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MARGINAL BAND SYSTEMS IN BLOOD CELLS OF INVERTEBRATES

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MARGINAL BAND SYSTEMS IN BLOOD CELLS OF INVERTEBRATES

by

IRIS NEMHAUSER

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1981

Iris Nemhauser

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Abstract of the Dissertation

Marginal Band Systems In Blood Cells Of Invertebrates

by

Iris Nemhauser

Advisor: Professor William D. Cohen

Two major objectives directed the studies described in this dissertation. The first was an attempt at surveying a wide range of invertebrate species in order to determine whether or not such organisms contained in their flattened, elliptical blood cells microtubule marginal bands (MBs) similar to those observed in the erythrocytes of nonmammalian vertebrates and mammalian platelets. The second objective, dependent on the first, involved use of MB-containing invertebrate erythrocytes and clotting cells as experimental tools for elucidation of the structure, function and assembly mechanism(s) of MBs. Use of a modified microtubule polymerization medium containing Triton X-100 facilitated accomplishment of the first objective by providing a means for rapidly assaying the cells of various species for the presence of MBs. This was made possible by the clearing of respiratory pigment and other soluble cellular components from the cells in the lytic medium. Development of a technique for simultaneously lysing and fixing cells prior to embedment made possible the ultrastructural examination of lysed erythrocytes in which the relationships of MB-system components were apparently preserved.

Light microscopic examination of blood from a variety of invertebrates revealed the presence of MB-containing erythrocytes or clotting cells in four phyla comprising six classes. Experiments on the blood cells of selected species, using indirect immunofluorescence with antibulin antibody, demonstrated that these MBs contained tubulin.

MBs are bundles of microtubules circumscribing the cells beneath the plasma membrane in the plane of cell flattening. Light and electron microscopic observation revealed the MB (as in vertebrates) to be a component of a system comprised of the band and a filamentous network, the latter lying subjacent to the plasma membrane and enclosing the other cellular contents. In addition, under the light microscope, phase-dense dots were seen associated with the MBs of the erythrocytes examined.

Members of the Arcidae, or blood clams, were found to have MB-containing erythrocytes that were useful in exploring questions of MB assembly and function. The Arcidae MBs are cold-labile and the phase-dense dots in these species were observed, in thin sections, to be centrioles. Furthermore, cold-disassembled MBs of one of these clam species, *Noetia ponderosa* were observed, using phase-contrast and electron microscopy as well as indirect immunofluorescence, to reassemble in association with the centrioles when rewarmed. The cells of this species were therefore used in experiments designed to study the possible role of centrioles in MB assembly. Time course rewarming experiments on these erythrocytes demonstrated that during MB reassembly the microtubules grow from the vicinity of the centrioles. This is the first demonstration of centrioles acting as microtubule organizing centers (MTOCs) in MB formation. The findings suggest a model for MB assembly in the Arcidae. The model is supported by results obtained in colchicine experiments on *N. ponderosa* erythrocytes as well as by the discovery of naturally occurring aberrant MBs in one blood clam species. The proposed model also suggests the means whereby the nascent MB and the network might interact to generate the native flattened elliptical cell shape.

MBs of amebocytes, or clotting cells, of *Homarus americanus* were shown, using the indirect immunofluorescence technique, to undergo natural disassembly as the cells were morphologically transformed to the amoeboid state during activation. These findings thus provide evidence of a cytoskeletal function for MBs in these cells. It is suggested that invertebrate clotting cells, obtainable in quantity, might serve as useful models for the study of the possible role of MBs in various types of clotting cells including platelets.

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INTRODUCTION

I. Microtubule Systems

Although the existence of filamentous structures, or fibrils of 200-400Å diameter had previously been observed with the electron microscope in neural tissue (Rozsa et al., 1950; DeRobertis and Franchi, 1953) and 250Å wide "tubular elements" in mitotic spindles and "microtubules" (180Å in diameter) in interphase cells had been noted by Porter (1954) and Slautterback (1963) respectively, it was not until the development of glutaraldehyde fixatives that the ubiquity of these structures in eukaryotic cells was established (De-Thé, 1964). The presence of microtubules (MTs) in diverse cells and specialized organelles thereof has attracted the interest of many investigators, and led to studies elucidating their structure and multifarious functions.

In addition to their obviously motile function in cilia and flagella, demonstrated by Satir (1968) and Summers and Gibbons (1971), and their somewhat controversial role in mitotic and meiotic spindles (Inoué and Sato, 1967; McIntosh et al., 1969; Margolis et al., 1978), MTs have been credited with involvement in activities ranging from intracellular vesicle and granule translocation (Smith et al., 1975; Byers and Porter, 1977; Schliwa and Euteneuer, 1978; Howard and Aist, 1980) to development and maintenance of form or asymmetry in a wide variety of organisms and cell types. Examples are: neurulation (Karfunkel, 1971), developing neurites (Yamada et al., 1971) and muscle (Fischman, 1967), neutrophil orientation (Malech et al., 1977), formation and maintenance of heliozoan axopodia (Tilney, 1968) and shape formation and/or maintenance in nonmammalian vertebrate erythrocytes (Fawcett and Witebsky, 1964) and mammalian platelets (Behnke, 1967). Despite their widespread occurrence, the manner in which MTs exert their effect on cellular morphology has not been fully explained.

Two additional major questions arise with regard to MT systems in general. The first is the problem of spatial regulation of MT assembly, that is, identification of control mechanisms whereby cells determine the position and number of microtubules during the various phases of the cell cycle, and elucidation of the control mechanism itself. The second, and related problem, is that of temporal control; that is, mechanisms by which the timing of MT assembly and/or disassembly is regulated. With regard to the spatial control mechanisms higher plants have, associated with their mitotic and meiotic spindles, membrane-rich (Hepler, 1980) organelle free polar caps (McLaughlin, 1971), whose possible function as microtubule-organizing centers (MTOCs) is problematical. In other eukaryotes, the presence of specialized regions or organelles associated with mitotic and meiotic spindle poles has stimulated investigation into the possibility that these structures are MTOCs. The presumptive MTOCs are a morphologically heterogeneous group ultrastructurally, and their evolutionary relationship, if any, is unclear. Tilney (1971) demonstrated that a granular electron-dense mass, the centroplast, nucleates the assembly of axonemal MTs in the protozoan Raphidiophrys. Mitosis in the cellular slime mold, Polyspondylium violaceum, is characterized by the conversion of a disc-shaped, multilayered, electron-dense "nucleus-associated body" (NAB) with MTs protruding from granular "nodules," to an unstructured, ring-shaped spindle pole body (SPB). The latter serves as the focal point for astral and spindle MTs (Roos, 1975). In some fungi diverse organelles known collectively as spindle pole bodies (SPBs) have been observed in association with mitotic spindles. In the zygomycete, Pilobolus crystallinus, the intranuclear SPB is an electron-dense region intimately connected to an apparently specialized area of the nuclear membrane (Bland and Lunney, 1975). In the yeast, *Saccharomyces cerevisiae*, the SPB is comprised of a layered structure inserted into the nuclear membrane (Petersen and Ris, 1976), while in *Fusarium oxysporium* the extranuclear spindle pole body appears as a "fibro-granular region" (Aist and Williams, 1972) attached to the nuclear membrane. The ability of *S. cerevisiae* SPBs to nucleate MTs was demonstrated in lysed spheroplasts incubated in the presence of exogenous tubulin (Borisy *et al.*, 1975).

In the Basidiomycetes *Boletus rubinellus* (McLaughlin, 1971) and *Armillaria mellea* (Motta, 1969) extranuclear spherical electron-dense bodies, composed of granular and fibrillar material, are associated with the spindle poles of mitotic and meiotic cells. Other structures shown to be capable of spindle MT nucleation are the kinetochores of mitotic chromosomes (McGill and Brinkley, 1975; Telzer *et al.*, 1975). In many cell types, including those of metazoan animals, ciliary, flagellar, spindle and cytoplasmic MTs are frequently observed in proximity to cylindrical structures, 150µm x 500µm called "centrioles" (Amano, 1957; Szollosi, 1964; Taylor, 1965), or electron-dense amorphous "pericentriolar material" or "satellites" associated with the latter (Szollosi, 1964; Pickett-Heaps, 1969; De-Thé, 1964). Centrioles typically occur in orthogonally arranged pairs, located at or near the cell center during interphase. They display a nine-fold symmetry related to the nine microtubule triplets that comprise their walls. Early ultrastructural examination of animal cells fixed with glutaraldehyde demonstrated the "connection" of centriolar satellites with cytoplasmic MTs (De-Thé, 1964). Basal bodies, which are structurally very similar to centrioles (Dustin, 1978), have long been implicated as the organizers of ciliary and flagellar axonemes (Sotelo and Trojillo-Cenóz, 1958).

In some organisms the presence of centrioles may alternate, during various phases of the life cycle, with other types of presumptive MTOCs. In the slime mold Physarum polycephalum there are plasmodial anastral mitotic figures which appear to be organized by amorphous, electron-dense fibrous or granular structures (Sakai and Shigenaga, 1972; Tanaka, 1973) while the myxameobae have centrioles and astral mitoses (Tanaka, 1973). In the unicellular flagellate Ochromonas, basal bodies, which organize the flagella, coexist with the kineto-beak and rhizoplast. The latter structures were shown to nucleate the cytoplasmic MTs that confer upon the organism its anterior asymmetry and tail shape, respectively (Brown and Bouck, 1973). During the mitotic cycle, de novo formation of protocentrioles (electron-dense bodies containing the cartwheel structures seen in the proximal end of some centrioles), has been observed in the slime mold Labyrinthula, while in the same organism de novo centrioles are formed from protocentrioles during meiosis (Perkins, 1970).

There have been numerous reports of experimental data supporting the theory that centrioles, and/or their associated material, can be considered as MTOCs. Tilney and Goddard (1970) showed that cold-disassembled cytoplasmic MTs of ciliated ectodermal cells in Arbacia blastulae re-formed in contact with basal body-associated pericentriolar material. This study thus provided evidence not only for the ability of centrioles to function in nucleation of MT assembly, but also the capacity of the same organelle to act as both centriole and basal body. Additional evidence for the dual capability of centrioles and their associated material was obtained by Schliwa et al. (1979) in angelfish melanophores. They demonstrated that the amounts of pericentriolar material varied directly with MT numbers, which decrease during the aggregated and increase during the dispersed phases of pigment granule distribution. Furthermore, the presumptive MTOCs nucleated assembly of astral arrangements of MTs from exogenous tubulin in numbers dependent upon whether or not the cells had been in the dispersed or aggregated state at the time of lysis. Centrioles washed free of pericentriolar material effected the assembly of MTs in a pattern similar to that found in axonemes, that is, the microtubules grew out of the end of the centriole, which was thus behaving like a basal body. Similar results were obtained in Chinese hamster cells by Gould and Borisy (1977).

Weisenberg and Rosenfeld (1975) had previously demonstrated association of newly assembled MTs in artificially activated Spisula eggs with a dense cylinder that in later stages contained a centriole and pericentriolar material, the latter apparently becoming progressively dissipated as aster formation proceeded. Berns and Richardson (1977) showed a lack of interference with mitosis when centrioles were damaged with laser beam irradiation, while Berns et al. (1977) demonstrated that normal mitosis was disrupted when the pericentriolar cloud was destroyed. The latter study provided evidence that the pericentriolar material may be necessary for the assembly of interpolar spindle MTs.

The ability of isolated basal bodies to nucleate MTs has been reported by Snell et al. (1974) who assembled microtubules in vitro onto isolated basal bodies from Chlamydomonas. Heidemann and Kirschner (1975) injected basal bodies from the same source as well as from Tetrahymena into Xenopus unfertilized eggs, demonstrating the induction of aster formation by these organelles.

As a correlate to their function in determining the site of MT nucleation, centrioles are involved in the directionality of that assembly, a finding with profound implications for models of spindle function (Margolis and Wilson, 1978; Euteneuer and McIntosh, 1980). The intrinsic polarity of MTs has been demonstrated by Haimo et al. (1979) using in vitro dynein binding and by Heidemann and McIntosh (1980) who used an assembly medium that causes the formation on MTs of hooked appendages, with directionality related to MT polarity. The apparent polarity of MT assembly in vivo has been reported by Osborn and Weber (1976) in studies on 3T3 cells, while measurement of H^3-GTP incorporation and loss by Margolis and Wilson (1978) and kinetic studies of Bergen and Borisy (1980) demonstrated in vitro that assembly occurs preferentially at one (+) end of a growing MT while disassembly prevails at the opposite (-) end.

Dentler et al. (1974) showed the unidirectional assembly of chick brain tubulin onto chick brain neurotubule seeds. In studies in which flagella were used as seeds, the distal end of the MT was shown to be the (+), or more rapidly growing end (Binder and Rosenbaum, 1973; Binder et al., 1975; Bergen and Borisy, 1980). Furthermore, Bergen et al. (1980) concluded that centrosomal MTs grow at the ends distal to the centrioles. Heidemann et al. (1980) using the tubulin assembly technique that produces hooked appendages, likewise deduced that spindle MTs assemble by addition of subunits onto the ends of the MTs distal to the MTOC.

The lability of certain types of MTs, such as those comprising cytoplasmic and spindle microtubules, at low temperatures and in the presence of microtubule inhibitors like colchicine and vinblastine, has been an indispensable tool in experiments designed to test the conditions and controls of microtubule assembly. The lability of these MTs is attributed to their existence in dynamic equilibrium with cellular pools of unassembled tubulin dimers (Inoué and Sato, 1967). The work of Wilson and Friedkin (1967), and Weisenberg et al. (1968), demonstrated that colchicine binds tubulin dimers tightly, while Margolis and Wilson (1977) showed that the resulting "colchicine-tubulin complex" can block further assembly by binding to the (+) ends of MTs. Disassembly is presumed to occur in situations of dynamic equilibrium where the (+) ends of MTs are blocked and dimers continue to dissociate from the (-) end. The in vivo effect of inhibition by colchicine on MTs known not to be in a state of dynamic equilibrium, was demonstrated by Rosenbaum and Child (1967) in Tetrahymena. When intact, the flagella in these organisms are insensitive to the drug but do not re-form in its presence after amputation.

Thermodynamic studies show the assembly of MTs to be an endothermic, entropy driven reaction involving an increase in volume, thus accounting for the sensitivity of MT assemblies such as those in spindles to cold and high hydrostatic pressure (Inoué and Ritter, 1975). Roth (1967) used the characteristic of cold lability to demonstrate kinetochore nucleation of MTs in the amoeba Chaos carolinensis, and Tilney and Porter (1967) similarly demonstrated the requirement of microtubules for elongation and maintenance of heliozoan axopodia.

The second major problem in regard to MT assembly is that of temporal control. In view of the demonstrated requirement for low Ca^{2+} concentrations in in vitro MT assembly (Weisenberg, 1972), the possibility arises that the in vivo process might be dependent on temporal fluctuation of local Ca^{2+} concentration. Kiehart and Inoué (1975) showed by injection of 1 mM Ca^{2+} that Echinoderm egg spindle MTs could be caused to disassemble in the presence of elevated levels of the cation. They attributed the subsequent rapid recovery of spindle birefringence to a postulated ability of the cell to sequester the additional Ca^{2+}. Recently Hepler (1980), using a technique that specifically enhances staining of the nuclear envelope and endoplasmic reticulum, has shown the close proximity of these membranes to the mitotic apparatus of barley (Hordeum vulgare). The membranes were observed to be concentrated near the spindle poles and along the kinetochore microtubule regions where, according to at least one theory of chromosome movement, it is assumed that MT disassembly takes place during anaphase (Margolis et al., 1978). Parallels were therefore drawn between the similarity in appearance and possible function of these membranes to the sarcoplasmic reticulum of muscle.

Evidence has also been presented for the localization, by antimonate precipitation, of Ca^{2+} in the MA-associated membranes of a water fern and barley (Wick and Hepler, 1980). Similarly, Wolniak *et al.* (1980) have demonstrated the presence of membrane-bound calcium between chromosomes and poles in the mitotic apparatuses (MAs) of *Haemanthus* using the Ca^{2+} chelator chlorotetracycline as a fluorescent probe. Extraction of cellular membranes with Triton X-100 resulted in loss of MA-localized fluorescence. The requirement for membranes to buffer Ca^{2+} concentration in the vicinity of spindles was shown recently by Salmon and Segall (1980). Their membrane extracted, isolated spindles were unable to recover birefringence after disassembly in the presence of Ca^{2+}.

Finally, indirect immunofluorescence has revealed the presence of calmodulin in the chromosome to pole region of the MA (Welsh *et al.*, 1978), and the ability of this Ca^{2+}-binding regulatory protein to effect the disassembly of MTs, in a Ca^{2+}-dependent manner, has been shown *in vitro* (Marcum *et al.*, 1978). *In vivo* involvement of calmodulin in the regulation of MT assembly/disassembly has yet to be demonstrated.

II. Marginal Bands

The MT system with which this dissertation is concerned is the marginal band (MB), a structure found in the red blood cells of nonmammalian vertebrates and in mammalian platelets. MBs are continuous circumferential bundles of microtubules running beneath the plasma membrane in the equatorial plane of the flattened, elliptical, nucleated cells. They were originally described as filamentous structures in the erythrocytes of nonmammalian vertebrates such as chickens (Dehler, 1895) and amphibians (Meves, 1904). Their microtubular structure was noted by Fawcett (1959) during electron microscopic investigation of toadfish erythrocytes. Since then, MBs have been found in the red blood cells of all nonmammalian vertebrates examined. MBs were discovered in the erythrocytes of Amphibia (Salamandra maculosa; Trotter, 1956), Reptilia (Dipsosaurus dorsalis; Maser, 1963), Chondrichthyes [dogfish (Mustelus canis) and skate (Raja erinacea); Cohen et al., 1977] and Agnatha [hagfish (Eptatretus stoutii); Sekhon and Maxwell, 1970]. Erythrocytes of additional species within the above classes were subsequently shown to have MBs (Table III). In addition, the circulating discoid or elliptically shaped platelets of mammals (Haydon and Taylor, 1965; Sandborn et al., 1966; Behnke, 1970), and immature anucleate Camelidae erythrocytes (Jolly, 1920; Barclay, 1966; Goniakowska-Witalińska and Witaliński, 1977; Cohen and Terwilliger, 1978), as well as primitive erythrocytes (van Deurs and Behnke, 1965) and, possibly, definitive erythroblasts of mammals (Grasso, 1966; Repasky and Eckert, 1979) were reported to contain MBs.

Prior studies of MT structure and function have focussed on complicated systems such as spindles which are difficult to analyze experimentally because of the large numbers of MTs involved, in addition to chromosomes and other components. Similarly, flagella are structurally complex because of the association of various types of proteins such as dynein and nexin with the microtubules, and their relative stability (Dustin, 1978) further limits their experimental usefulness. Furthermore, the motility functions of both systems impose an additional order of complexity. MBs were thus chosen for study in the present work because it seemed that such relatively simple structures would lend themselves readily to experimental manipulation and structural analysis. In addition, since MBs are present, as noted above, in the primitive erythrocytes of mammals, as well as in platelets, they are of interest from a medical point of view. The "Cabot rings" seen in cases of severe anemia (Jordan et al., 1930) may possibly be marginal bands persisting in immature circulating red blood cells. With regard to comparative hematology, mammals, with their anucleate, MB-lacking erythrocytes are an exception among the vertebrates, a finding that is of great interest from an evolutionary point of view. Elucidation of MB function might shed light on the reasons for their disappearance from definitive mammalian red blood cells. Another reason for choosing MB-containing blood cells is their ready availability in large enough quantities for various kinds of experimentation.

III. Objectives and Initial Approach

Two major objectives were formulated with regard to the present work. The first concerned the question of whether or not MBs were present in blood (internal fluid) cells of invertebrates. The literature on invertebrate blood described the existence of cells containing respiratory pigments and/or clotting cells in various invertebrate types as primitive as the Nemertines, or proboscis worms (Andrew, 1965), but a search of the literature revealed virtually no modern information on these cell types. A subsequent search, after this work was underway, brought to light only two clear demonstrations of MBs in invertebrate blood cells, one in the hemocytes of the cockroach Periplaneta americana (Baerwald and Boush, 1970) and another in the hemocytes (erythrocytes) of the sea cucumber Cucumaria miniata (Fontaine and Lambert, 1972). There was no record of an attempt to determine whether or not MB-containing blood cells were widespread among invertebrates. Therefore, exploration of this area of ignorance appeared to be a worthwhile pursuit in its own right.

Other matters that could be addressed during a survey of invertebrate blood cell types involved questions concerning the possible presence of MBs in invertebrate clotting cells analogous to those seen in mammalian platelets, as well as whether or not there are MBs in erythrocytes containing respiratory pigments other than hemoglobin. One could also ask whether or not a connection could be found between MB presence and an organism's habitat, i.e., does possession of an MB confer osmotic resistance to the cells of an organism that is routinely subjected to osmotic stress? Determination of whether or not there was evidence for a correlation, in invertebrates, between MT number in the marginal band and cell size, as had been shown for vertebrates (Goniakowska-Witalińska and Witaliński, 1976) could confirm the role of the MB in shape maintenance or resilience.

The second major objective, dependent on the first, was that of attempting to find MB-containing cell types that might prove experimentally useful, with the intention of employing them to address basic questions about MB nucleation and assembly. Aside from descriptions of erythropoiesis, and demonstrations in some cell types of the cold-lability of MBs and their capacity to reassemble in mammalian platelets (Behnke, 1970), there was no literature on MB assembly, particularly where control mechanisms are concerned. Very little was known about the three-dimensional structure of MBs, i.e., whether or not they were composed of one or more MTs wound around the cell periphery numerous times, a number of microtubule hoops, or sections of tubules arranged in overlapping arrays, in such a manner as to give the impression of a continuous marginal band. Furthermore, the manner in which MBs exert their effect on blood cell morphogenesis was unknown. Although in vertebrate erythrocytes the MB was shown to be one component of a cytoskeletal system that also included a network (Cohen, 1978), there were no data concerning the possible interaction of the two in effecting the shape of the mature erythrocyte. There were thus numerous interesting problems that might be resolved through experimentation on appropriate MB-containing cells.

