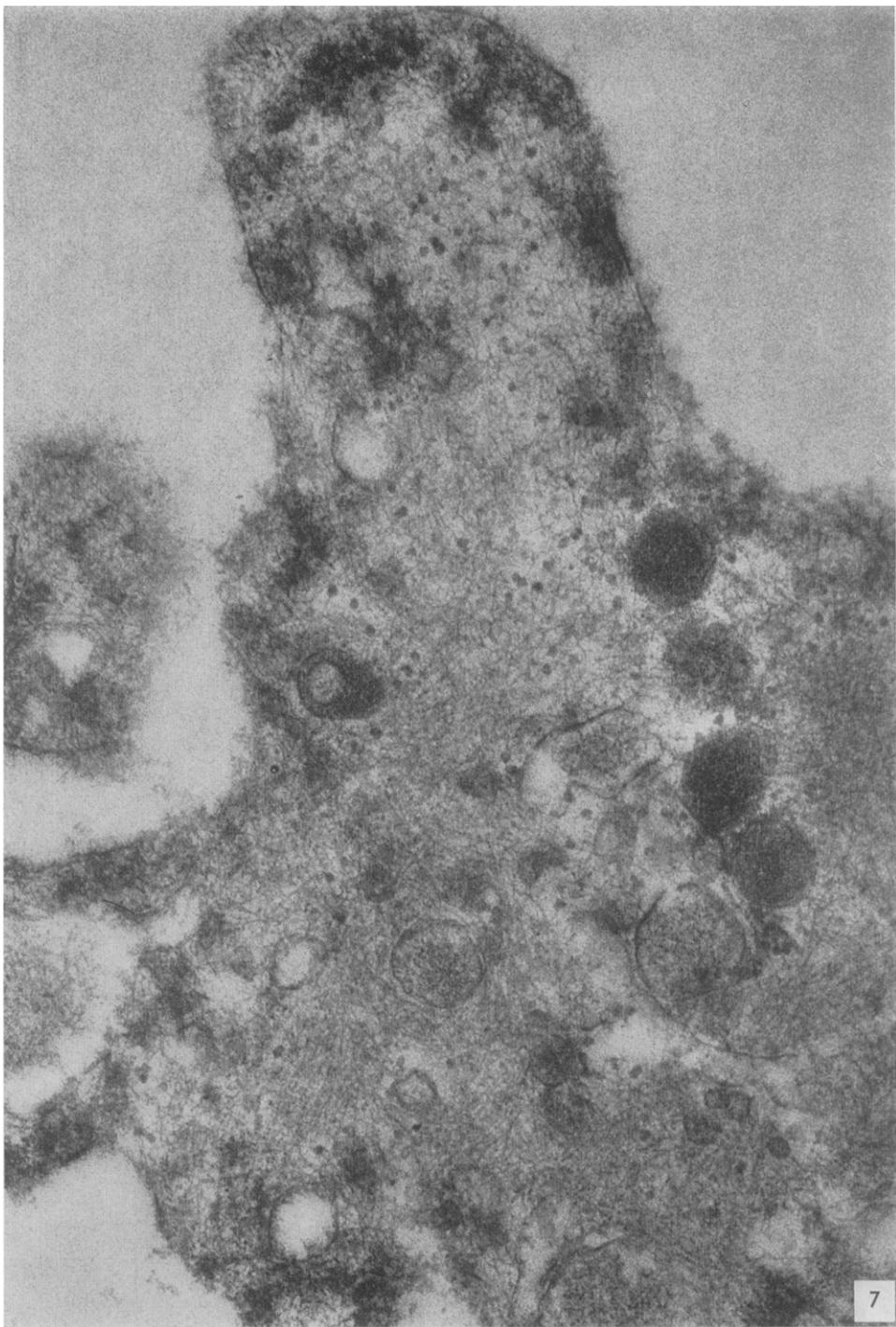


TABLE V
VINCA ALKALOIDS

Cell	Microtubules	Cell Shape	Crystals
Chick erythrocytes	Not present	Disks	Not observed
Chick thrombocytes	Not present	Irregular and spheres	Present
Rat blood platelets	Not present	Spheres	Present
Human blood platelets	Not present	Spheres	Present

Cell shape. After such treatment chick erythrocytes retained their disk shape; chick thrombocytes were irregular or spherical; platelets were predominantly spherical (Fig. 6).