In approaching the first objective, it was possible to take advantage of a new method whereby blood samples can be rapidly assayed for the presence of MBs (Cohen, 1978). The technique involves lysis of the blood cells with Triton X-100 in a modified microtubule polymerization medium (Weisenberg, 1972), resulting in the clearing from the cell of soluble components such as respiratory pigment which otherwise obscure internal structures. Phase-contrast microscopy of such preparations reveals the presence of a phase-dense band (the MB), with shape and circumference corresponding to that of the intact cell. A nucleus and other organelles are present, and in favorable views of erythrocytes a transparent sheetlike structure can be observed enclosing all of the above, thereby presumably preventing disorganization of the "cytoskeleton" into its component parts. The latter material is revealed as a network by negative staining for transmission electron microscopy (Cohen, 1978), and is the network component of the cytoskeletal system referred to above. Utilizing this technique, supplemented by indirect immunofluorescence and transmission electron microscopy (TEM), the phylogenetic distribution of MBs in invertebrates has been explored as described in this dissertation ("Results", Parts I and II). The existence of MBs has been demonstrated in blood, or circulatory fluid cells (including clotting cells), of a wide range of invertebrate species representing four phyla. Preliminary reports (abstracts: Cohen et al., 1977, 1978; Cohen and Nemhauser, 1979; Nemhauser et al., 1979; Nemhauser and Cohen, 1980), and two papers on the findings (Nemhauser et al., 1980 and Cohen and Nemhauser, 1980) have been presented.

The species and cell types studied have also proven valuable for further work, particularly with respect to the more general problem of spatial and temporal regulation of in vivo microtubule assembly into MBs.

Parts II and III of the "Results" in the present work describe the association of centrioles with the erythrocyte marginal bands of species of clams belonging to the family Arcidae (Cohen and Nemhauser, 1980), and the ability of these cold-labile MBs to reassemble in association with the centrioles upon rewarming (Nemhauser and Cohen, 1980). Observations were made using phase-contrast light microscopy and indirect immunofluorescence, and by examination of whole mounts and thin sections under the transmission electron microscope. Results obtained with all three methods were in agreement as to the presumed role of centrioles in MB assembly, and have permitted formulation of a model for MB formation. The model is supported by reassembly experiments in the presence of colchicine ("Results" Part IV).

In addition, a case of naturally occurring MB disassembly has been discovered in the coelomocytes, or clotting cells of the lobster Homarus americanus ("Results", Part V). Loss of MBs in this case correlates with cell shape transformation, and thus these cells could serve as a model system for the study of marginal band function in platelets. Furthermore, the system may prove useful in investigating the effect of Ca^{2+} on the mechanism of MB disassembly.

MATERIALS AND METHODS

I. Biological Specimens

Specimens of the sipunculan worm Phascolopsis (syn. Golfingia; Gormley et al., 1978) gouldii, the horseshoe crab Limulus polyphemus, and the blood clams Anadara ovalis (the blood ark) and Anadara transversa (the transverse ark), were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and maintained in running sea water at MBL, or in "Instant Ocean" (I.O.) in aerated sea tanks (Aquarium Systems, Inc., Eastlake, Ohio) at Hunter College, New York. In the latter case "Marine Invertebrate Diet" and "Trace Elements" (Hawaiian Manne Imports, Inc., Houston, Texas) were added to the water at regular intervals. Some specimens of Limulus were purchased from Shark River Marine Laboratory, Wall, New Jersey. Also obtained from the MBL and maintained in running sea water were representatives of the following species: the lobster Homarus americanus, and the crabs Libinia emarginita, Cancer borealis, C. irroratus and Carcinus maenas. Freshwater crayfish were also kindly supplied by the MBL and maintained for a brief period of days in a fresh water tank.

The following species were purchased from Pacific Bio Marine, Venice, California, and kept as described above in a 15°C sea tank at Hunter College: The sipunculan Themiste, the echiuroid worm Urechis, and the brachiopod Laqueus. The echinoderms (Holothuroidea) Molpadia (Caudina) arenicola and Cucumaria miniata, also acquired from Pacific Bio Marine, were kept at the MBL in running sea water.

The ponderous ark Noetia ponderosa, the cut-ribbed ark Anadara lienosa floridana, the incongruous ark A. braziliana, A. ovalis (southern spp.), Anadara spp.[1], the clam Cardita aloeidana floridana, and the sipunculan peanut worm Dendrostomum alutaceum were bought from Gulf Specimen Co., Inc., Panacaea, Fla., and kept in sea tanks at 18°C.

II. Experimental Procedures

Table I contains a summary of the species investigated, procedures followed, and blood cell types examined for the presence of MBs.

A. Phase-Contrast Light Microscopy

1) Sipunculans: Phascolopsis gouldii and Themiste: A 1 ml tuberculin syringe with 22 G needle was used to puncture the body wall of the worm and remove coelomic fluid containing primarily erythrocytes as well as gametes and amoebocytes. The fluid was placed directly in vaseline wells on slides for immediate examination. For observation of marginal bands (MBs), one drop of the fluid was mixed with 0.5 ml of lysing medium (LyM), a modified microtubule polymerization medium consisting of 100 mM piperazine-N-N'-bis[2-ethane sulfonic acid] (PIPES), 1 mM $MgCl_2$, 5mM ethyleneglycol-bis-[β-aminoethyl ether] N,N'-tetraacetic acid (EGTA), 10 mM p-tosyl arginine methyl ester HCl (TAME) and 0.4% (wt/vol) Triton X-100, brought to pH 6.8 with KOH (Cohen, 1978). EGTA reduces the Ca^{2+} concentration to a level that does not cause MT depolymerization (Weisenberg, 1972) while TAME inhibits proteolysis (Rebhun et al., 1974). A modified LyM, containing 10 mM EGTA and a final pH of 6.2 (LyM, pH 6.2) was used in some experiments in order to further minimize loss of microtubules. To avoid confusion of MBs with sperm tails, only female worms were used in the lysing experiments.

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[1] Identified by Gulf Specimen Co. as a juvenile form of A. lienosa, but I have seen sperm in these specimens, therefore classify them separately.

TABLE I

Species Examined and Procedures Used[1]

Species	Light Microscopy Lysed Phase contrast	Light Microscopy Lysed Immunofluorescence	Transmission Electron Microscopy Intact Cells	Transmission Electron Microscopy Sectioned Lysed	Transmission Electron Microscopy Sectioned Lyse-Fixed	Transmission Electron Microscopy Whole Mounts Lysed		Cell Type[4]
Sipunculans								
Phascolopsis gouldii	+	+	+		+	+	(Nemhauser *et al.*, 1980)	e[3]
Themiste spp.	-							e
Dendrostomum alutaceum	-							e
Mollusca								
Anadara transveras	+	+			+	+	(Cohen & Nemhauser, 1980)	e[2]
A. ovalis	+							e
A. brasiliana	+							e
A. lienosa floridana	+							e
A. spp.	+							e
Noetia ponderosa	+	+			+	+		e
Cardita aloeidana floridana	-							l (Andrew, 1965)
Brachiopoda								
Laqueus spp.	-							c[3] (Meglitsch, 1967)
Echiuroidea								
Urechis spp.	-							h[2] (Meglitsch, 1967)
Arthropoda								
Limulus polyphemus	+			+				a (clotting cells)
Homarus americanus	+	+						c (clotting cells
Cancer borealis	+							a
C. irroratus	+							a
Carcinus maenas	+							a
Libinia emarginata	+							a
Echinodermata								
Cucumaria miniata	+							h[2]
Molpadia arenicola	+							h

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[1] + = MBs observed, - = cells were examined, but MBs were not detected, no symbol = cells not examined. [2] contain hemoglobin. [3] contain hemerythrin. [4] e=erythrocytes, l = lymphocytes, c = coelomic fluid cells, h = hemocytes, a = amebocytes.

2) Arthropods: Amebocytes were obtained from Limulus polyphemus by slitting a chitinous leg joint with a razor blade. The fluid containing cells was either placed directly on slides for observation or treated with propranolol to prevent transition of the cells to the amoeboid form [cells were collected into silicone-coated test tubes containing 2 mM propranolol in 3% NaCl ($0^{o}C$)] (Levin and Bang, 1964; Mürer et al., 1975). For MB observation, blood was obtained in the same manner and immediately diluted 1:10 with LyM. In later experiments, 2 drops of blood were mixed into 0.5 ml crustacean Ringer's solution (MBL Formulae and Methods VI, 1956, Woods Hole, Mass.) in order to slow down morphological transition and clumping of cells prior to lysis; immediately two drops of the latter were added to 1 ml LyM.

Lobster coelomocytes were obtained and treated in the same manner. In order to facilitate observations on whole, unlysed cells, the hemolymph was sometimes diluted into Ringer's solution containing 0.1% glutaraldehyde, thus preventing morphological transition. Amoebocytes from various crab species were similarly obtained and handled. Crayfish coelomocytes were diluted into crayfish Ringer's solution (MBL Formulae and Methods, VI) first and then treated like those of the other Arthropods.

3) Arcidae: Arks were opened using a clam knife. The blood, containing primarily hemoglobin-bearing erythrocytes in addition to amoebocytes and gametes, was gently removed with a Pasteur pipette from between the mantle and shell and placed directly on a slide for observation. Alternatively, to prevent erythrocyte shape change to a crenated form, in those species in which the transition was observed, the blood was diluted 1:10 or 1:20 into molluscan Ringer's solution (MBL Formulae and Methods VI) or I.O. containing 0.1% glutaraldehyde. Cells were lysed for MB observation by direct addition of blood to LyM as described above for P. gouldii. Blood from C.

aloeidana was handled similarly.

4) Holothurians: Coelomic fluid was obtained through the body wall, via a tuberculin syringe, from C. Miniata and M. arenicola and observed as described above for P. gouldii. Alternatively, an entire animal was slit with a razor blade and coelomic fluid was removed with a Pasteur pipette.

5) Miscellaneous: Blood was obtained from Urechis by the methods described for the sipunculans, and treated similarly. Body fluid was withdrawn from Laqueus by piercing the body or the heart with a Pasteur pipette and withdrawing yellowish fluid.

Specimens were mounted on glass slides and observed through coverslips. Observations on all of the above were made with a Zeiss phase-contrast microscope equipped with Planachromat (16x, 25x) and Neofluar (40x, 100x) objectives. Photographs were taken with Kodak Plus-X film.

B. Indirect Immunofluorescence

1) Phascolopsis gouldii: A modification of the method of Lazarides (1976) was used. Coelomic fluid was obtained from a female worm with a tuberculin syringe. Small drops were placed on individual coverslips and the cells were permitted to settle for 3 min. The coverslips were rinsed twice by gentle perfusion with 1M sucrose, then drained. LyM was introduced dropwise onto the coverslips, cell lysis proceeding for 10-15 sec. This step was followed by a rinse with Lym. Fixation was accomplished by dropwise addition of 3.5% formaldehyde (dissolved in LyM without Triton), allowing the coversip to remain covered with this solution for 3 min. The preparation was rinsed three times with deionized, distilled H_2O. This was followed by rinses in 1:1 acetone: H_2O, acetone, and 1:1 acetone:H_2O (all at 4°C). After three more H_2O rinses the coverslips were air dried.

The remainder of the procedure was performed in the laboratory of Dr.

R.D. Goldman (Carnegie Mellon Institute). Rabbit antitubulin antibody was applied to the coverslips which were then incubated for 15-60 min at 37°C. After several water rinses, the coverslips were similarly treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (IgG), rinsed, and mounted on slides over water. Slides were examined and photographed with a Zeiss photomicroscope equipped with epifluorescence illumination. Images were recorded on Kodak Plus-X film developed with Diafine.

2) Anadara transversa: One drop of blood was transferred via a Pasteur pipette to 0.5 ml LyM. Single drops of this mixture were then placed on individual coverslips in moist chambers consisting of 100 mm Petri dishes containing water-soaked filter papers lining the bottoms. The cells were allowed to settle for 10 min. The coverslips were then washed dropwise with LyM, followed by LyM without Triton X-100 and Tame (LyM No T&T). They were subsequently placed in 30 mm plastic petri dishes and enough fixative consisting of 3.7% formalin in LyM No T&T (1 volume 37% formaldehyde: 9 volumes LyM No T&T) was added to cover the preparations. After an approximately 20 min fixation, the coverslips were rinsed dropwise with LyM No T&T, drained with bibulous paper, and rinsed by gentle back and forth movement in three successive 30 ml beakers of phosphate buffered saline (PBS; 6 mM Na^+-K^+ phosphate buffer, 171 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ at pH 6.8-7.0). The coverslips were then drained and put back into the moist chambers. Twenty µl of antitubulin antibody (diluted 1:10 with PBS), a gift of Dr. R.D. Goldman, Carnegie Mellon Institute, were spread over the area occupied by the original drop. The preparation was incubated for 30 min at 37°C, rinsed in PBS and drained as above. This was followed by application of 20 µl of fluorescein isothiocynate- conjugated goat anti-rabbit IgG (diluted 1:20 with PBS; Miles-Yeda, Rehovoth, Israel), and incubation as described above. The

coverslips were again rinsed with PBS, and wet mounts were made by inverting them over PBS on slides and sealing with "valap," a mixture of equal parts vaseline, lanolin and paraffin (Allen et al., 1979). Photographs were taken with the above mentioned Zeiss photomicroscope or with a Zeiss phase-contrast microscope, both equipped with epifluorescence. Antibody specificity had been tested by the Protein A-I ^{135} procedure against α-and β-tubulin, double immunodiffusion analysis against brain tubulin that had been cycled three times in vitro, and by using preimmune serum as a control (R.D. Goldman, personal communication). Images were recorded on Kodak Plus-X film developed with Diafine or Microdol-X.

3) Homarus americanus: The following experiments were done in collaboration with W.D. Cohen: Controls: One drop of hemolymph obtained with a Pasteur pipette was diluted into 0.5 ml crustacean Ringer's solution. One drop of the resulting suspension was then immediately mixed with 0.5 ml LyM. A sample of the latter was spread on acid-washed coverslips and processed for indirect immunofluorescence as described above for A. transversa. Spreading coelomocytes: One drop of hemolymph was diluted into 1 ml crustacean Ringer's solution at t_0 min, and the resulting cell suspension allowed to settle onto coverslips in moist chambers for one min. Coverslips were then placed, cells up, in 25 ml Ringer's and transferred to fresh Ringer's at $t_{2.5}$ and t_4 min. This was done to wash away the contents of clotting granules that might interfere with, or obscure, antigen-antibody interactions. Cells on one coverslip were lysed at t_5 min by dribbling LyM onto them. This was followed by a rinse with LyM No T&T, and further processing as described for A. transversa. The cells on additional coverslips were lysed at t_{10} and t_{20} min respectively, and handled in the same manner.

4) Noetia ponderosa: Antitubulin antibody staining of the MBs in

this species was performed as described above for A. transversa with the following modifications: Antibodies and preimmune sera were kindly supplied by Dr. E. Wang (Rockefeller University, N.Y.). They included fluorescein-conjugated goat anti-rabbit IgG (Antibodies, Inc.). This antibody had been diluted from 20 mg/ml to 0.5 mg/ml with PBS and adsorbed with formaldehyde-fixed and Triton-extracted normal chick embryo fibroblasts (CEF) in order to minimize nonspecific staining. The supernatant, obtained after the cells were centrifuged out, was supplied to us. Preimmune rabbit serum and antitubulin antibody had been diluted 1:10 with PBS and were used without further dilution. All observations were made with the Zeiss phase-contrast microscope described above. Two drops of blood were lysed in 1 ml LyM, and all PBS rinses contained 100 ml buffer.

The results of the above experiment served as controls for the following MB disassembly/reassembly experiment: Using blood from the same clam, a series of test tubes was set up, each tube containing 1 drop of blood and 0.5 ml I.O. The tubes were kept at 0-2°C overnight (approximately 23 hours), at which time all were returned to room temperature. The contents of one tube were lysed immediately (t_0) by removal of the overlying I.O. from the settled cells and introduction of 0.5 ml LyM. The suspension (t_0) was examined with the phase-contrast microscope. After disappearance of the MBs was confirmed, single drops of the suspension were placed on each of two coverslips which were then treated as those described above. At subsequent intervals of 30, 60 and 120 min after the start of rewarming, cells were similarly lysed, and drops placed on 4 coversips in each case. These too were incubated with antibodies as described.

C. Effect of Colchicine on MBs of *Noetia ponderosa*

Presence of colchicine during cold-induced MB disassembly and subsequent reassembly; Effect on reassembly. I.O. (4.5 ml) was added to a 12 ml conical centrifuge tube and 4.5 ml of the same solution containing 0.1 mM colchicine was added to a second tube. Both tubes were kept on ice. After addition of 0.5 ml blood from an *N. ponderosa* specimen to each, the contents of both tubes were divided into pairs of ice cold test tubes and were kept at 0°C. After a 20 min incubation at 0°C, one pair of tubes was centrifuged in the cold in an IEC clinical centrifuge, at top speed for 15 sec. The supernatant was then removed and replaced with 0.5 ml LyM, pH 6.2, in which the cells were resuspended and lysed. The suspension was then inspected for the presence or absence of MBs under the phase-contrast microscope. The same procedure was repeated for a second pair after all remaining tubes had been at 0°C for one hour, at which time it was determined that there were no MBs present in the tubes that did not (NC) as well as those that did (C) contain colchicine. Subsequently, tube pairs were lysed after 1 hr in the cold, followed by an I.O. wash, and either 1 or 19.5 hours at ambient temperature. The contents of pairs of C and NC tubes were then inspected for the presence or absence of MBs, and if present, their relative appearance.

Cold-induced MB disassembly followed by reassembly in the presence of colchicine: A series of test tube pairs was set up, each containing 0.5 ml I.O. at 0°C and 1 drop of blood. After the tubes had been at 0°C for 2 hr, 0.06 ml of I.O. was added to one member of each pair of tubes (NC), and an equal volume of the salt solution containing 1 mM colchicine (C = approx. 0.1 mM colchicine) was added to the other member, and all tubes were rewarmed to ambient temperature. At rewarming intervals between 1 to 3 hr thereafter, small samples of settled cells were removed from the bottoms of tube pairs for microscopic examination of cell shape. This was followed by removal with a Pasteur pipette of as much as possible of the overlying I.O., its replacement with an equal volume of LyM, pH 6.2, and subsequent microscopic observation for the presence of MBs.

D. Electron Microscopy

1) Intact Erythrocytes: Phascolopsis gouldii. Coelomic fluid was removed with a Pasteur pipette from a worm that had been longitudinally slit open with a razor blade. To 0.5 ml of the fluid, containing erythrocytes (primarily), amoebocytes and gametes, in a 12 ml conical centrifuge tube, 0.1 M sodium phosphate buffer, pH 6.8, containing 1% glutaraldehyde was added to a total volume of approximately 6 ml. The tube contents were mixed by shaking. After a 1.5 hr fixation period the cells were centrifuged in the clinical centrifuge (swinging bucket rotor) for 5 min at setting no. 7. The supernatant was aspirated and the cells were rinsed twice in the same buffer without glutaraldehyde, with centrifugation in between at setting no. 3. Post-osmication was for 1 hr in 1% OsO_4 in the same buffer. After three washes in buffer the cells were dehydrated through an ethanol series, infiltrated with propylene oxide and embedded in Epon. All procedures were performed at ambient temperature.

Thin sections were cut on a Sorvall MT-2 ultramicrotome (DuPont Instruments, Sorvall DuPont Co., Newtown, Conn.) with a diamond knife. Sections with gold, silver and gray interference colors were picked up on 200 and 300 mesh copper grids, stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate, and examined with a Hitachi HS-8 transmission electron microscope operated at 50 kV.

Unstained sections were carbon coated, and electron-dense membrane particles and cellular inclusions were subjected to X-ray microprobe analysis and X-ray scanning using a JEOL JEM-100 CX analytical TEMSCAN equipped with a

KEVEX 7000 analytical spectrometer.

2) Alternate fixation method: P. gouldii: An attempt was made to fix P. gouldii erythrocytes prior to embedment under conditions that would minimally distort cell shape (Mathieu et al., 1978) and provide maximum preservation of MBs, together with the relationship of the latter, if any, to other cell components. Preliminary observations had indicated that the coelomic fluid had a pH of about 8, and that the fluid, cleared of cells by centrifugation, was 962 mOsm. Furthermore, the cells retained their normal appearance when fixed in coelomic fluid containing 0.1% glutaraldehyde (Fig. 13). Therefore, cells were fixed in the following manner: To 1 ml of P. gouldii coelomic fluid in a 12 ml conical centrifuge tube, 0.1 ml of 10% glutaraldehyde was added. Fixation was for one hour at 15°C. Subsequent centrifugation for 5 min at setting no. 4 yielded a 0.4 ml pellet of cells. These were resuspended 3 times in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.6 M sucrose (mOsm=959) with centrifugation as above after each step. Cells were resuspended in 0.1 M sodium phosphate buffer, pH 6, containing 0.61 M sucrose and 0.1% OsO_4 (972 mOsm) at 0°C, and postfixed for 0.5 hr. This post-osmication procedure was followed in order to maximize preservation of actin filaments that might be present in the network (Maupin-Szamier and Pollard, 1978; Cohen, 1978). Cells were washed as described above in the same buffer without OsO_4 at 0°C, and twice in 30% ethanol at room temperature, before pre-staining in 1% uranyl acetate in 30% ethanol. The remainder of the procedure was as described above. Osmolality measurements were made with an Advanced Osmometer (Advanced Instrument Co., Needham Heights, Mass.). Sample osmolalities were determined by comparison with a standard curve.

3) Triton-Lysed Preparations

a) P. gouldii: Simultaneous Lysis and Fixation. Three ml of LyM, pH 6.24, containing 10 mM EGTA and 2.5% glutaraldehyde, in a 12 ml conical centrifuge tube was mixed with 0.3 ml of the coelomic fluid of a female worm. The fluid had been withdrawn through the body wall of the animal with a 1 ml tuberculin hypodermic syringe and a 22 G needle. After a 1 hr fixation at 15-16°C the cells were centrifuged for 3 min (IEC clinical centrifuge, setting no. 5). The fixative was replaced with two 3 ml rinses of 0.1 M sodium phosphate buffer, pH 6 at 0°C, with centrifugation in between as described above. The lysed cells were post-fixed in 0.1% OsO_4 in the pH 6 phosphate buffer at 0°C for 0.5 hr (Maupin-Szamier and Pollard, 1978). The suspension was pre-stained in 1% aqueous uranyl acetate for 0.5 hr and dehydrated through an ethanol series. All steps were performed at 0°C, and all centrifugations were done in the cold. The preparation was allowed to come to room temperature in the last dehydration step, and all subsequent steps were performed at ambient temperature. The remainder of the embedment procedure was as described above for the intact cells.

b) P. gouldii: Lysis in the Presence of ATP. TEM on Whole Mounts of Isolated MBs. Approximately 0.5 ml of blood was obtained by slitting a female worm with a razor blade. The blood was suspended in 4.5 ml of 0.53 M NaCl (isotonic to sea water), and the preparation was permitted to stand until the eggs settled out. The supernatant including the erythrocytes was then pipetted into a 15 ml conical centrifuge tube, and centrifuged for 1 min at maximum speed. The supernatant and buffy zone atop the erythrocytes was removed and the remaining erythrocytes were resuspended in 4.5 ml NaCl and divided into two tubes. After centrifugation, the packed volume of cells in each tube was determined to be approximately 0.03 ml. LyM (1 ml) was added to the cells in one tube, and LyM containing 25 mM ATP was added to the other. The lysates were examined with phase-contrast microscopy to ascertain that the MBs in the ATP-treated samples had separated from the nuclei. Drops of the lysed cell suspension in ATP were placed on 200 mesh formvar-coated grids and allowed to settle for approximately 3 min. Fluid was drawn off with filter paper and replaced with wash medium (Lym No Tame) or H_2O. These were then replaced by 1% glutaraldehyde in wash medium. Fixation was for 2 min, and was followed by a H_2O rinse and staining with 1% aqueous uranyl acetate.

c) Limulus polyphemus: Lysed Amebocytes. Blood was obtained from an individual animal by the slitting of a chitinous leg joint with a razor blade. One volume of the amoebocyte-containing fluid that emerged was immediately added to 9 volumes of LyM, pH 6.8 in a silicone-coated 15 ml conical centrifuge tube. Lysis was quickly followed by addition of an equal volume of LyM containing 2% glutaraldehyde. After 3-1/2 hr of glutaraldehyde fixation the cells were rinsed twice in 0.1 M sodium phosphate buffer, pH 6.8, and post-fixed in 1% OsO_4 in the same buffer for 0.5 hr. Further processing and preparation for electron microscopy were as described above for P. gouldii "Intact Cells." All procedures were performed at room temperature.

d) Anadara transversa: Simultaneous Lysis and Fixation. Three specimens were pried open, and blood was withdrawn from between the mantle and shell with Pasteur pipettes used to puncture the former. In this manner a total of 0.8 ml of blood was pooled into a 12 ml conical centrifuge tube. The blood was centrifuged at setting no. 1 in the clinical centrifuge for 5 min, yielding a 0.2 ml pellet of cells. The supernatant was drawn off and replaced with 0.8 ml LyM, pH 6.8, containing 1% glutaraldehyde in which the cells were resuspended. After fixation for 1 hr at 16°C, the preparation was post-fixed for 1 hr with 0.1% OsO_4 in 0.1 M sodium phosphate buffer, pH 6, at

0°C. This was followed by two H_2O rinses, pre-staining with 1% uranyl acetate in 30% ethanol and ethanol dehydration series. The remainder of the procedure was as described for "*P. gouldii*: Simultaneous Lysis and Fixation."

e) *Noetia ponderosa*: Simultaneous Lysis and Fixation. Tannic Acid as a Mordant, and Serial Sectioning. Blood was obtained from two animals as described above for *A. transversa*. Nine volumes of LyM with 1% glutaraldehyde were mixed with one volume of blood, and the suspension was immediately centrifuged (at setting no. 3) for 7 min. The resultant pellet was washed twice in LyM without Triton and TAME and with 0.5 M PIPES, pH 6.8, (LyM No T&T-0.5P) containing 1% glutaraldehyde with 3 min centrifugations in between. This formulation of the fixative was used because preliminary trials had indicated that a precipitate formed when tannic acid was mixed with Triton or 0.1 M PIPES, but that the mordant was apparently compatible with 0.5 M PIPES at the concentration employed. One-half hr after the start of the procedure, fresh LyM No T&T-0.5P with 1% glutaraldehyde containing 0.2% tannic acid (Mallinckrodt; added as a powder just before use) was used to resuspend the pellet of lysed cells (Begg *et al.*, 1978; Goldman *et al.*, 1979). Fixation was continued for an additional 1.5 hr, at which time the lysed preparation was washed once with LyM No T&T-0.5P, followed by a wash with 0.1 M sodium phosphate buffer, pH 6. All procedures up to this point were performed at room temperature. Postfixation was accomplished with 1% OsO_4 in the same buffer at 0°C for 1 hr. Further processing was as described above for "*P. gouldii*: Simultaneous Lysis and Fixation." Ribbons of sections with a gray interference color were picked up with formvar-coated loops and transferred to slot grids. Staining and viewing procedures were as described above.

f) N. ponderosa: MB Disassembly/Reassembly. Blood from two specimens was pooled and 20 drops were added to a 12 ml centrifuge tube containing 10 ml LyM, pH 6.85, with 1% glutaraldehyde at 20°C (control tube, C). One drop of blood containing intact cells was fixed for microscopic observation by addition to 0.5 ml I.O. containing 1% glutaraldehyde (9 vol I.O.:1 vol 10% glutaraldehyde). For MB disassembly, 120 drops of blood were pipetted into 60 ml of I.O. at 0°C. After 1 hr of fixation at 20°C, the pellet in tube C was twice resuspended in 0.1 M sodium phosphate buffer, pH 6, with centrifugations in between, at the maximum setting on the clinical centrifuge, for 5 min (all subsequent centrifugations were the same speed and duration). The tissue was left overnight in the second rinse at 4°C. The intact cells in I.O. (0°C) were resuspended by swirling and 10 ml aliquots were rapidly pipetted into test tubes which were stored overnight (approx. 17 hr) at 2°C.

By the following day, the cells had settled to the tube bottoms. The overlying supernatant was aspirated from one tube (t_0) and immediately replaced by 10 ml of LyM, pH 6.85, with 1% glutaraldehyde in which the cells were resuspended. Lysis and absence or presence of MBs were confirmed by phase-contrast microscopy. From another tube (I) 0.5 ml of intact cell suspension was fixed by addition to 0.06 ml of 10% glutaraldehyde. Immediately afterward all tubes, including I, were transferred to a 25°C water bath. Fifteen min later a second tube was simultaneously lyse-fixed (t_{15}) at 20°C as described above, and 120 min later a third tube (t_{120}) was similarly treated. At each of the above time points intact cells from tube I were fixed as described and observed with phase-contrast microscopy.

All fixations for electron microscopy were for 1 hr at 20°C and the tube contents were processed in an identical fashion with tube C. After the second wash in pH 6 buffer, the contents of all tubes containing lyse-fixed material were post-fixed in 0.1% OsO_4 in sodium phosphate buffer, pH 6 at 0-2°C for 45 min. Two washes in distilled deionized H_2O at 0°C were followed by dehydration and embedment as described above for "P. gouldii: Simultaneous Lysis and Fixation."

Subsequently, a similar experiment was performed in which reassembly was interrupted at earlier intervals during rewarming, e.g., 2 min and 5 min so that results were obtained for a control (C), t_0, t_2, t_5, and t_{120}. The procedure was as described above, except that rewarming of all tubes was not simultaneous. In addition, prior to simultaneous lysis-fixation, samples were removed from the tubes, lysed in LyM, pH 6.8, and observed with the phase-contrast microscope without fixation. Intact cells were also examined at intervals without prior fixation.

g) N. ponderosa: Negatively-Stained Whole Mounts. Immediately upon removal from the animal, one drop of blood was lysed in 0.5 ml LyM, pH 6.8, (control) and 1 drop was added to 0.5 ml I.O. to which had been added 0.06 ml of 10% glutaraldehyde (approx. 0.1% final concentration). The remaining blood was diluted 1:4 (vol/vol) into I.O. at 0°C and distributed into test tubes in 0.5 ml aliquots. The tubes were incubated at 0°C, until replacement of the supernatant from one tube by 0.5 ml LyM revealed the absence of MBs (t_0). At this time, approximately 1.5 hr after incubation had begun, all remaining tubes were transferred to a 25°C (approx.) H_2O bath. At intervals of 2, 5, 15 and 120 min samples were lysed, and reassembly of the MBs during these time periods was followed by examination of the samples under the phase contrast microscope. Intact cells from companion tubes were fixed at the same time points by addition of 0.06 ml of 10% glutaraldehyde.

Negatively-stained whole mounts were made of the lysed controls and t_{120} samples as previously described (Cohen and Nemhauser, 1980). Samples were placed on formvar-coated grids and allowed to settle for 20 min, at which time the fluid was drawn off with filter paper. The latter was successively replaced by LyM without Triton and TAME (wash medium), wash medium containing 2.5% glutaraldehyde for 12 min, wash medium, H_2O and 2% aqueous uranyl acetate, using filter paper for removal each time. The grids were air dried and examined in a Phillips 300 TEM operated at 60 kV.

RESULTS

I. Marginal Band Systems in Marine Invertebrates: Distribution

A survey of marine invertebrates from four phyla yielded the results presented in Tables I and II. Representatives of three of the phyla are depicted in Figure 1.

II. Erythrocytes: MB System Morphology

A. Phylum Sipuncula: *Phascolopsis gouldii*. The coelomic fluid of the sipunculan worm *Phascolopsis* (syn. *Golfingia*; Gormley *et al.*, 1978) *gouldii* contains a variety of cell types including erythrocytes, amoebocytes and gametes. The erythrocytes, which make up the majority cell type, are flattened, biconcave and generally elliptical, with long axes measuring from 20 to 30 μm (Fig. 2). When the cells were lysed with Triton X-100 in modified microtubule polymerization medium (LyM), permeabilization of the plasmalemma resulted in release of the respiratory pigment hemerythrin, making it possible to observe the cell interior. Under the phase-contrast microscope a thin, relatively phase-dense MB, was seen to be circumscribing the remaining cellular contents including the nucleus and various cellular organelles (Fig. 3). The position and circumference of the MB are coincident with the circumference, in the plane of flattening, of the formerly intact cell, leading to the tentative conclusion that it normally lies just under the cell membrane. In addition, in many instances the MB assumed a twisted figure-8, or saddle shape, in which it was possible to observe that the MB itself was enclosed in a sheet-like structure that is otherwise invisible under the light microscope (Fig. 3b). When the vaseline well was omitted, a coverslip placed directly over the suspension flattened the lysed

TABLE II

Marginal Band System Distribution in Invertebrates

Phylum and species	Cell Type[1]	MB-Associated Centrioles[2]
Sipuncula		
Phascolopsis gouldii	erythocyte	+
Mollusca		
Anadara transversa	erythrocyte	++
Anadara ovalis	erythrocyte	+
Anadara brasiliana	erythrocyte	+
Anadara lienosa floridana	erythrocyte	+
Anadara spp.	erythrocyte	+
Noetia ponderosa	erythrocyte	++
Arthropoda		
Homarus americanus	coelomocyte	N.D.
Limulus polyphemus	amebocyte	N.D.
Libinia emarginata	amebocyte	N.D.
Cancer borealis	amebocyte	N.D.
Cancer irroratus	amebocyte	N.D.
Carcinus maenas	amebocyte	N.D.
Periplaneta americana	hemocyte	N.D.[3]
Echinodermata		
Cucumaria miniata	erythrocyte	+
Molpadia arenicola	erythrocyte	+

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[1] The echinoderm blood contained numerous cell types; those containing MBs are presumed to be erythrocytes (see Clifford, 1969 and Fontaine and Lambert, 1972).

[2] ++ = centrioles observed with TEM; + = "dots" seen with LM; N.D. = not determined.

[3] Baerwald and Boush (1970).

Figure 1. Representatives of Three Invertebrate Phyla Surveyed for the Presence of MBs. (*a*) The blood clam, *Anadara transversa*. (*b*) The green crab, *Carcinus maenas*. (*c*) The peanut worm, *Phascolopsis gouldii*. In the species shown in *a* & *c*, MBs were found in erythrocytes that contain hemoglobin and hemerythrin, respectively. In the crab, amebocytes, or clotting cells, have MBs. x1

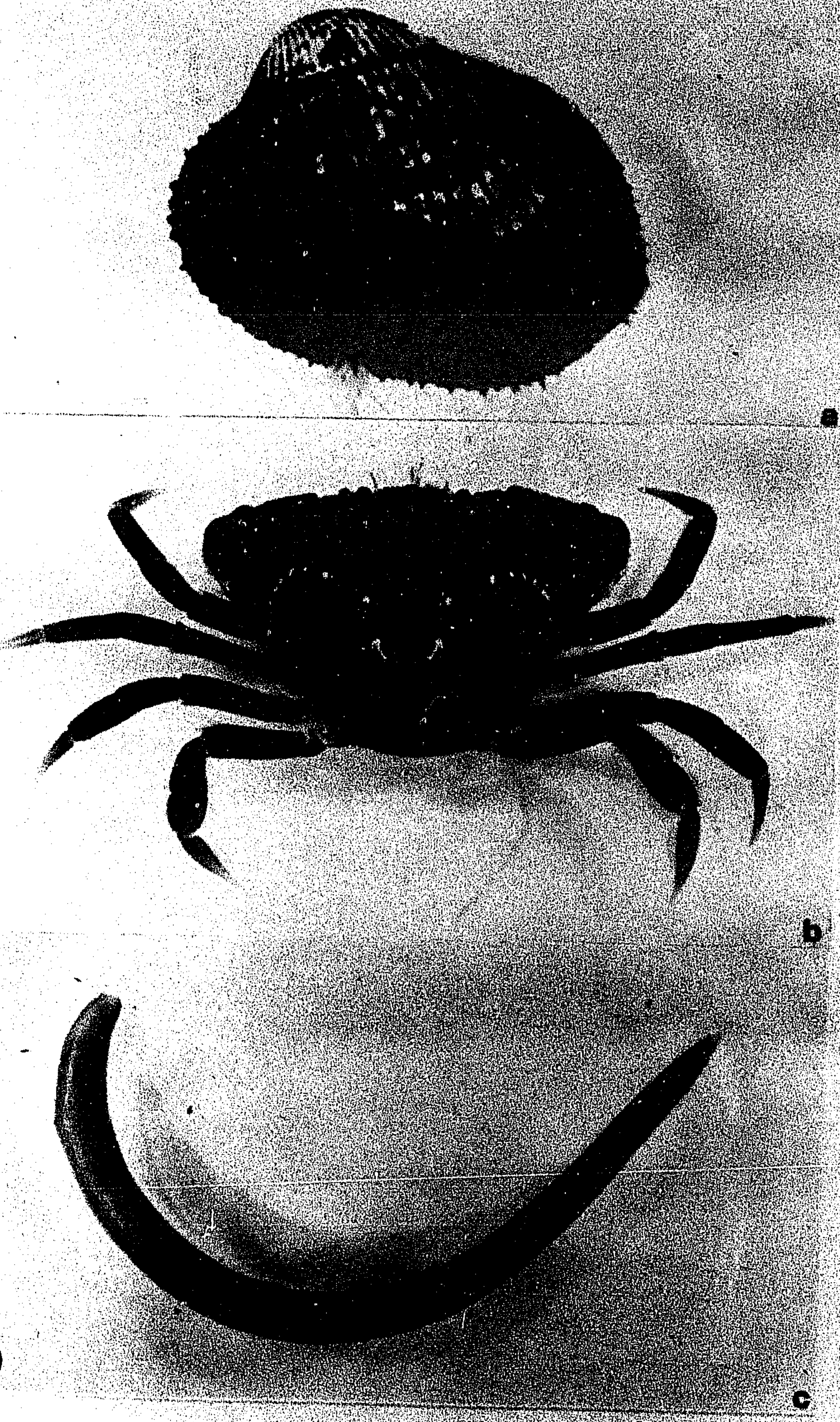

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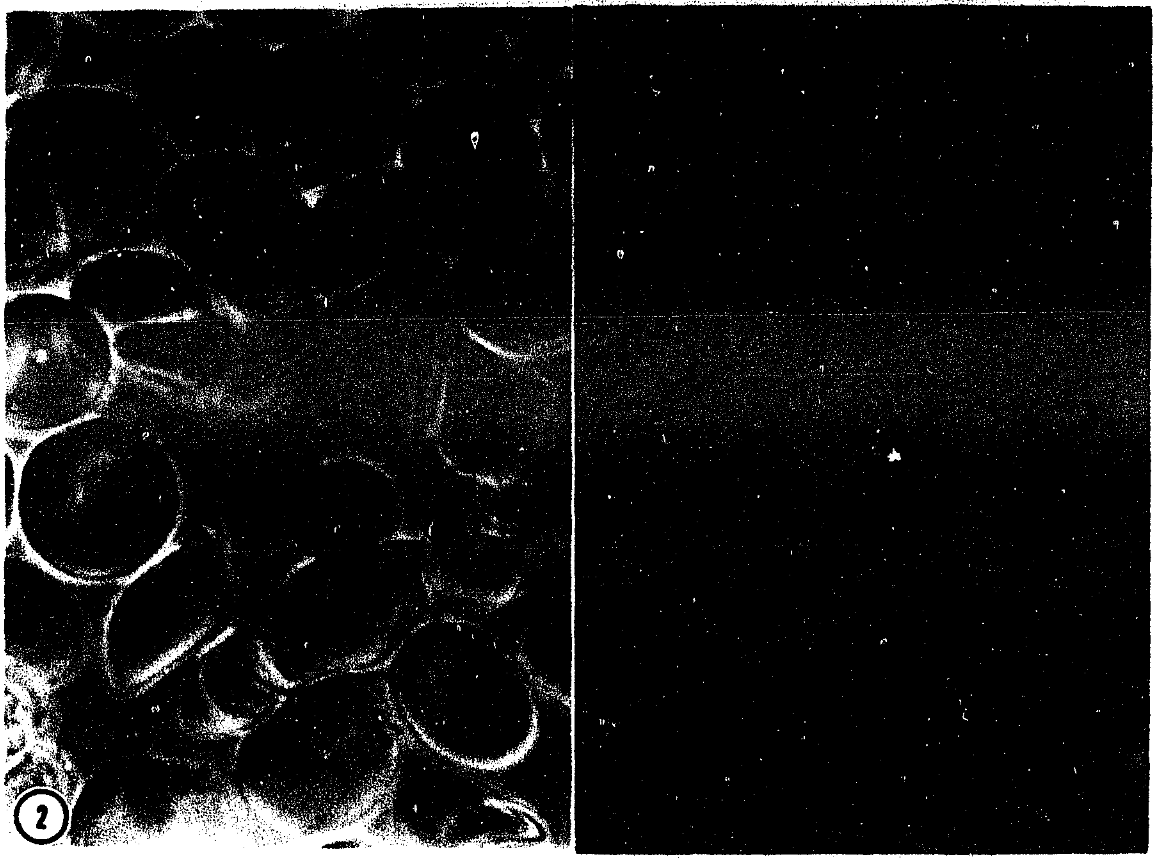
Figure 2. Living Blood Cells of P. gouldii. (a) The majority of cells are circular to elliptical erythrocytes. In (b) an edge on view of two cells illustrates their flatness and biconcavity. e = erythrocyte, n = nucleus. Phase contrast. (a) x960, (b) x2,450

Figure 3. Lysed Erythrocytes of P. gouldii. When the cells are lysed in LyM, what remains behind is the nucleus (N), remnants of other cellular organelles, and a phase-dense marginal band (MB) circumscribing these cell contents. Because of the figure-8 configuration of the MB in b, a sheet-like material can be seen to enclose the contents of the former cell (arrow). In c, the MB (arrow) is seen to conform to the shape and size of the intact cell. The lysed cell in a has been flattened with a coverslip for ease of visualization of the dots (arrowhead) associated with the MB. Focussing showed them as pairs of dots unlike the "dots" at the lower left of this MB. Phase contrast. (a) x2,150, (b & c) x900



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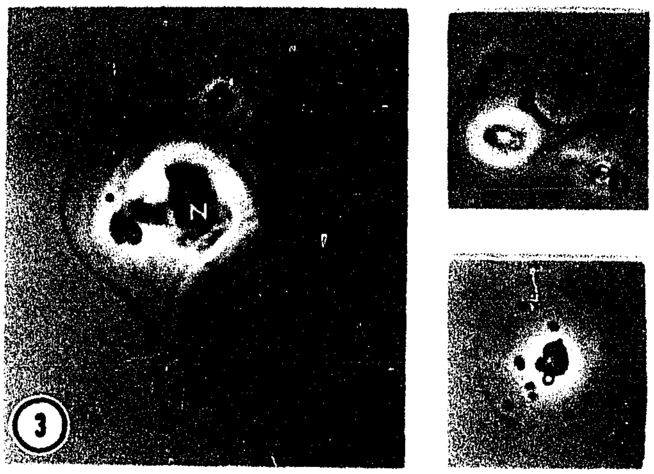

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cells, revealing the presence, in at least some instances, of two relatively phase-dense dots associated with the MB (Fig. 3a, arrowhead).