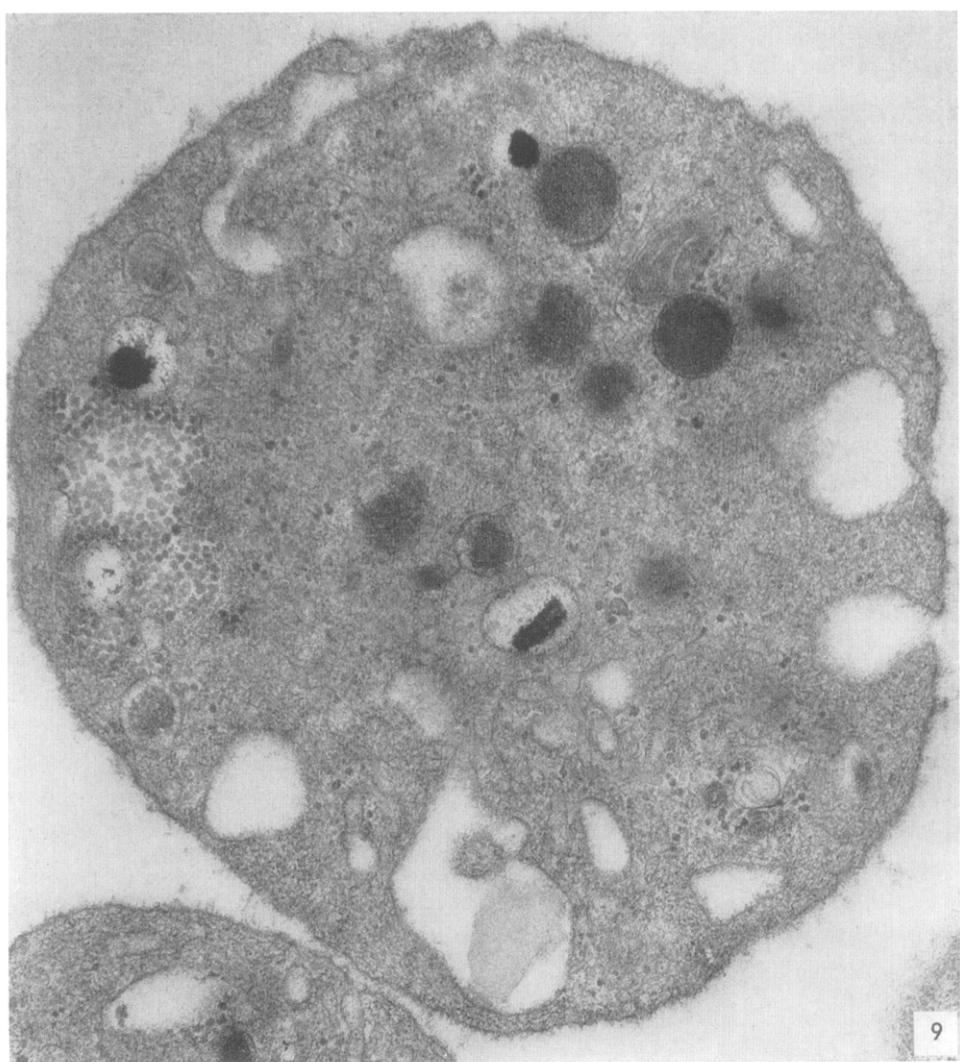
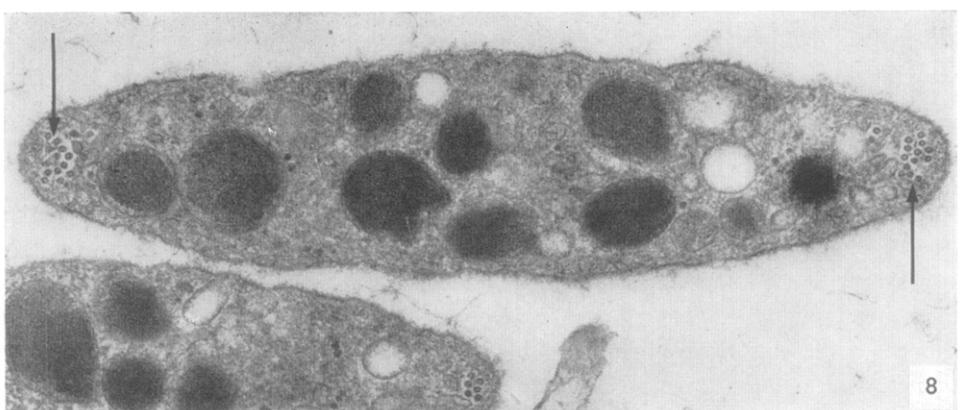
Crystalline inclusions. After 4 hours of incubation, crystalline inclusions were present in the cytoplasm of platelets (Fig. 6) and chick thrombocytes, but crystals were not visible in chick erythrocytes. The crystalline inclusions were morphologically identical to those described previously in platelets (45) and in other cells (7, 8) after treatment with *Vinca* alkaloids.