Themiste spp. Blood from this species appeared similar to that obtained from P. gouldii, however the erythrocytes tended to buckle, assuming a triangular shape, and MBs were not observed when these cells were lysed.

B. Phylum Mollusca. The erythrocytes of the various members of the class Arcidae observed in the present study are flattened, more or less elliptical nucleated cells that in all cases examined, save Noetia ponderosa, tended to crenate[2] rapidly after the animals were opened (cf. Figs. 4a and 5a). This necessitated suspension of the cells in molluscan Ringer's solution containing 0.1% glutaraldehyde in order to preserve the native shape of the erythrocytes long enough for them to be photographed. This treatment did, however, cause the cells to shrink somewhat and appear refractile (Figs. 5, 6 and 7).

When the erythrocytes were lysed, as in P. gouldii, MBs were seen conforming to the original cell size and shape and surrounding the remaining cellular organelles (Figs. 4, 5, 6 and 7). When the cell suspensions were flattened with coverslips every MB that could be seen in its entirety was observed to have dots associated with it. Usually there were two dots; occasionally only one dot could be seen clearly, probably because it was overlying and obscuring the second one of the pair (Fig 7e). The dots were usually adjacent, but sometimes separated by distances as great 1.3 μm (Figs. 5d & e; 7g &h). In some instances the portion of the MB between separated dots appeared less phase dense than the remainder of the MB (Figs. 5d & 7g).

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[2] The term crenate, as used here, refers to a wrinkling of the cell membrane and is not employed in the same sense for which it is used when describing the appearance of mammalian erythrocytes placed in hypertonic solution.

Figure 4. Erythrocytes of the blood clam, *Noetia ponderosa*. (a) Intact cells. The arrows indicate two cells that come to a point at one end. the majority of cells are elliptical. (b-e) *N. ponderosa* red blood cells lysed in LyM. d & e are photomicrographs of the same cell at different focussing levels. Arrowheads point to the MB-associated dots. The latter are usually located at or near the ends of the ellipses. In d & e a fiber (f) runs from the region of the dots toward a distant location on the MB. The MB in c has assumed a figure-8 configuration. Phase contrast. (a) x960, (b-e) x2,320

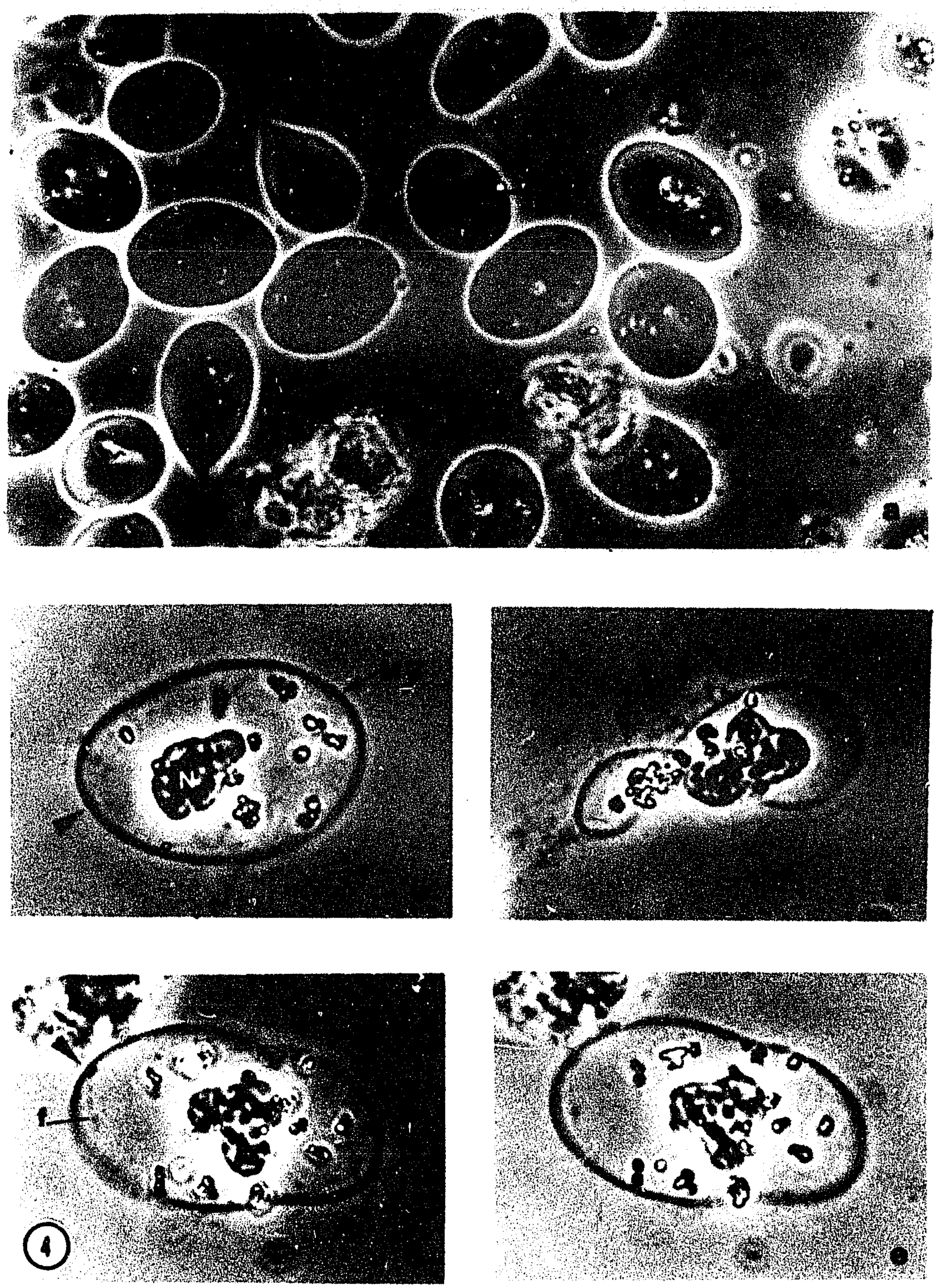
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Figure 5. Erythrocytes of the Blood Clam *Anadara ovalis*. The majority of the living cells in *a* have crenated; only two cells in the field have not yet been transformed and are seen to have an elliptical shape. The cells in *b*, fixed in molluscan Ringer's containing 0.1% glutaraldehyde, have the native elliptical shape, but are refractile as a result of fixation. (*c*-*f*) Lysed erythrocytes. Double arrowheads indicate separated dots. In *d* the area between the dots appears less dense than the rest of the MB. In *d*, *e* and *f* fibers (f) extend from the dots to more distant regions of the band. The dots are located at (*d*-*f*) or near (*a*) the ends of the ellipses. Phase contrast. (*a* & *b*) x1,500, (*c*-*f*) x2,250

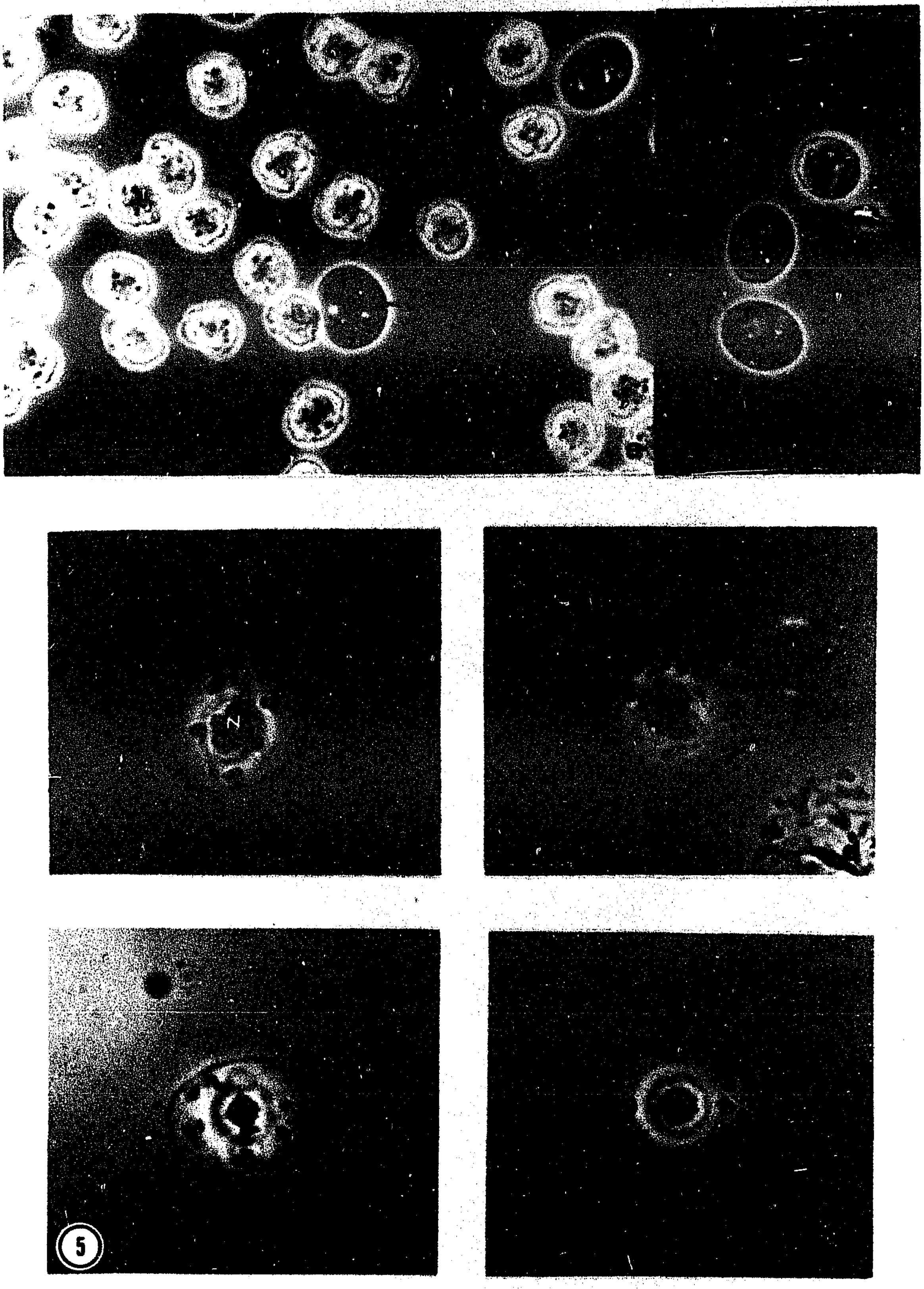
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Figure 6. *Erythrocytes of the Blood Clam Anadara transversa*. The intact cells in *a* were fixed in molluscan Ringer's solution containing 0.1% glutaraldehyde. (*b* & *c*) Lysed cells. The arrows point to the dots. Phase contrast. x1,350

Figure 7. *Erythrocytes of Anadara spp*. In this species a significant number of cells have points at one end. A comparable number of MBs with points at one end are seen when the cells are lysed (*c*-*f* and *h*). The dots are located at the points. The double arrowheads in *g* and *h* indicate widely spaced dots. In *g*, the area between the dots appears less dense than the rest of the MB. In *e* and *f*, focussing on different levels of the same cell, reveals an MB with the configuration of a tennis racket. The cell in *h* was lysed by pouring blood directly from the animal into LyM in order to eliminate the possibility that the points were artifacts caused by pipetting of the blood. The intact cells in *i* were glutaraldehyde-fixed *in vivo*. Phase contrast. (*a*-*g*) x2,250, (*h*) x2,400, (*i*) x810

In favorable preparations thin fibers could be observed radiating from one or both dots toward a more distant point on the MB (Figs. 4d; 5d, e, & f; 7a). In one species of the Arcidae (Anadara spp.) a significant number (8-51%) of erythrocytes were pointed at one end (Fig. 7i) as compared with a similar count in N. ponderosa of 0.17% (Fig. 4a), and lysis revealed a similar number of pointed MBs (13-21%; Fig. 7c-f & h) in the former species. Almost without exception the dots were located at the tips of the points, sometimes adjacent to one another, at other times clearly separated (Figs 7c-f & h). The MB in Figure 7h was seen in blood that was poured directly into the lysing medium to eliminate the possibility that pipetting caused formation of the points. Similarly, the blood cells in Figure 7i were fixed in vivo. In addition, occasional cells were seen in which the MBs, observed at different focussing levels, appeared to be shaped like tennis rackets (Fig. 7e & f). That is, in these cells a portion of the MB passed centripetally to the point containing the centrioles.

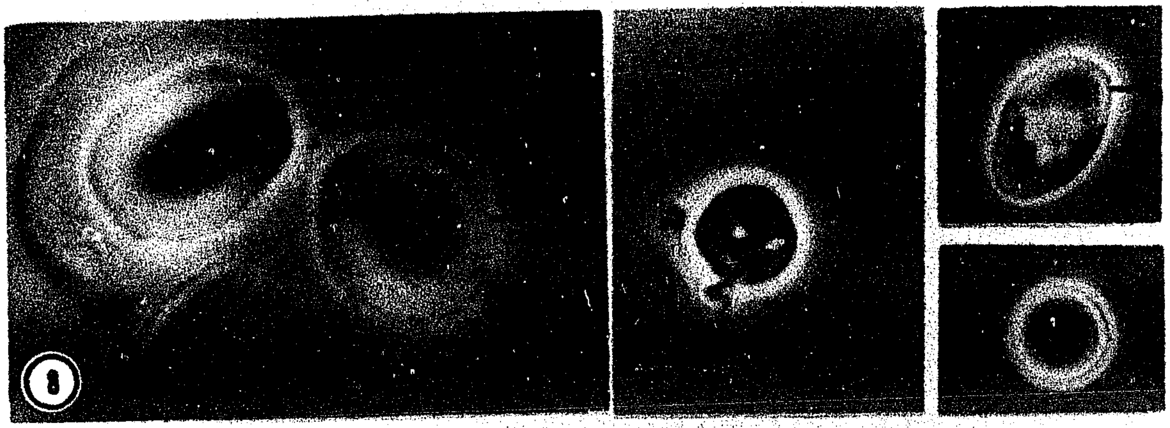
C. Phylum Echinodermata. In two other species of invertebrates, the sea cucumbers Molpadia arenicola and Cucumaria miniata, the presence of MBs with associated phase-dense dots was confirmed in cells presumed to be erythrocytes (Fig. 8). In Figure 8d a morula cell, one of numerous additional blood cell types in these species, is depicted (Clifford, 1969).

D. Indirect Immunofluorescence: The nature of both the MBs and their associated dots was investigated in two ways, with indirect immunofluorescence using antitubulin antibody and by ultrastructural analysis. The positive results obtained with immunofluorescence indicated, at the light microscope level, that the MBs were composed of microtubules and that the dots might be centrioles.

In lysed P. gouldii erythrocytes, indirect immunofluorescence demonstrated that the MBs bind antitubulin specifically (Fig. 9). Although structures corresponding to dots were not shown to be fluorescent, amorphous bodies that

Figure 8. Erythrocytes of Sea Cucumbers. (*a*) *Caudita arenicola*. The lysed erythrocytes shown have MBs and associated dots. (*b*-*d*) *Cucumaria miniata*. The MB of a lysed erythrocyte is shown with associated dots; an erythrocyte (e) is shown in *c* and a morula cell in *d*. Phase contrast. (*a*) x2,500, (*b*-*d*) x746

Figure 9. Indirect Immunofluorescence Using Antitubulin Antibody Stains the MBs of *P. gouldii*. *a* and *b* are a phase-immunofluorescent pair illustrating the ease with which these thin MBs, barely visible under phase contrast, are visualized with the fluorescent technique. (*c*-*e*) In areas where the MB has unravelled, its fibrous nature is revealed. The arrow in *c* indicates an amorphous, brightly fluorescent body typically adjacent to the nucleus. In *d* and *e* similar bodies are seen, the one in *e* located some distance from the faintly discernible nucleus. (*a* & *b*) x190, (*c*) x2,875, (*d* & *e*) x1,875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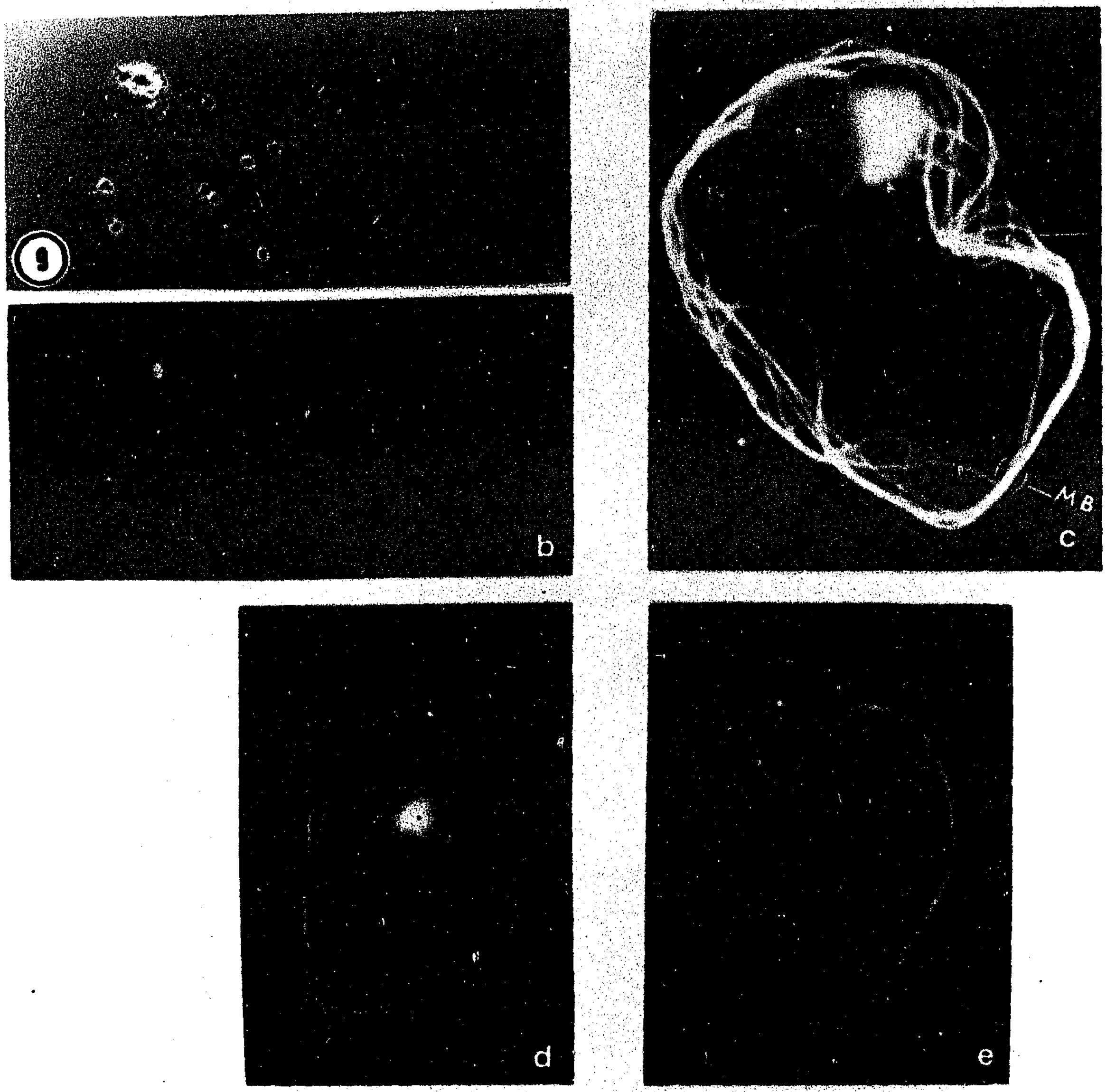
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fluoresce at least as brightly as the MTs were frequently seen, often in association with the faintly outlined nucleii (Fig. 9c-e). Figure 9a & b are a phase-fluorescent pair of the same coverslip area at low magnification which demonstrate the greater ease of visualization of these thin MBs using this technique.

Phase-fluorescence photomicrograph pairs of Anadara transversa lysed erythrocytes show that both the MBs and their associated dots bind antitubulin, the latter fluorescing more brightly than the former (Fig. 10). Similar results were obtained in the case of N. ponderosa (Fig. 23). Neither the nucleii nor any other organelles bound the antibody.

E. Transmission Electron Microscopy

1) P. gouldii

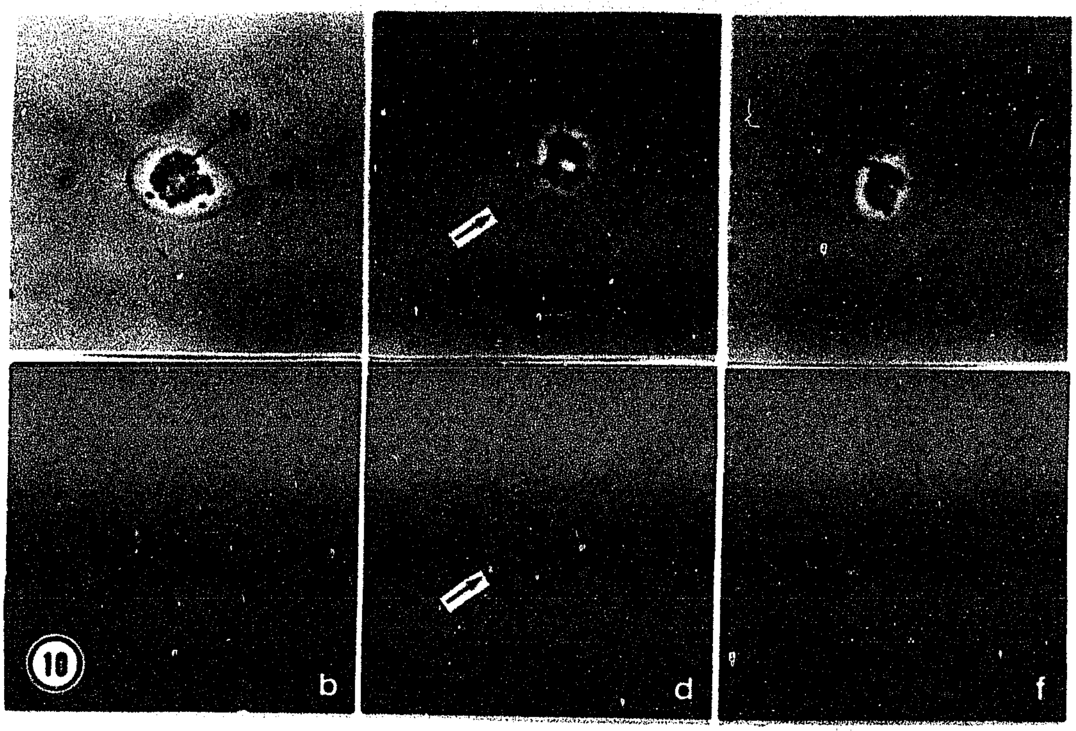
a) Intact Cells

MT bundles can be seen in thin-sectioned intact erythrocytes of P. gouldii. Favorably sectioned material shows MT bundles at opposite poles of the erythrocyte as would be expected of a fibrous structure circumscribing the cell beneath the plasma membrane, in the plane of flattening (Figs. 11 and 12). The MBs consisted of from 2 to 9 MTs, measuring 21 nm in diameter. Also seen were the homogeneously distributed respiratory pigment hemerythrin, the nucleus, mitochondria and various membrane-bound vacuolar structures. The nucleus and other structures, presumably corresponding to the membrane-bound organelles seen with TEM, could also be discerned in phase-contrast photomicrographs of the Epon-embedded whole mounts (Fig. 12a).

Frequently, electron-dense structures measuring 40 nm by 45 nm were observed apparently embedded in the plasma membrane (Fig. 13a & b). In addition, similarly dense masses were observed in membrane enclosed vacuolar structures within the erythrocytes (Fig. 14a & c). When such structures were

Figure 10. Phase Contrast-Immunofluorescence Pairs of Lysed Erythrocytes of A. transversa. The dots, indicated by arrows in c & d, fluoresce more brightly than do the MBs. The MB in a and b has widely separated dots associated with it. x1,160

Figure 11. A Thin-Sectioned Intact Erythrocyte of P. gouldii. The MB in a, outlined by squares at each pole of the cell, is shown at higher magnification in b and c where it is observed to consist of 8 MTs. The erythrocyte (a) is filled with hemerythrin (he) and contains a nucleus (N), a mitochondrion (m) and some membrane bound vesicles (mo), the latter probably remnants of the endoplasmic reticulum. ne = nuclear envelope. TEM. (a) x5,800, (b) x46,300, (c) x45,600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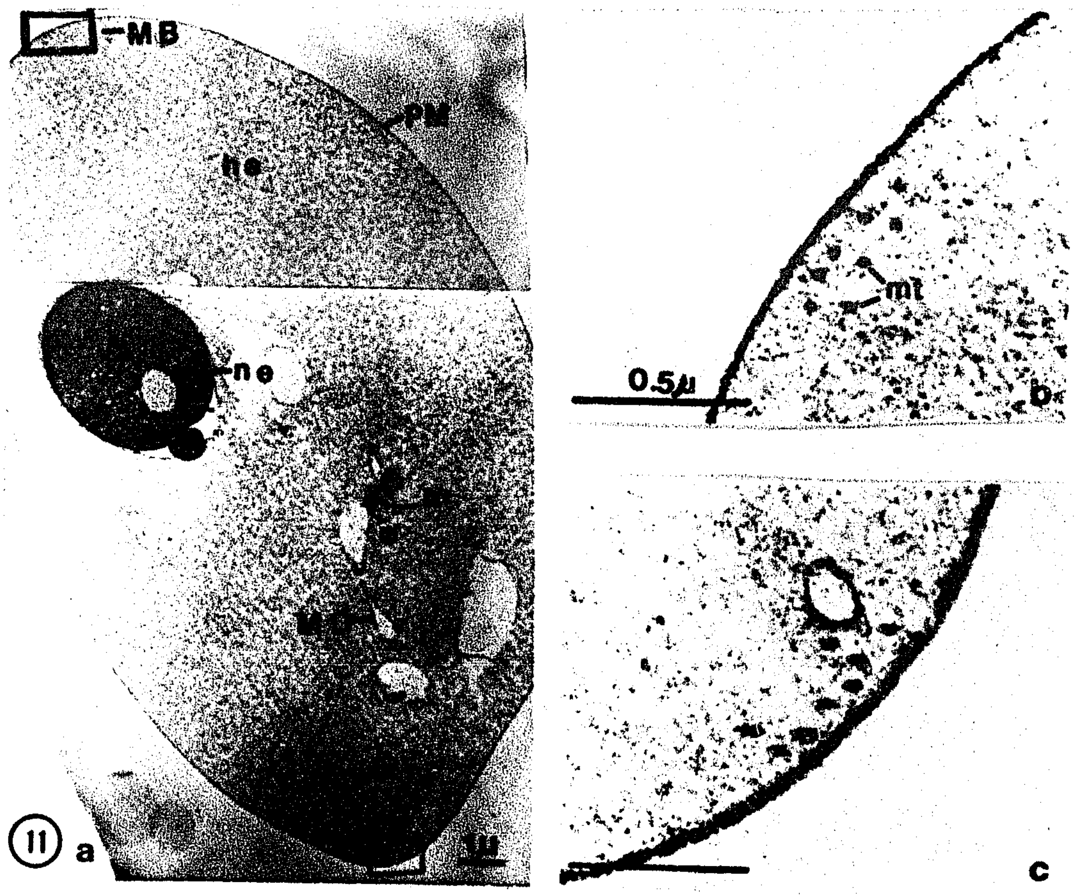
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Figure 12. P. gouldii. Sectioned Intact Erythrocytes. (a) A whole mount of P. gouldii erythrocytes embedded in Epon. The nucleus (N) and various organelles are visible in these intact cells. (b) The MB seen in cross-section at one pole of the cell is shown at higher magnification in c to be composed of 7 MTs. np = nuclear pore. (a) Phase contrast. (b and c) TEM. (a) x470, (b) x13,000, (c) x43,300

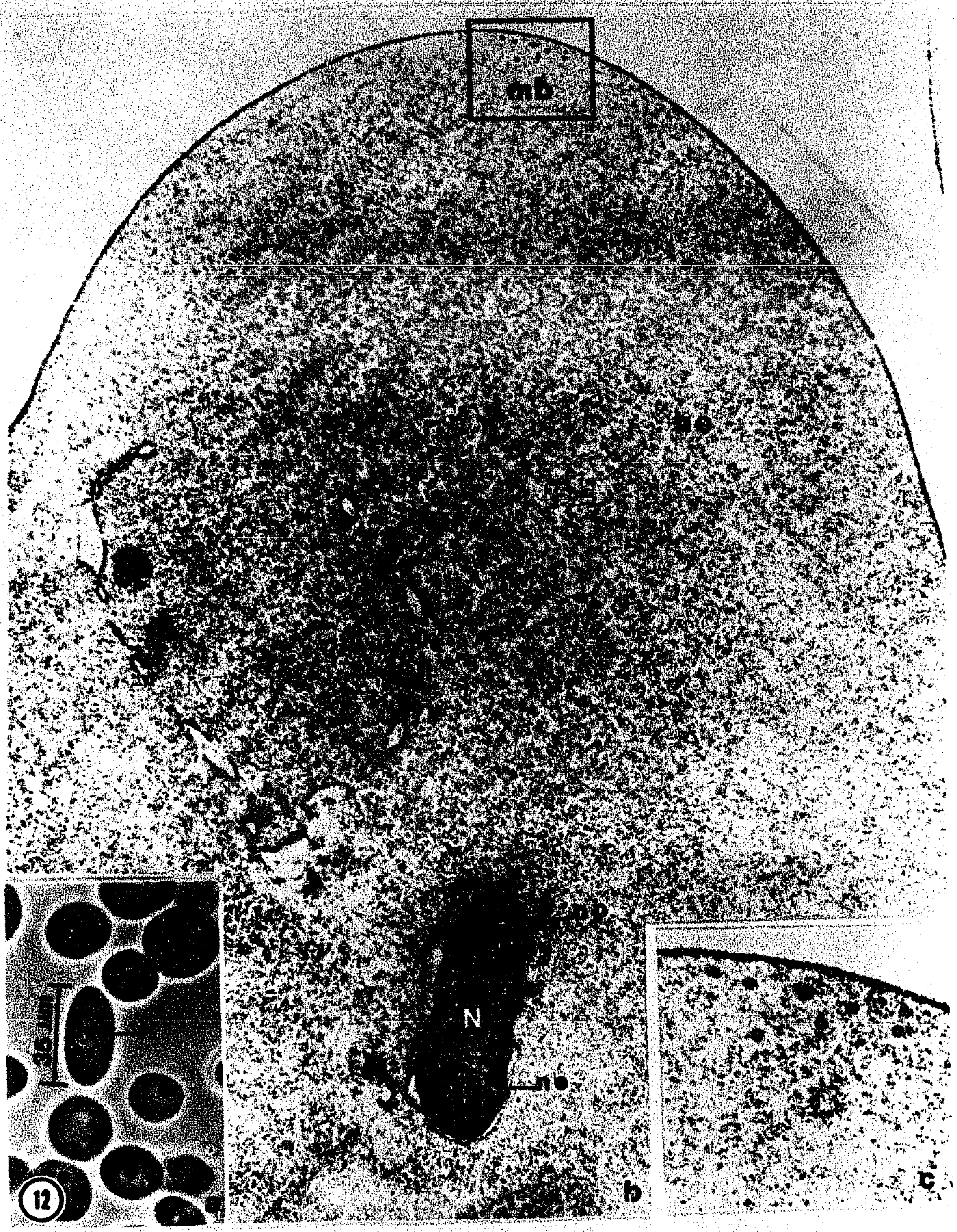
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Figure 13. Electron-Dense Granules (g) Embedded in the Erythrocyte Membrane of P. gouldii. The granules are seen in tangential (a) and cross (b) sections of the membrane. X-ray microprobe analyses of the cell in b reveals the presence of Fe in the granules. As controls, the electron beam was directed elsewhere in the cell (d) and at the Epon outside of the cell (e). TEM. (a) x47,000, (b) x100,000

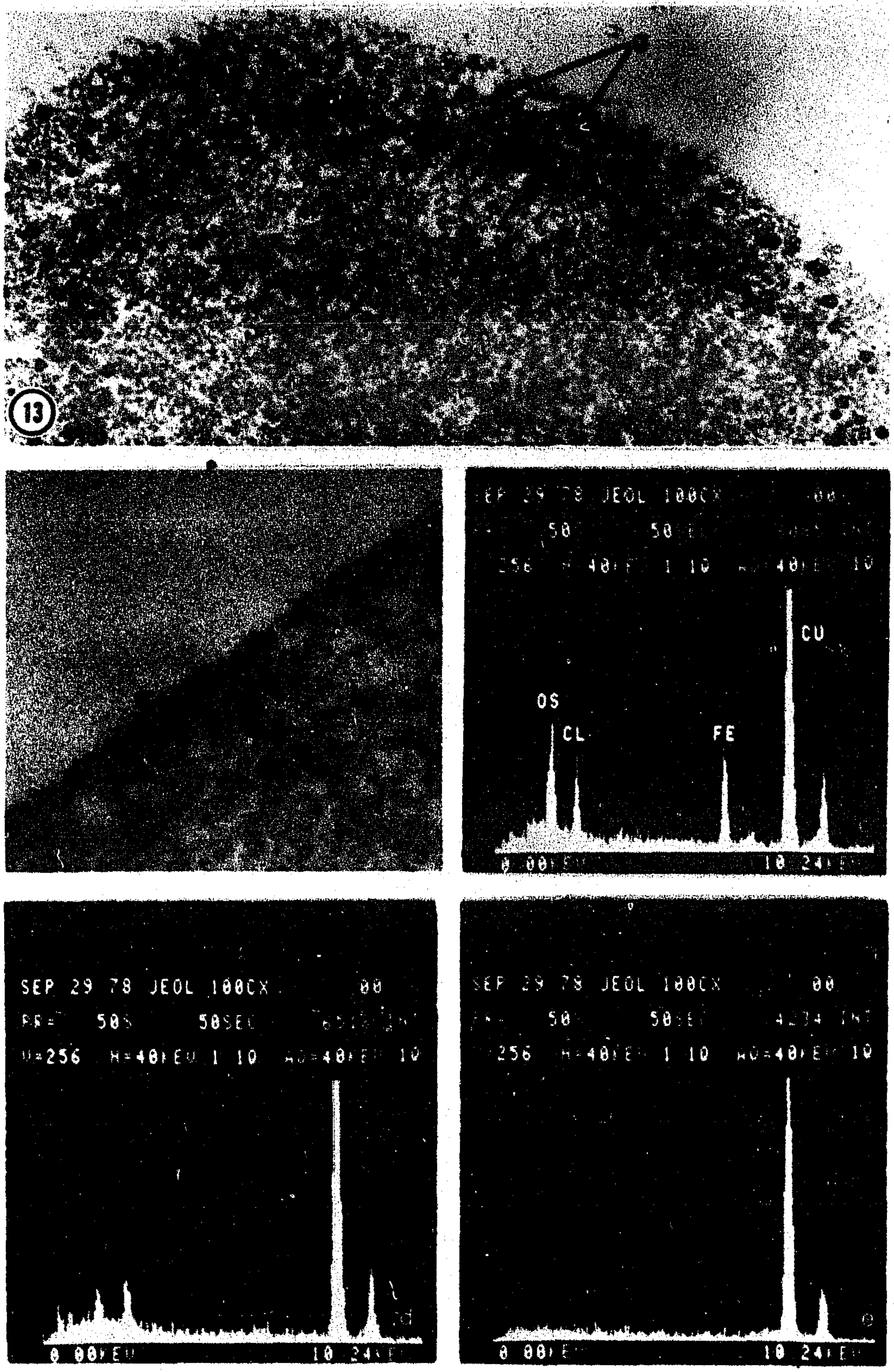
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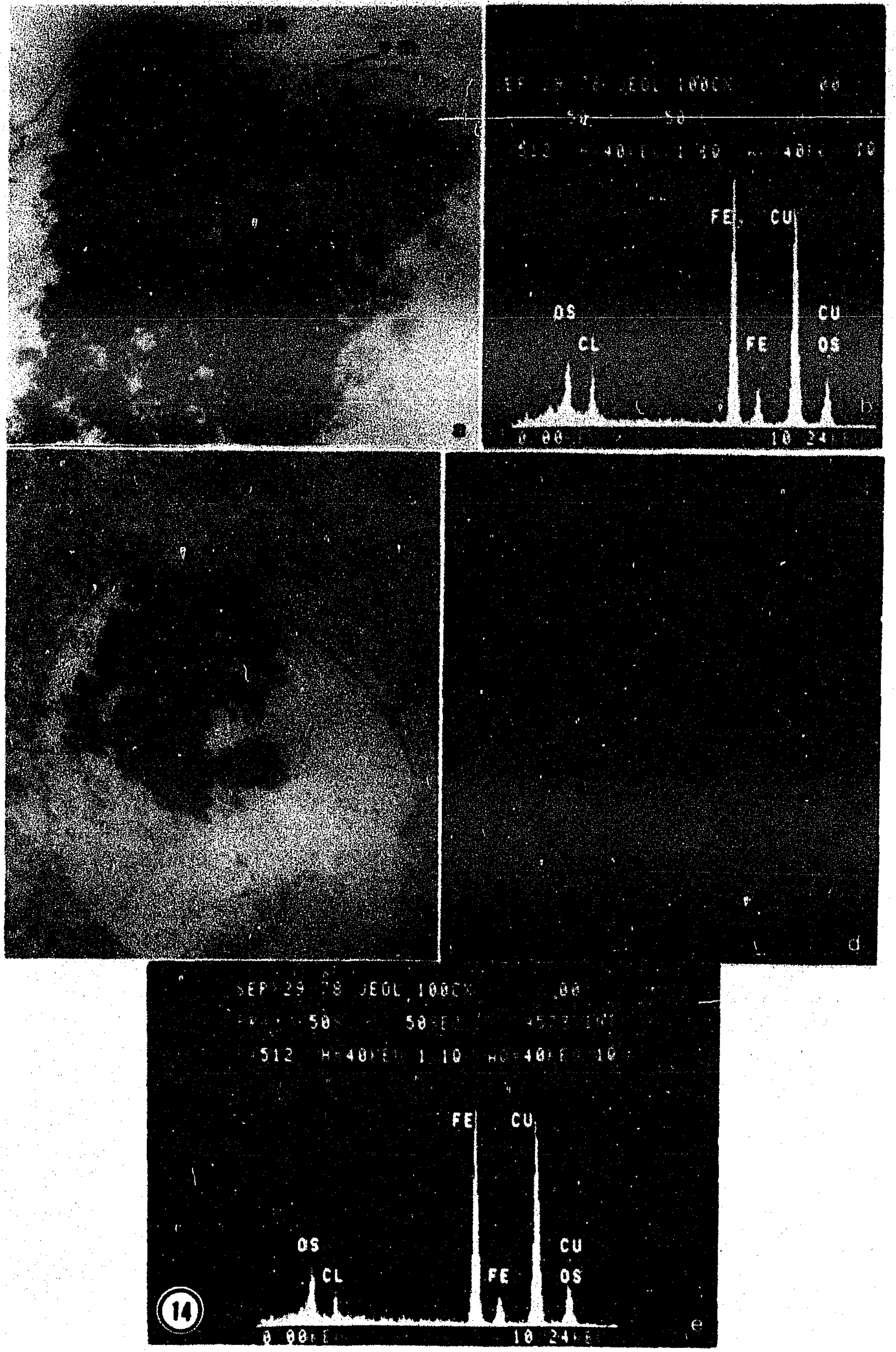
Figure 14. Electron-Dense Masses in Erythrocytes of P. gouldii. X-ray microprobe analysis (b) of the electron-dense mass (dm) surrounded by a vacuolar membrane (vm) in a shows that that mass contains Fe. When the mass in c was scanned for Fe the matching profile in d was obtained. (e) X-ray analysis of a single point in the dense mass in c also shows the presence of Fe. TEM. (a) x146,800, (c) x50,000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subjected to X-ray microprobe analysis, they were shown to contain Fe (Figs. 13c-e; 14b, e). Furthermore, when a membrane-bound organelle in which the dense material is contained was scanned for the presence of Fe, profiles such as those shown in Figure 14 (c & d) were obtained in which the outline of the dense substance corresponded to the Fe scan. From the foregoing, it would appear that the dense material bound to the plasma membrane, and contained in vacuoles, represents a storage form of Fe that is eventually incorporated into the respiratory pigment. The Cl observed in the spectra (Fig. 13c & d; and 14b & e) is a cellular component, and therefore not present in the Epon (Fig. 13e; Olins et al., 1979).

In some instances fixation did not appear to be ideal, the cells appearing swollen and sometimes having empty spaces (Fig. 11 and cf. Figs. 12a and 2). Since the fixatives used were hypotonic (226 mOsm) to the coelomic fluid (962 mOsm), attempts were made to devise a fixation method that would preserve the cells in a milieu isotonic with their natural environment. Preliminary work seemed to indicate that the erythrocytes could be preserved in essentially native shape when 10% glutaraldehyde was diluted 1:10 (vol/vol) into the coelomic fluid in which the cells were suspended (Fig. 15, cf. Fig. 1).