DISCUSSION

The data presented make it difficult to sustain the unifying hypothesis that a marginal bundle of microtubules is the main factor responsible for maintenance of cell shape in *all* cell types where such a structure is found. The observation that chick erythrocytes remained disk-shaped in spite of the disappearance of microtubules as morphologically identifiable structures argues against the interpretation of the marginal bundle as an important cytoskeletal structure in these cells. In platelets, however, the arrangement of microtubules appeared to be an important factor in determining cell shape: disk-shaped platelets always had marginal bundles, and when platelets became spindle-shaped after rewarming (Table I), their microtubules were arranged in the long axis of the spindle.

One could argue, of course, that the microtubules do not *determine* cell shape in platelets but that the disk or spindle shape is caused by as yet unidentified factors and that the arrangement of microtubules is determined by the shape of the cell. Though I find this unlikely, the objection cannot be overcome at present. Moreover,

FIG. 8. Normal human platelet. The marginal bundle of microtubules is indicated by arrows. $\times 35,000$.
FIG. 9. Human platelet treated with Colcemid for 1 hour. Microtubules are not visible, and the platelet is spherical. $\times 41,000$.



some evidence that other factors do in fact influence platelet shape comes from the experiments with cold and Colcemid: platelets were predominantly spheres after treatment with Colcemid whereas platelets were predominantly irregular after storage at 0°C.

The cytoplasmic microtubules of fully differentiated cells, the microtubules of the spindle of dividing cells, and the microtubules of the axoneme of cilia and flagella appear to be homologous structures as studied morphologically. However, microtubules in individual cells respond differently to various chemical agents and to changes in cellular environment (6, 12, 43) providing evidence that all microtubular elements are not the same. The marginal bundle microtubules of disk-shaped blood cells also showed differences in their response to experimental treatments. The microtubules of frog erythrocytes were unaffected by cold whereas the microtubules of the other cell types disappeared, but at different rates. Microtubules were still present in chick thrombocytes after 1 hour of cooling whereas they had disappeared from platelets. Furthermore, microtubules reappeared in chick erythrocytes during prolonged cold treatment, but there was no such reformation in chick thrombocytes or in mammalian platelets.

Whether the differences in response actually reflect differences in the microtubules themselves in the various cell types or reflect different cellular responses cannot be decided on the present evidence alone. If cold affects microtubules directly, then 3 kinds of microtubules must be assumed to exist in these cells: (a) stable microtubules, (b) very labile microtubules, and (c) moderately labile microtubules. Furthermore it seems, from the experiments with chick erythrocytes, that some labile microtubules can convert into a stable variety, and, if so, this would raise the question why such an adaptive conversion takes place only in chick erythrocytes, and not in thrombocytes or mammalian platelets?