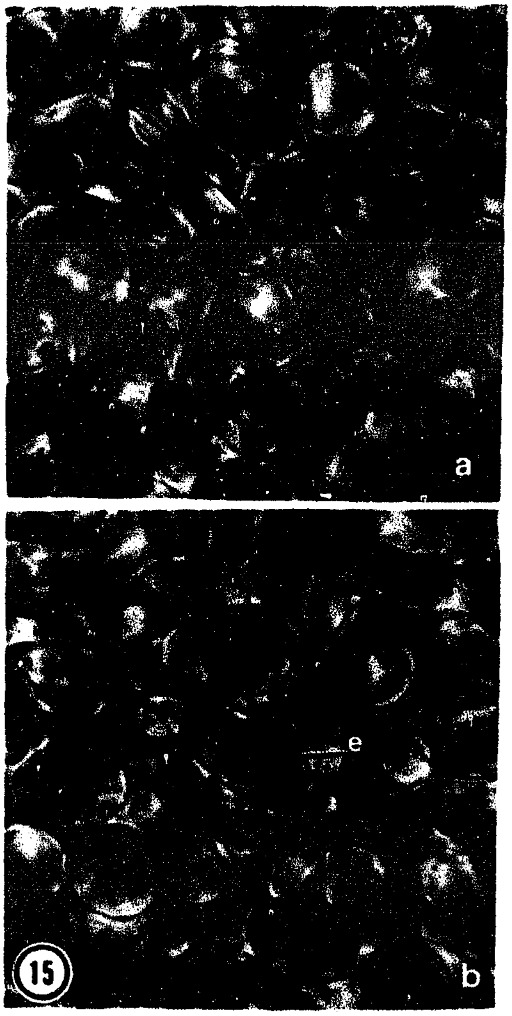
The alternate fixation method described in "Materials and Methods" was therefore used. However, the results (not shown) were disappointing for the purposes of the experiment; the hemerythrin was densely packed making it impossible to discern MT profiles.

b) Lyse-Fixed Cells

Because of the above mentioned problems, and a desire to be more certain about the native ultrastructure of the MB, i.e., what holds it together as well as the nature of the sheet-like material barely discerned with the LM, lysed material was fixed and processed for TEM observation. When this method proved

Figure 15. Erythrocytes of P. gouldii fixed with 1% glutaraldehyde in the animal's coelomic fluid. e=erythrocyte. Phase contrast. x1,230

Figure 16. Thin Sections of Lyse-Fixed P. gouldii Erythrocytes. The cross-sectioned MB in a consists of 13 MTs (mt) associated with a filamentous network (net). The longitudinally-sectioned MB in b is similarly associated with filamentous material. Arrowheads point to fine links between MTs. TEM. (a) x46,200, (b) x81,400



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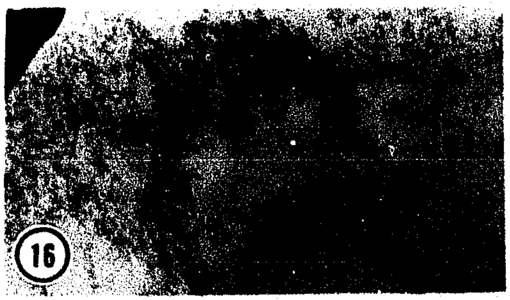

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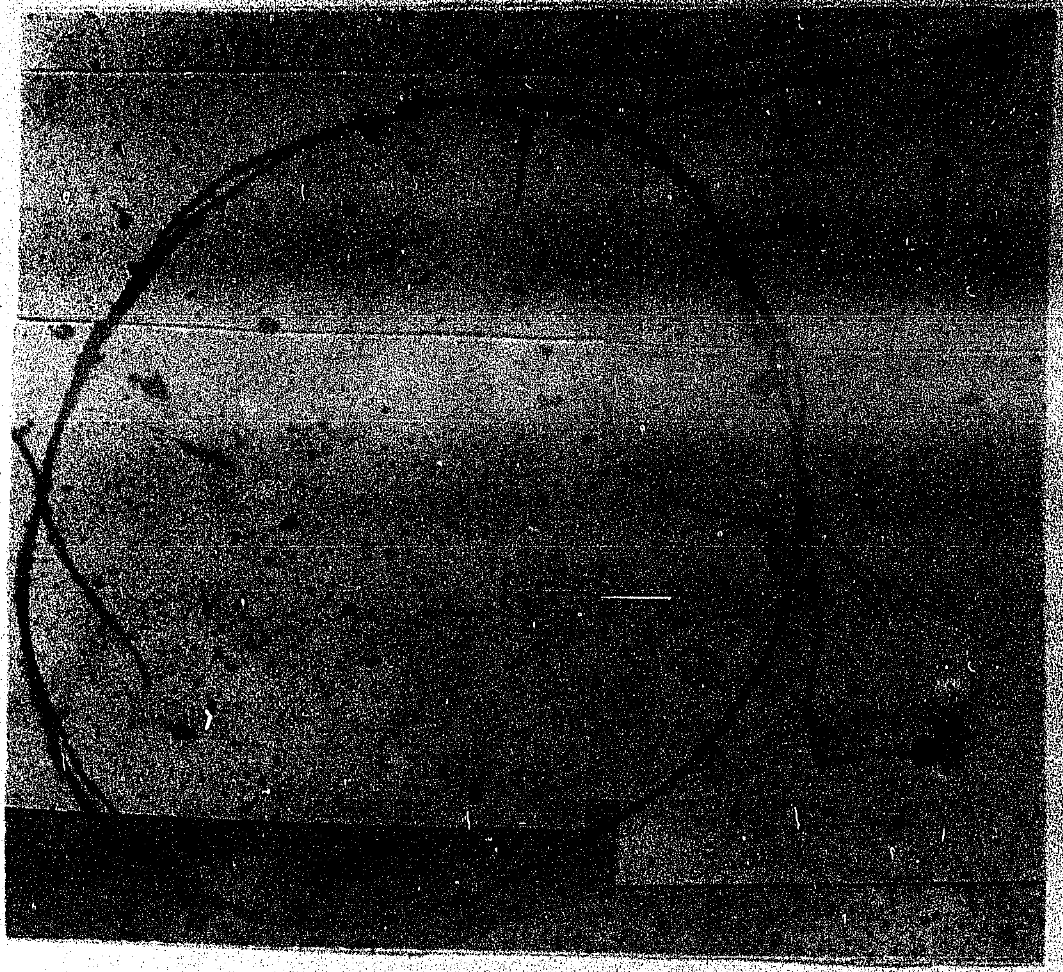
unsatisfactory because it resulted in apparent disruption of cellular organization, a technique was devised whereby the cells were simultaneously lysed and fixed (=lyse-fixed; see "Materials and Methods"). The results, shown in Figure 16, demonstrated close association and interconnections among the microtubular components of the MB and between them and a filamentous meshwork. The latter is presumed to correspond to the sheet-like material seen with the LM, and to underly the plasma membrane.

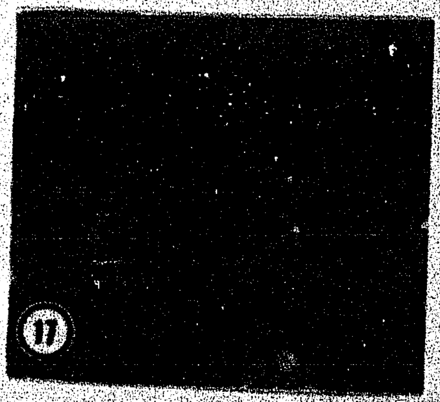
c) Isolated MBs

MBs of erythrocytes lysed in the presence of ATP were revealed, under phase-contrast microscopy, to have separated from their nuclei and other organelles within 10-15 min of lysis, while the control cytoskeletons, lysed in LyM only, remained intact. Transmission electron microscopy of whole mounts demonstrated that ATP treatment of the cytoskeletons resulted in isolated MBs, bereft not only of nuclei and other cellular organelles, but of the network as well. Furthermore, in the absence of the network, the MBs, when viewed with phase-contrast as well as electron microscopy, assumed a more circular configuration than they did when the cytoskeleton was intact (Fig. 17; cf. with Figs. 2 and 20). These results indicated that elliptical cell shape may be the result of interaction between the MB and network.

In addition, one of the proposed models of MB structure may now be eliminated. Close inspection under high magnification of the MB depicted in Figure 17 as well as a thinner isolated MB, two microtubules thick (results not shown) in which it was possible to follow individual MTs virtually the entire distance around the band, led to the conclusion that the MBs are not composed of short segments of overlapping microtubules. However, the results could not distinguish among models based on one continuous tubule wound around the cell a number of times, numerous MT hoops, or other possible alternatives.

Figure 17. *P. gouldii* MB Isolated as a Result of Lysis in the Presence of 25 mM ATP. Phase-contrast (*b*) and TEM (*a*) micrographs of the same MB. Arrows indicate points where MTs appear to be peeling away from this 4-6 MT thick band. (*a*) x4,100, (*b*) x980

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2) A. transversa

a) Lyse-fixed cells

Lyse-fixed and thin-sectioned erythrocytes of A. transversa clearly demonstrated not only the close association of the MB and network, but also that the dot-like structures seen under phase-contrast LM apparently correspond to centrioles. The centrioles were observed in cross- or longitudinal section, either singly or in pairs, the latter often at right angles (Figs. 18 and 19). The MTs, measuring 24 nm in diameter, in some instances appeared in cross- and longitudinal section to have connections to neighboring tubules, and (those that were peripherally situated) to the network (Fig. 19). There was an association between the MTs and centrioles as well, with some of the tubules apparently inserting into, or running through, the amorphous electron-dense material surrounding the centrioles (Figs. 18 inset; 19b & c). The cross-sectioned MB in Figure 19d contains 52 MTs, which is typical for this species.

b) Whole Mounts

Uranyl acetate-stained whole mounts (prepared in collaboration with Dr. W.D. Cohen) provided an alternate method of examining lysed specimens of the same material. With this technique the MB was readily visualized together with the associated electron-dense centrioles and the encompassing network in which were suspended the nucleus and various other organelles (Fig. 20). In Figure 20e deliberately underexposed photographs of MB-associated centrioles are shown to demonstrate their size (0.17x0.25 µm) and hollow cup-shaped structure (Cohen and Nemhauser, 1980). The centrioles here, too, are either adjacent or situated apart (Fig. 20f-k) and the space between the separated centrioles appears to contain fewer MTs than the remainder of the MB (cf. this Fig. h-k and Figs. 5d and 7g).

Figure 18. A. transversa. Thin Section of a Lyse-Fixed Preparation. The network (net) encloses the remaining cellular contents, including the nucleus (N), and MB. The latter is seen in near cross section in the area outlined by the box. The outlined area is shown at higher magnification in the inset where the microtubules (mt) comprising the MB and their associated centriole (ce) are clearly seen. The electron-dense, amorphous material, in which the nine MT triplets of the centriole are embedded, extends some distance to make contact with nearby MB microtubules. Peripheral MTs are in contact with the network. TEM. x16,600, inset x47,000

18

Figure 19. A. transversa. Thin Sections of Lyse-Fixed Material. (a) The longitudinally sectioned MTs lie at the cell periphery and follow its contours as defined by the network. The arrowhead indicates the location of inter-MT cross-bridges. (b & c) Longitudinally-sectioned MB-associated centrioles, the pair in b in the typical orthogonal configuration. MTs can be seen in contact with the amorphous material surrounding the centrioles. (d) Cross section of an MB containing 52 MTs. Cross bridges are seen between some MTs, while peripherally located tubules appear linked to the network. TEM. (a & b) x47,000, (c) x46,300, (d) x61,700

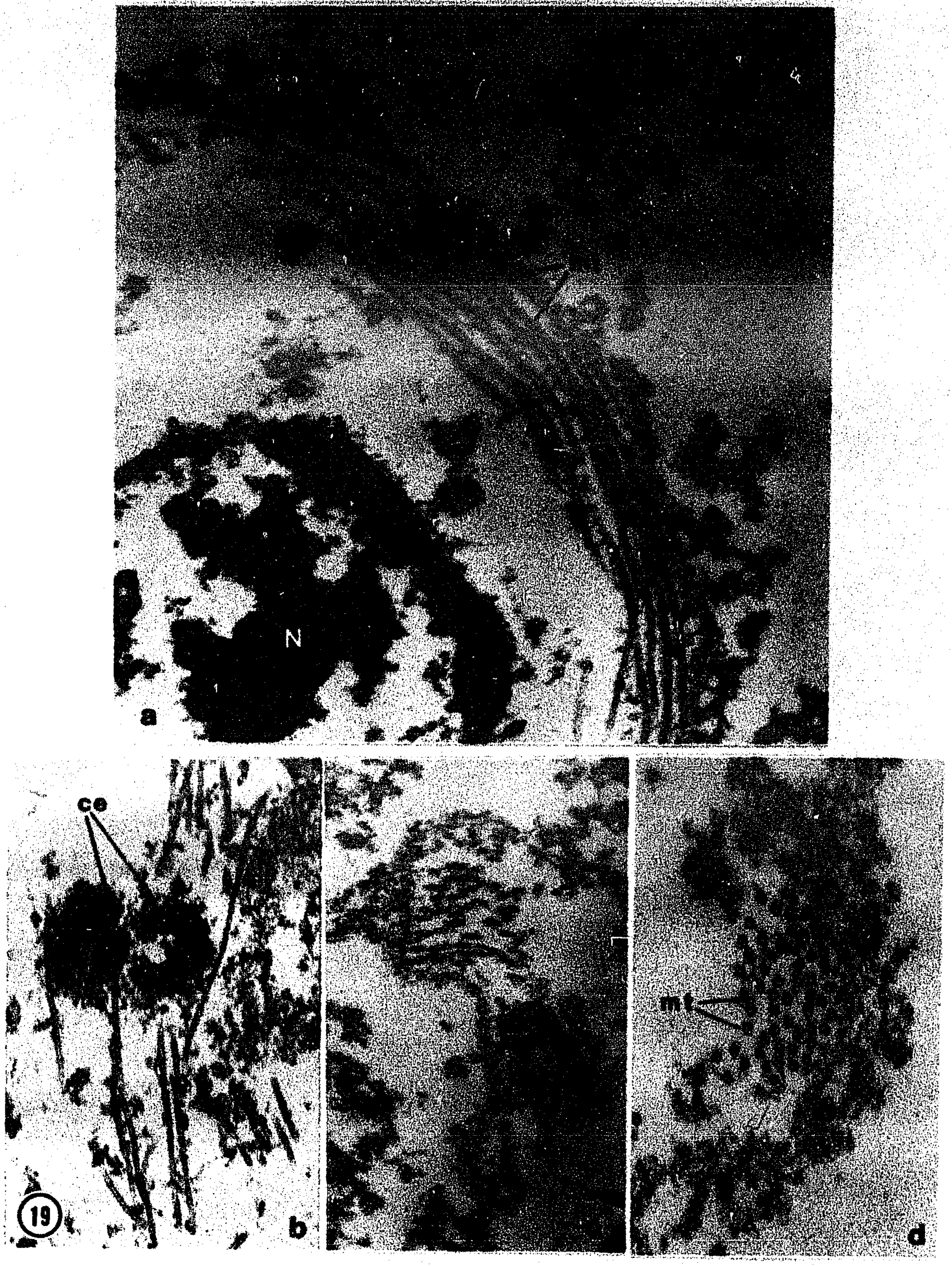
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Figure 20. *A. transversa*. Whole Mounts of Lysed Erythrocytes. In *a* an entire lysed erythrocyte is shown. The arrow points to the centriole pair associated with the MB that has assumed a figure-8 configuration. The network (Net) can be clearly seen in the region where it is folded over. (*b*) In another cell photographed at higher magnification, extra-MB MTs can be seen radiating from the vicinity of the centrioles. The meshwork-like nature of the network is also evident. (*c* & *d*) Centriole pairs in right-angle configuration. (*e*) Underexposure of the centriole pair in *d* to demonstrate their cup-shape structure. (fl) is a sperm flagellum inserted for size comparison. In *f* - *k* centriole pairs in which the relative positions of the pair members with respect to one another ranges from adjacent to separated by 2µm. TEM. (*a*) x12,000, (*b*-*e*) x54,000, (*f*-*k*) x18,000

3) N. ponderosa: Serial Sections

The relationship between marginal band MTs and centrioles was further investigated in serial sections of lyse-fixed N. ponderosa erythrocytes (Fig. 21). In the example shown in the figure, MTs insert into, or at least end at, the amorphous electron-dense material around one or the other of the centrioles in the right-angle pair (Fig. 21b & e). Other MT profiles are continuous in this region, generally passing peripherally between the centrioles and the network. Even in the section lying above the plane of the centrioles a centripetally located MT comes to an abrupt stop (Fig. 21a, arrowhead) at a point where, two sections later (Fig. 21b, arrowhead), similarly located tubules run into electron-dense material overlying a centriole. No MTs were seen running between the centrioles, the space between them appearing empty except for some fine filaments connecting the two structures (Fig. 21c, arrow). The "empty" space may correspond to the relatively less phase-dense areas seen between separated dots with the LM (Figs. 5d and 7g) and in TEM whole mounts (Fig. 20h-k). In Fig. 22, the filamentous nature of the tangentially sectioned network may be seen. Tannic acid treatment did not enhance the filamentous appearance of the network or of the links between the MTs, possibly because the application of the mordant was not simultaneous with fixation (Tilney et al., 1973).

III. MB Disassembly/Reassembly: N. ponderosa

1) Indirect Immunofluorescence

Experiments were performed to test whether the MBs of various species of Arcidae are cold-labile for, if so, they could provide an ideal system for analyis of the role of centrioles in MB assembly. Indeed, MBs were no longer visible in lysed erythrocytes examined under phase contrast within 1-1/2 hr after the cells

Figure 21. N. ponderosa. Serial sections of Lyse-Fixed Material. b - e are successive sections in the series, while there is one section missing between a and b. The arrowhead in a points to an MT that comes to an abrupt stop at a point where in b MTs are seen inserting into dense amorphous material (arrowhead) associated with a centriole seen a section later in c. The space between the centrioles is generally devoid of MTs, but in c fine filaments (arrow) are seen between them. MT profiles are observed in all sections in the region between the network and centrioles. In e, a grazing section of the lower centriole shows MTs inserting into the amorphous material associated with that organelle. TEM. x44,600

Figure 22. N. ponderosa. A Grazing Section of the Network. The filamentous nature of the network is clearly seen. TEM. x44,600

had been incubated in a $1^{o}C$ water bath. Furthermore, when the erythrocytes of N. ponderosa were allowed to rewarm to ambient temperature, they retained their native shape, and when these cells were lysed and observed with phase-contrast microscopy, the MBs, with their associated centrioles, had reappeared. Experiments were therefore performed to determine whether or not the MB-associated centrioles were responsible for nucleating, or in some way controlling, band assembly. To this end, MB disassembly/reassembly was followed in N. ponderosa erythrocytes with phase-contrast and immunofluorescent LM as well as TEM.

Figure 23a-d shows phase-fluorescent pairs of two lysed cells incubated with rabbit antitubulin antibody followed by FITC-conjugated goat anti-rabbit IgG. In these controls the MBs and centrioles were observed to stain specifically, the latter fluorescing more brightly in some instances (Fig. 23b). Neither the nuclei, the networks, nor any of the other organelles could be seen to bind antibody in detectable amounts. Immunofluorescence controls, in which preimmune serum (Fig. 23e & f) or FITC-conjugated goat anti-rabbit IgG alone (Fig. 23g & h) were used, served to demonstrate the specificity of the staining.

Cells stored for 23 hr at $0-2^{o}C$, then lysed, appeared to have no MBs when observed under phase contrast (Fig. 24a & c). This impression was confirmed when the same cells were examined for immunofluorescence (Fig. 24b & d). There was diffuse staining within the cell ghost, possibly the result of adventitious binding of tubulin to the network. The fluorescent sperm tails and unstained sperm nucleii in Figure 24 (c and d) served as internal controls.

After 30 min of incubation at ambient temperature, the MBs reappeared (Fig. 25). Where centrioles were visible, fluorescent fibers (presumably MTs) were observed to radiate from their vicinity (Fig. 25a & b). The ragged appearance of the forming MBs may be the result of a paucity of microtubule

Figure 23. *N. ponderosa.* Phase-Fluorescence Pairs. Indirect immunofluorescence using antitubulin antibody on lysed erythrocytes (*a* & *b* and *c* & *d*) of controls. Arrowheads point to dots, which in *b* fluoresce more brightly than does the MB. No other organelles are stained. (*e* & *f*) A control using pre-immune serum. (*g* & *h*) Another control in which only the secondary, FITC-conjugated, antibody was used. x2,400

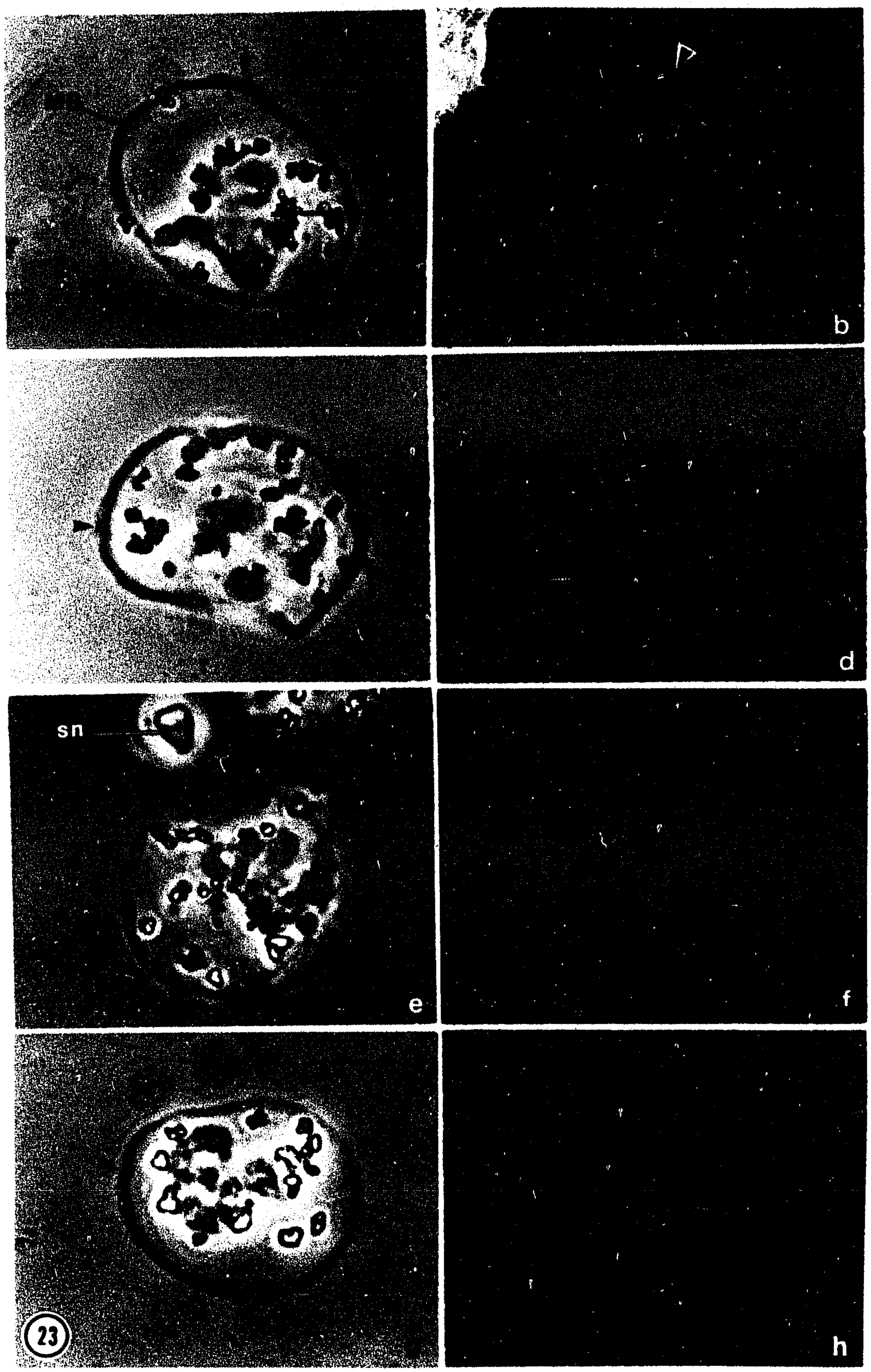
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Figure 24. *N. ponderosa*. Phase-Fluorescence Pairs of Erythrocytes Cooled to 0°C. The cells had been kept at 0-2°C for 23 hours, then lysed. In *a* and *b* the nuclei are surrounded by remnants of cellular organelles, but no MBs are evident. In *b* and *d*, a diffuse fluorescence is observed outlining the cell ghosts. The sperm tails which fluoresce brightly serve as internal controls (*c* & *d*). x2,400

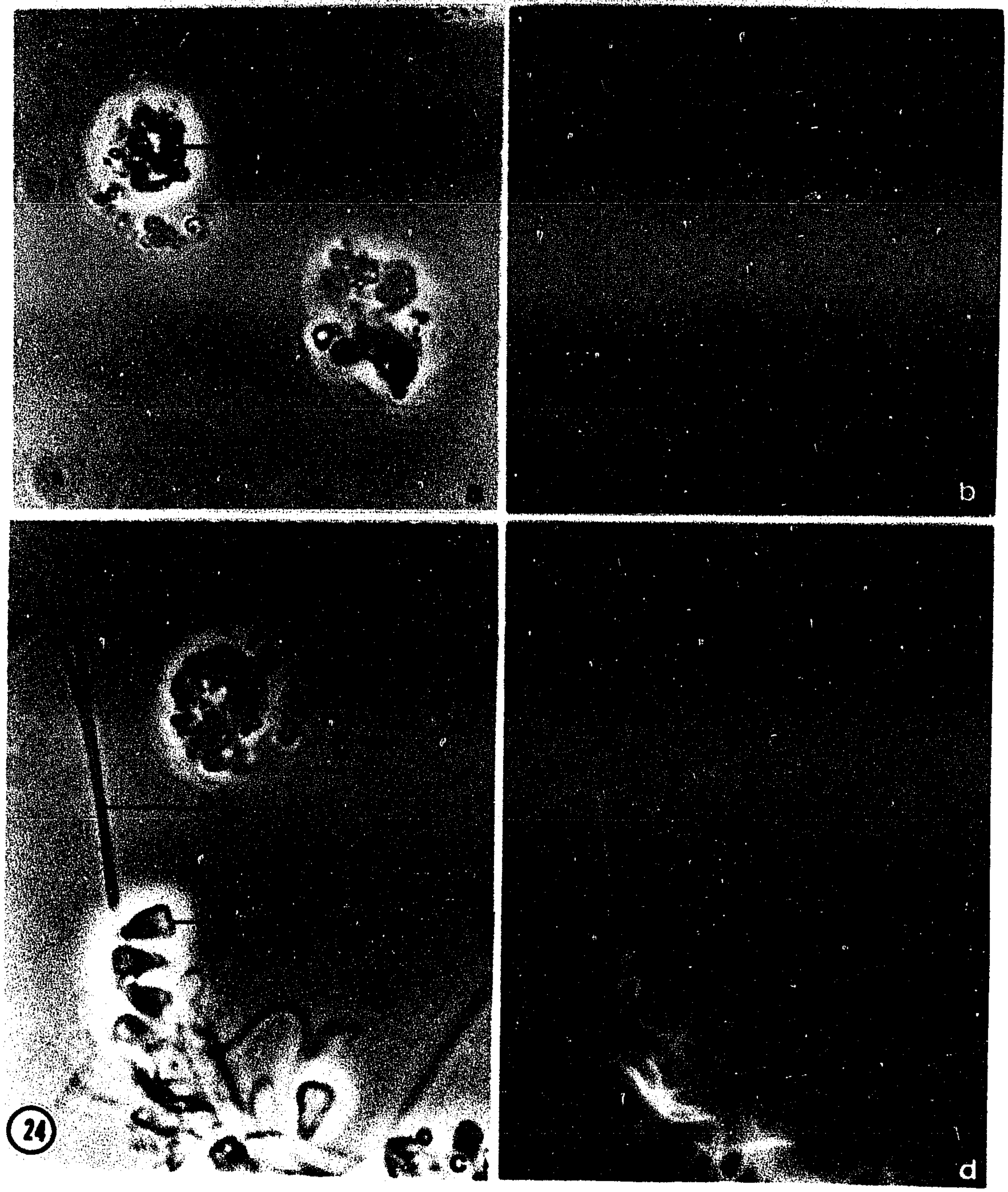

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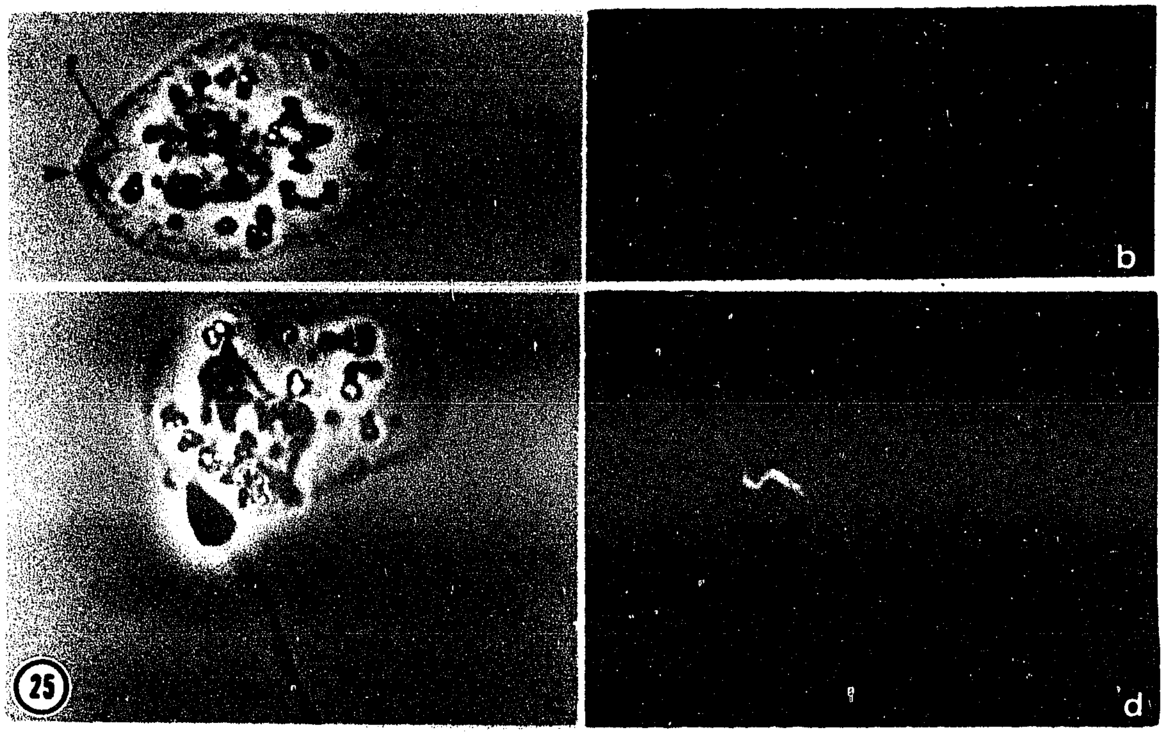
Figure 25. *N. ponderosa*. Phase-Fluorescence Pairs After a 30 Min Rewarming. After 30 min at ambient temperature the MBs have begun to reappear. In *a* and *b* tubulin-containing fibers (f) are seen emanating from the centriolar region (arrowhead in *a*). x2,400

Figure 26. *N. ponderosa*. Phase-Fluorescence Pairs After a 60 Min Rewarming. MBs are seen ranging in appearance from those that are similar to the 30 min rewarmed MBs (*a* & *b*) to those closer to completion (*c* & *d*). Long fibers (f) radiate from the centrioles (arrowhead in *c*) and extend toward more distant points on the MB. x2,400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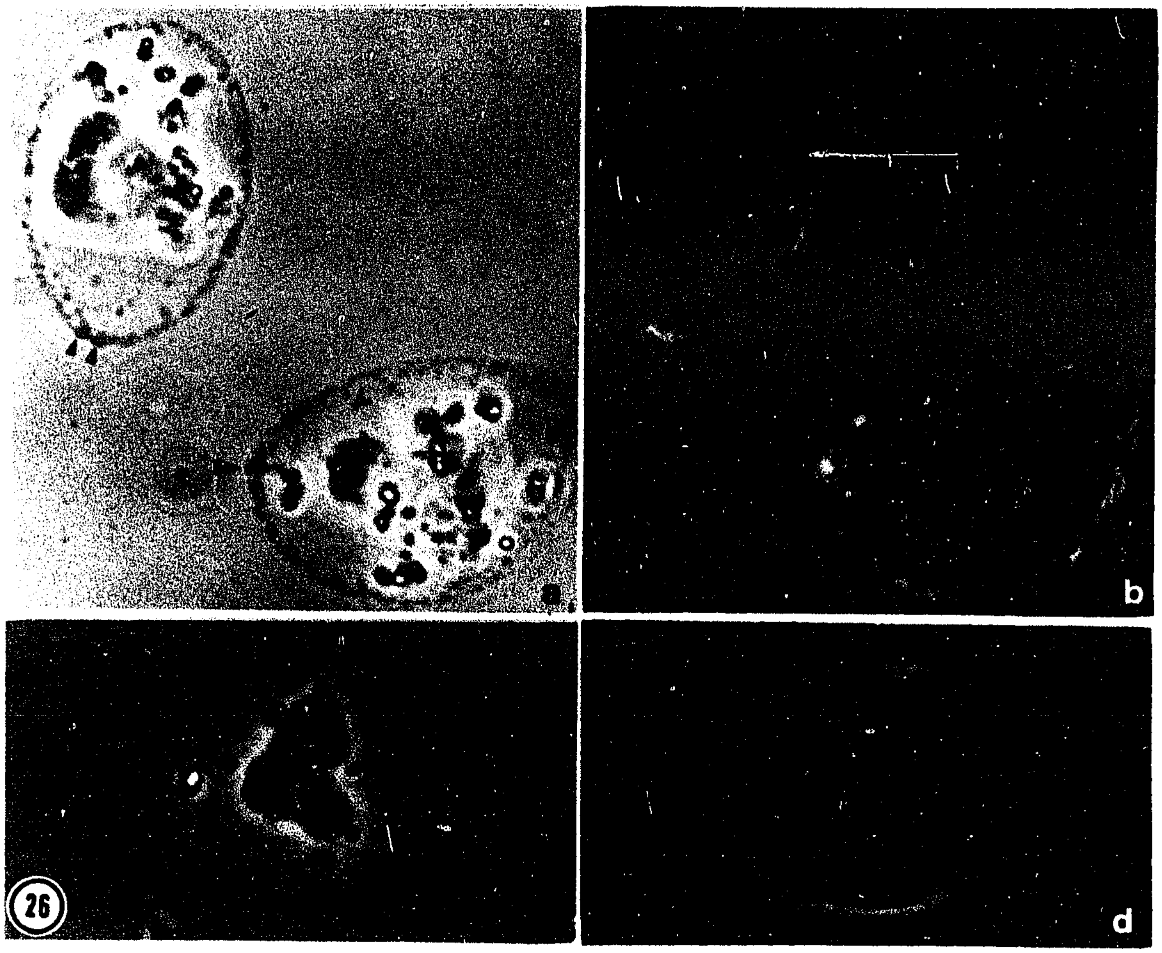
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associated proteins (MAPS), making them vulnerable to the suboptimal conditions that may have obtained during the experiment (see "Discussion"). In addition, although coverslips were carefully cleaned, the possibility of interaction of substances adherent to them with the MBs cannot be ruled out.

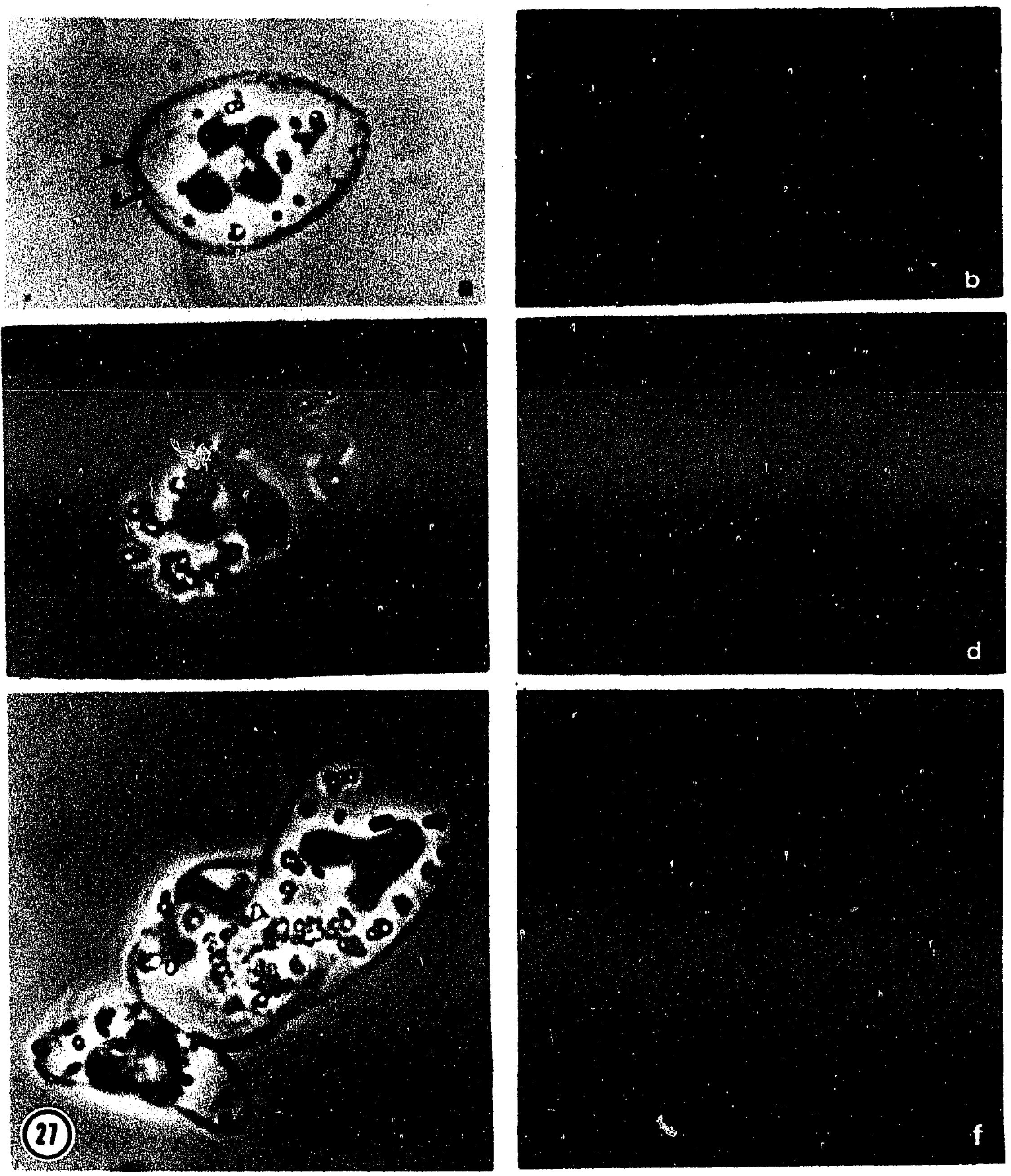
Erythrocytes rewarmed for 60 min before lysis contained MBs that appeared similar to those seen after 30 min rewarming (Fig. 26a & b). More solid appearing bands in the process of assembly were also seen (Fig. 26c & d). In the latter case, long tubulin-containing fibers were observed radiating from the region of the centrioles and eventually joining the MB. There was also a fairly solid stretch of MB running peripheral to the centrioles, and a similar tract on the opposite side.

After a 120 min incubation period at ambient temperature, relatively dense MBs were formed (Fig. 27a-d), often with thick fibers running from the centrioles toward the bands (a & b). Some MBs were virtually complete (cf. Fig. 27e & f and Fig. 23a-d). As the period of rewarming increased and the MBs advanced toward completion of assembly, there appeared to be a progressive decrease in nonspecific fluorescence, probably reflecting increased incorporation of tubulin dimers into the bands (cf. Figs. 24b & d, 25b & d, 26b & d and 27b,d & f).

Four-and-one-half hr of erythrocyte rewarming resulted in MBs such as those shown in Figure 28. These latter were not treated with antibodies and may be compared with freshly drawn lysed cells (Fig. 4). While the reassembled MBs appeared quite solid, they did not seem to be completely normal, i.e., not as dense as controls, an indication that either a longer incubation period may be necessary, or that perhaps it is not possible under the experimental conditions to re-form completely normal MBs.

Figure 27. *N.* *ponderosa*. *Phase-Fluorescence Pairs After 120 Min Rewarming*. The MBs are relatively more solid than those seen at earlier time points with MBs like those in *e* and *f* appearing virtually normal. x2,400

Figure 28. *N.* *ponderosa*. *MBs Rewarmed for 4.5 Hr*. The bands appear solid, but somewhat less dense than controls. x2,400



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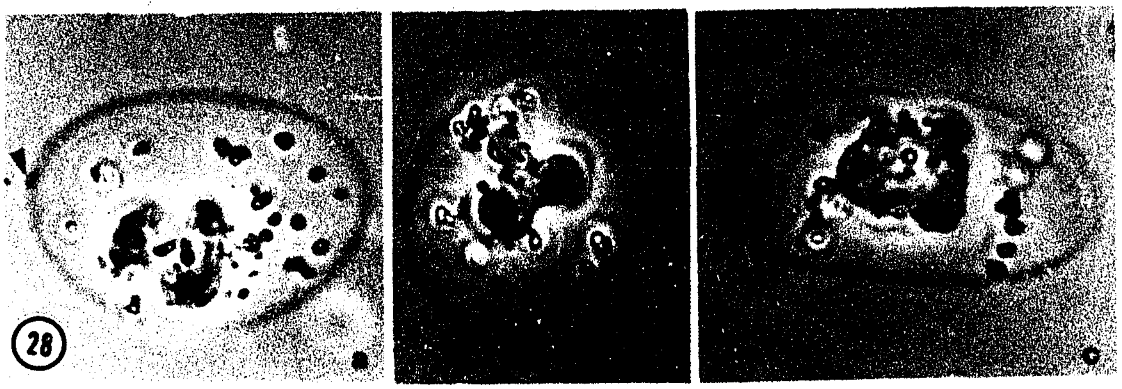

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2) Phase Contrast LM and TEM

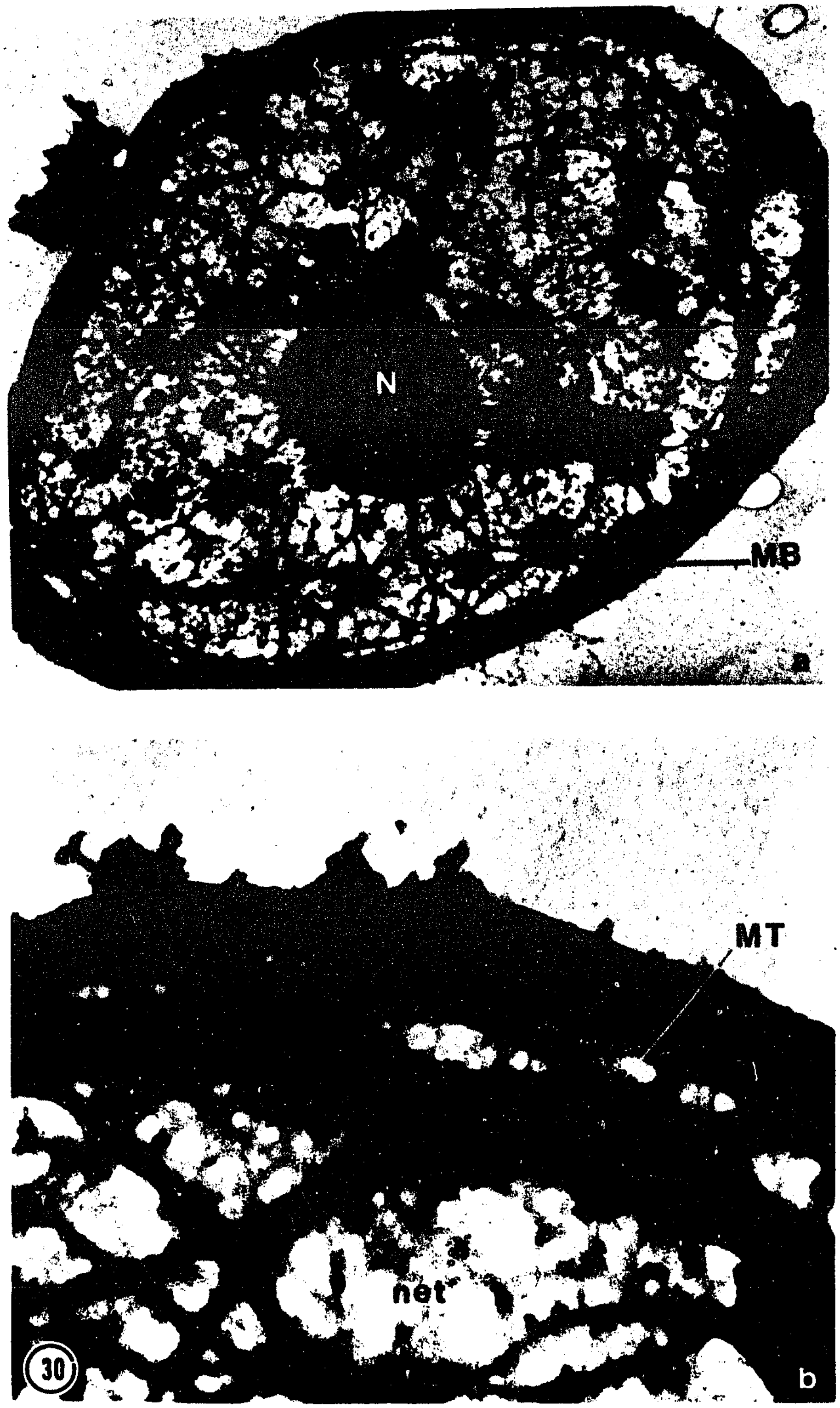
Additional disassembly/reassembly experiments were done in order to obtain confirmation of the above results as well as more information on the mode of MB formation and the possible role of centrioles in that process. An experiment (I) was done in which cells were cooled overnight, rewarmed at intervals, lyse-fixed and embedded for TEM observation of thin sections. A similar time course experiment (II) was done in which cells were cooled for 1-1/2 hr at $0^{o}C$; the rewarmed samples were lysed and examined under the phase-contrast microscope. Whole mounts of untreated controls and 120 min rewarmed lysed cells were examined with TEM as well. In the final experiment (III) both of the above mentioned methods were combined. Cells cooled overnight and allowed to rewarm for various intervals, were lyse-fixed and embedded for TEM after samples had been removed and photographed under phase-contrast LM. The results obtained for comparable time intervals of rewarming were essentially the same in all three experiments.