Alternatively, if cold primarily affects some hypothetical "control system" in the cells, then we are forced to postulate 3 control systems and, moreover, that chick erythrocytes can switch from one control system to another.

It appears that the most simple interpretation to the observations presented is to assume chemical differences of the subunits of microtubules in the different cell types. Some support for this view comes from work on other systems of microtubules; it has been reported that the central pair of microtubules and the peripheral doublet microtubules in *isolated* ciliary and flagellar axonemes show differences in solubility (19, 32, 36) and that the A and B tubules of the outer doublets show some chemical differences (41).

N-Ethylmaleimide is a powerful and specific sulfhydryl inhibitor. Sulfhydryl groups have been demonstrated histochemically in areas of cells with a high density of cytoplasmic microtubules (2) and in the mitotic apparatus (24; see also review in

28), and the organic mercurial sulfhydryl inhibitors *p*-chloromercuribenzoate (34, 47) and Salyrgan (34) have been shown to dissolve the isolated mitotic apparatus. In mammalian platelets inhibition of sulfhydryl groups with NEM resulted in disappearance of microtubules whereas microtubules looked normal in chick erythrocytes. It cannot be concluded, however, that the microtubules of chick erythrocytes are insensitive to NEM, a possible interpretation of the lack of any visible effect on chick erythrocytes may be that NEM did not pass into the cells. The profound overall effect of NEM on platelet and thrombocyte morphology indicates serious cell damage by the compound, which is reflected also in its inhibition of various platelet functions *in vitro* (14, 20).

Alkaloids obtained from the periwinkle plant, *Vinca rosea* Linn., are inhibitors of mitosis (13, 18) and cause the disappearance of microtubules from the spindle of dividing cells (7), from the cytoplasm of human platelets (45), from fibroblasts and leukocytes (7, 8), and from mouse spinal ganglia neurons *in vitro* (23).

The characteristic crystals appearing in the cytoplasm of cells treated with *Vinca* alkaloids have recently been described by Bensch and Malawista (8). These authors consider the crystals as a sequestration of microtubule protein caused by changes induced in the protein by the alkaloids. If so, the absence of crystals from chick erythrocytes despite disappearance of microtubules might indicate that the microtubule protein in these cells is different from that of platelets and thrombocytes.

Colcemid caused the disappearance of platelet microtubules, but not of microtubules of nucleated cells. Colchicine binds to a protein subunit of both the central and peripheral microtubules of cilia and flagella (36, 37). It also binds to a protein subunit of the labile spindle microtubules (9) and to a protein subunit, probably of microtubule origin, in interphase cells (10). It remains unexplained, however, why spindle and some cytoplasmic microtubules disappear during colchicine treatment while those of cilia and flagella remain morphologically intact. On the other hand, colchicine inhibits the polymerization of flagellar microtubules after deflagellation in *Chlamydomonas* (30) and *Tetrahymena* (33), presumably by reacting with the microtubule precursor protein. Once the bonds between the subunits are established in the microtubules, however, they seem to be stable and unaffected by colchicine.

These conflicting data might tentatively be explained by assuming that colchicine never causes breakdown of already formed microtubules, but that it acts only by binding to a microtubule monomer protein. In the dividing cell Inoué and collaborators (e.g., 22) have proposed a pool of microtubule protein monomer in equilibrium with linearly aggregated polymers, the microtubules, and that this equilibrium is readily shifted. If colchicine acts by shifting the equilibrium toward the monomer by blocking the monomer, then depolymerization would be seen only in equilibrium systems. These would then include spindles of living cells, platelet marginal bundle

microtubules (46), axonemes in the arms of heliozoa (42), and cytoplasmic microtubules in a variety of other cells (6, 25, 31, 43). The axonemes of cilia and flagella would be nonequilibrium systems, and it is pertinent in this argumentation to note that flagellar regeneration (30, 33) is dependent on protein synthesis, any sizable pool of microtubule precursor protein does not seem to be present.

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