Thin sections of untreated controls revealed the presence of intact MBs which in cross-section contained from 51 to 85 MTs (Fig. 29). In favorable views of the entire cell it was possible to observe the MB at opposite poles of the cell, just beneath the network (Fig. 29a). Peripherally located MTs appeared to be in close association with the network and in some instances filamentous bridges were seen extending from individual MTs toward elements of the network (29a inset). Bridges appeared between neighboring MTs as well (29a inset, b & c). The negatively stained whole mounts in Figure 30 illustrate the mesh-like nature of the network. Centrioles, as expected, were associated with the MBs (Fig. 29d & e) and once, a cell without MBs was seen (Fig. 29f). The latter most likely corresponds to the occasional cell observed under the LM with no discernible MB.

N. ponderosa intact erythrocytes incubated at $0^{o}C$ until they had lost their

Figure 29. N. ponderosa. Lyse-Fixed Erythrocytes. MB Disassembly/Reassembly Controls. (a) The section contains a profile of a cell in which the transected MB is seen to be positioned so that it appears to stretch the overlying network, thus causing the flattened shape of the cell. The inset contains the boxed area at higher magnification and shows the cross-sectioned MTs, in some cases with links between them and associations between peripheral tubules and the network. b & c show two more MBs in cross section in protrusions of the network. In b inter-MT cross-links are seen, but not observed as readily in c where the MTs are also spaced further apart from one another. Centrioles are associated with the MBs in d & e. The centriole pair in f is located in a cell in which no MTs were seen. TEM. (a) x16,700, inset x48,000, (b & c) x59,000, (d-f) x33,700

Figure 30. N. ponderosa. Whole Mounts. MB Disassembly/Reassembly Controls. In b individual MTs can be discerned as well as the filamentous network (net). TEM. (a) x11,000, (b) x44,200



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MBs, retained their native elliptical shape, but their edges appeared somewhat wrinkled when the cells were fixed immediately with 1% glutaraldehyde (Fig. 31a). Unfixed erythrocytes wrinkled moderately on initial rewarming, but soon regained their native shape (results not shown). Microtubules were not observed anywhere in thin sections of the lysed cells (Fig. 31), nor were MBs detected under the LM. Centrioles were observed (Fig. 32) completely denuded of MBs and associated with electron-dense flocculent material (Fig. 32b & c). The networks, apparently no longer stretched by the MBs, assumed a random configuration (Figs. 31b & c; 32c).

When cells were rewarmed for 15 min and then fixed intact, they had smooth edges and occasional points at one end of the ellipse (Fig. 33a). Under phase contrast, lysed cells appeared to have continuous MBs that were often somewhat pointed (Fig. 33b-c). Some bands did not have points (Fig. 33d). The cell in Figure 33c was photographed through an air bubble, facilitating the visualization of fine threads radiating from the centrioles. Cross and longitudinal thin sections confirmed the reassembly of discrete MBs in this material as well as their association with centrioles (Fig. 34). The MBs were seen in cross section to contain fewer MTs than the controls with counts ranging from 33 to 38. In addition, in both cross and longitudinal sections there appear to be fewer connections between adjacent MTs and between peripheral microtubules and the network than were seen in untreated controls.

Nearly normal-appearing, usually elliptical, MBs were recovered when N. ponderosa erythrocytes were rewarmed at 25°C for 120 min (Fig. 35b-e). This was especially true when the cells were cooled for 1-1/2 hr (Fig. 35d & e) instead of overnight (Fig. 35b & c). The unfixed intact cells appeared normal also (Fig. 35a). A whole mount of the lysed material examined with TEM revealed an intact MB and a network (Fig. 36b; cf. Fig. 30). Discrete MBs were seen in thin

Figure 31. N. ponderosa. Erythrocytes Cooled to 0-2°C. (a) Intact cells, fixed in 0.1% glutaraldehyde, retain their elliptical shape, but have wrinkled edges. (b) and (c) No MTs are seen anywhere in this lyse-fixed material and the networks leave assumed a random configuration. (a) Phase contrast. (b & c) TEM. (a) x960, (b) x30,800, (c) x14,500

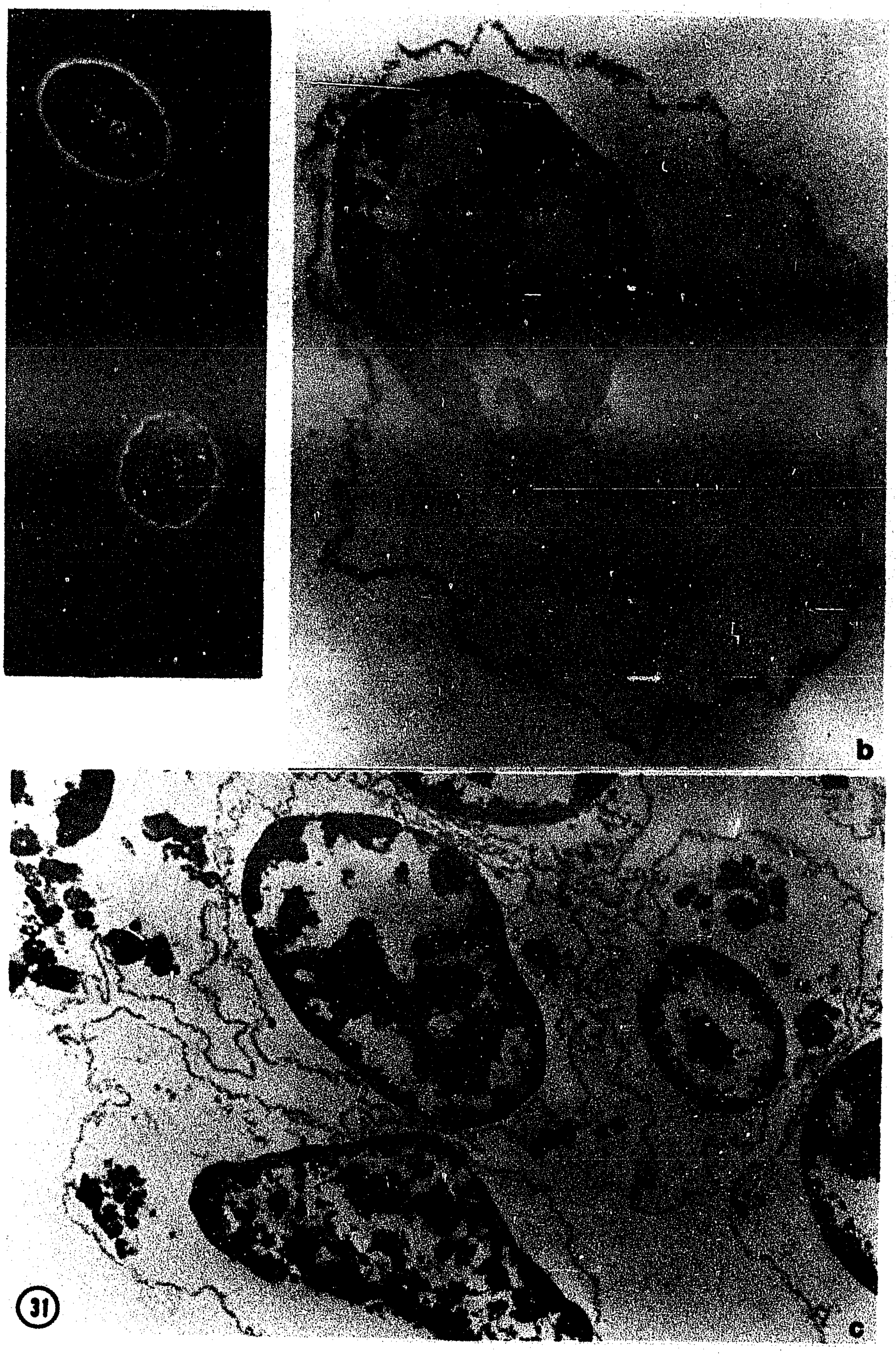
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Figure 32. *N. ponderosa*. Erythrocytes at 0°C. The phase-contrast image in (a) shows a nucleus surrounded by cell organelle remnants and two phase-dense dots (arrowhead). In b and c centrioles devoid of MTs are seen in the sectioned material. The higher magnification inset of c shows the centriole embedded in dense flocculent material. (a) Phase Contrast. (b & c) TEM. (a) x1,700, (b) x46,200, (c) x27,300, inset x45,400

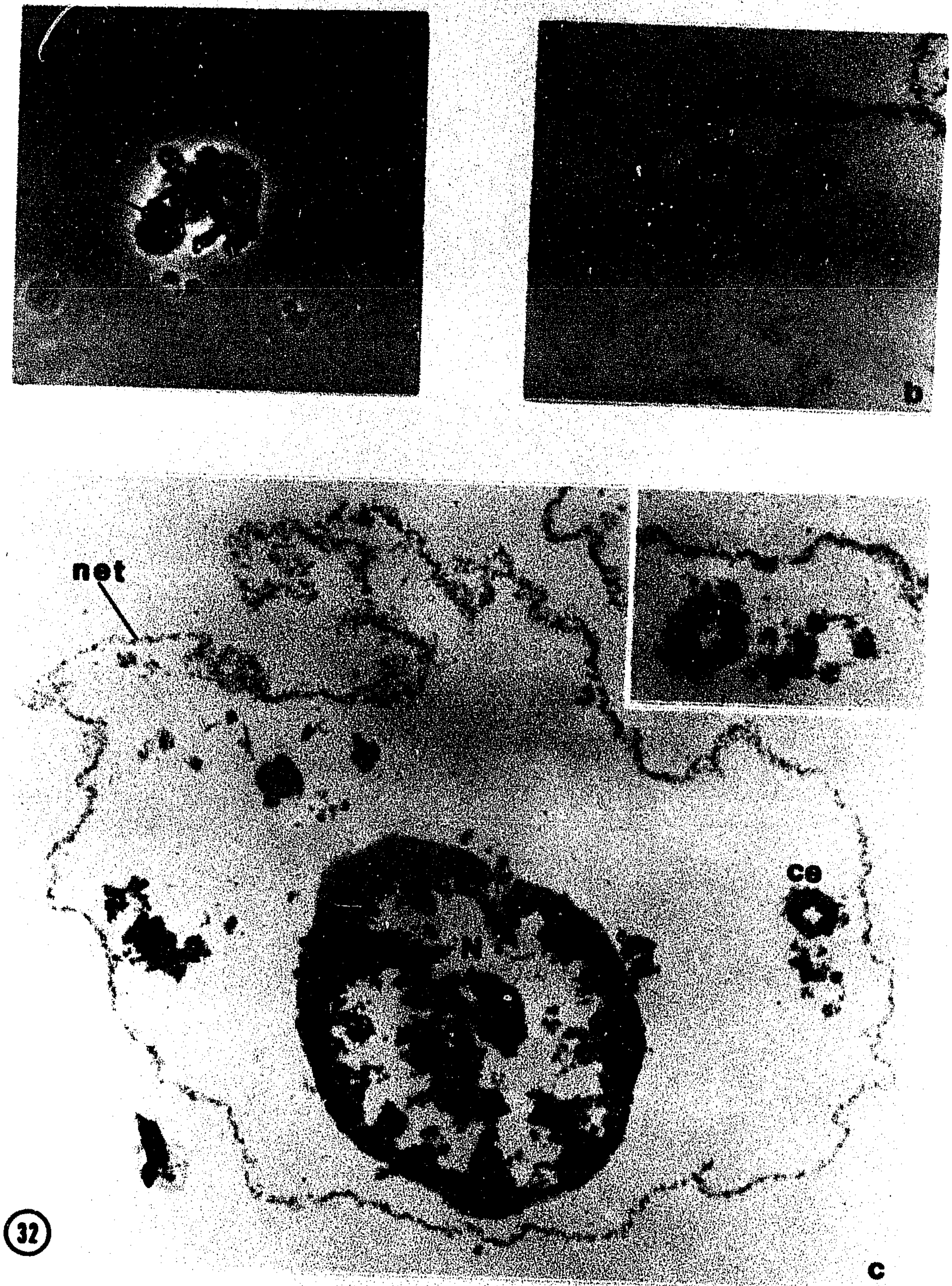

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Figure 33. N. ponderosa. Erythrocytes Rewarmed for 15 Min. The intact cells in a have smooth outlines and usually come to a point at one end of the ellipse (arrow). The lysed cells in b and c have centrioles at the MB points. The cell in c was photographed through an air bubble facilitating visualization of the fibers emanating from the centriolar region. Rarely, MBs like those in d were encountered in which the dots are not located at points. Phase contrast. (a) x960, (b-d) x1,700

Figure 34. *N*. *ponderosa*. *Erythrocytes Rewarmed for 15 Min*. MBs consisting of loosely-packed MTs are seen in cross section in *a*, *b*, *d*. and *e*. Few if any cross bridges are detected. (*c*) In longitudinal section the long parallel MT profiles appear also to have few cross links. The MBs are typically observed in protrusions of the network and are associated with centrioles (*d*, *e* and *f*). The arrow in (*f*) indicates the fibrous network that can be seen to advantage in this grazing section. TEM. (*a*) x46,500, (*b*) x37,200, (*c* & *d*) x46,300, (*e*) x37,400, (*f*) x37,300

Figure 35. N. ponderosa. Erythrocytes Rewarmed for 120 Min. The unfixed intact cells in a are smooth contoured ellipses. The MBs of lysed erythrocytes are usually elliptical (c-e), rarely coming to a point graced by dots (b). In the elliptical MBs the dots are located at (d & e) or near (c) the ends of the ellipses. In d and e fibers emanate from the vicinity of the centrioles, joining the MBs some distance away. In thin sections at low magnification MBs are seen in all network protrusions. The arrows indicate MB associated centrioles. (a-e) Phase contrast. (f) TEM. (a) x630, (b-e) x1,700, (f) x18,250

section to be located in network protrusions similar to those seen in controls (Figs. 35f and 36a), giving the distinct impression that the MB is involved in conferring flattened shape on the erythrocytes via its influence on the network. Connections could be seen between MTs (Fig. 36a). Centrioles were observed in association with MT bundles (Figs. 35f; 36c-e), and the number of MTs per MB ranged from 31 to 41. In addition, in Figure 36e the filaments comprising the network are readily observed. The foregoing results demonstrated that the MBs reassembled in association with centrioles, but did not provide evidence for a role of the centrioles in that process.

3) Very Early Stages of Reassembly

The key to obtaining such evidence turned out to be the examination of cells after much shorter rewarming periods. In experiments II and III rewarming was therefore interrupted after 2 and 5 min, as well as after the above-mentioned time intervals. After a 2 min rewarming at 25°C, the intact cells fixed in 1% glutaraldehyde had become somewhat less wrinkled, usually with a smooth outline along one end of the ellipse (Fig. 37a & b). Observed under phase contrast, lysed cells were seen to have centrioles with threads emanating from their vicinity (Fig. 37c-e). In some instances the threads could be seen to end at distances of 3.5 to 8 μm from the centrioles (Fig. 37c & d). Thin-sectioned material provided ultrastructural confirmation of the LM findings. Short lengths of MTs were seen near the centrioles and gave the appearance of emanating from or focussing on the latter (Fig. 37f; Fig. 38). While in one instance MTs were observed to extend approximately half way along the length of the cell (Fig. 37f), on no occasion were microtubules seen at the end of the cell opposite the centrioles. Cell outlines, as defined by the network, were similar to those seen in the 0°C sample.

Five minutes of rewarming resulted in intact cells with one very smooth

Figure 36. N. ponderosa. Erythrocytes Rewarmed for 120 Min. (a) and (c-f) are higher magnification views of the MBs. The arrow in a indicates a pair of MTs with a bridge between them. Centrioles are associated with the MBs in c-e. In e the fibrous network (arrows) is seen in tangential section. (b) Part of a reassembled MB is seen as a whole mount. TEM. (a) x39,900, (b) x5,600, (c) x33,100, (d) x45,900, (e) x45,200

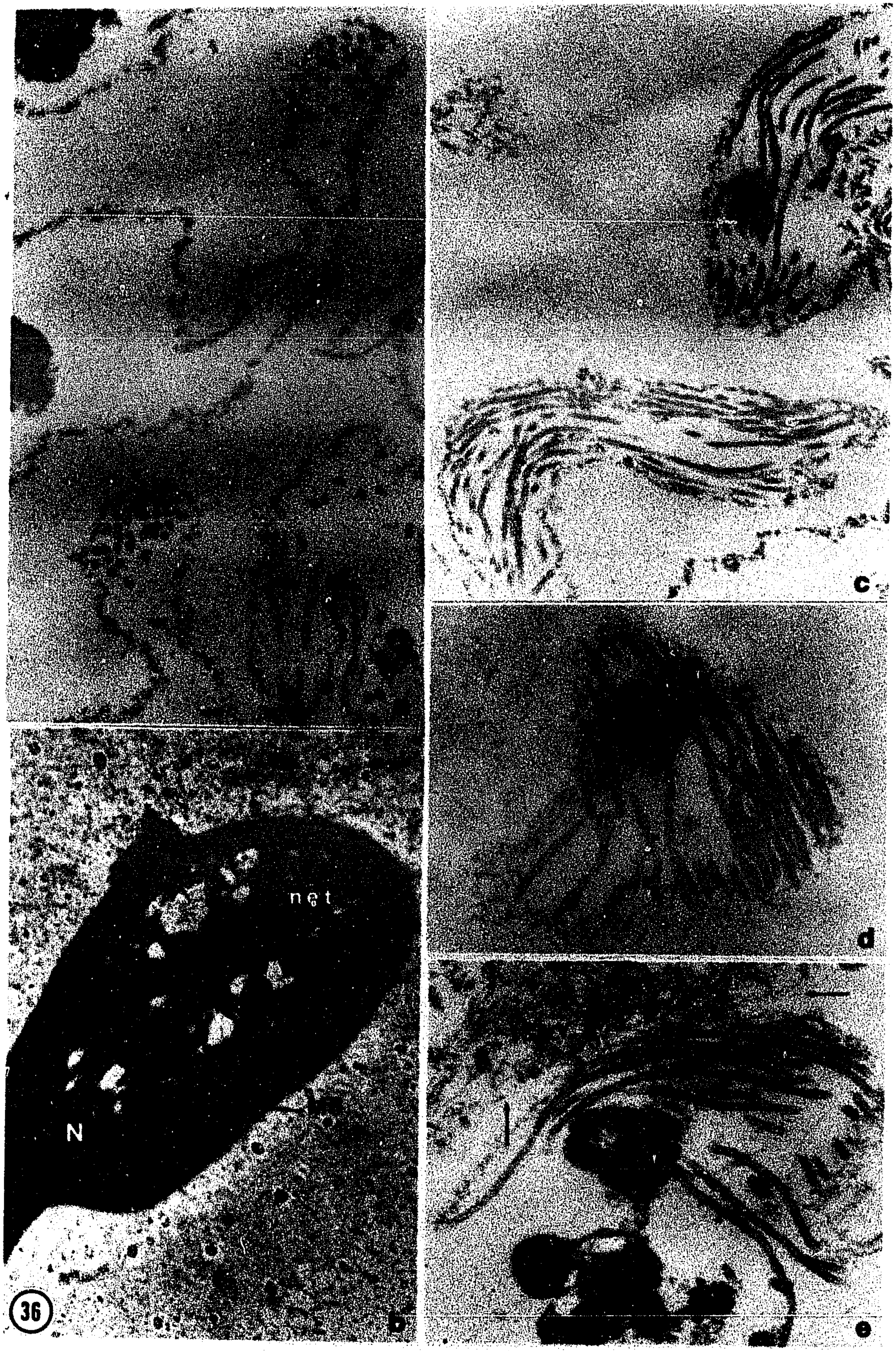
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Figure 37. N. ponderosa. Erythrocytes Rewarmed for 2 Min. The cells depicted in a and b are smooth at one end of the ellipse and have a slightly wavy contour along the remainder of the cell. (c-e) Threads (arrows) focussing on the dots come to a point in the vicinity of the latter. In c and d the threads do not circumscribe the cell, but can be seen to end at a distance of 3.5 to 8 µm from the dots. (f) In thin sections short lengths of MTs are observed radiating from the region of the centriole. Short MT profiles are observed up to about half the distance from the centriole to the opposite end of the cell, but no further. (a-e) Phase contrast (f) TEM. (a & b) x960, (c-e) x1,700, (f) x17,200

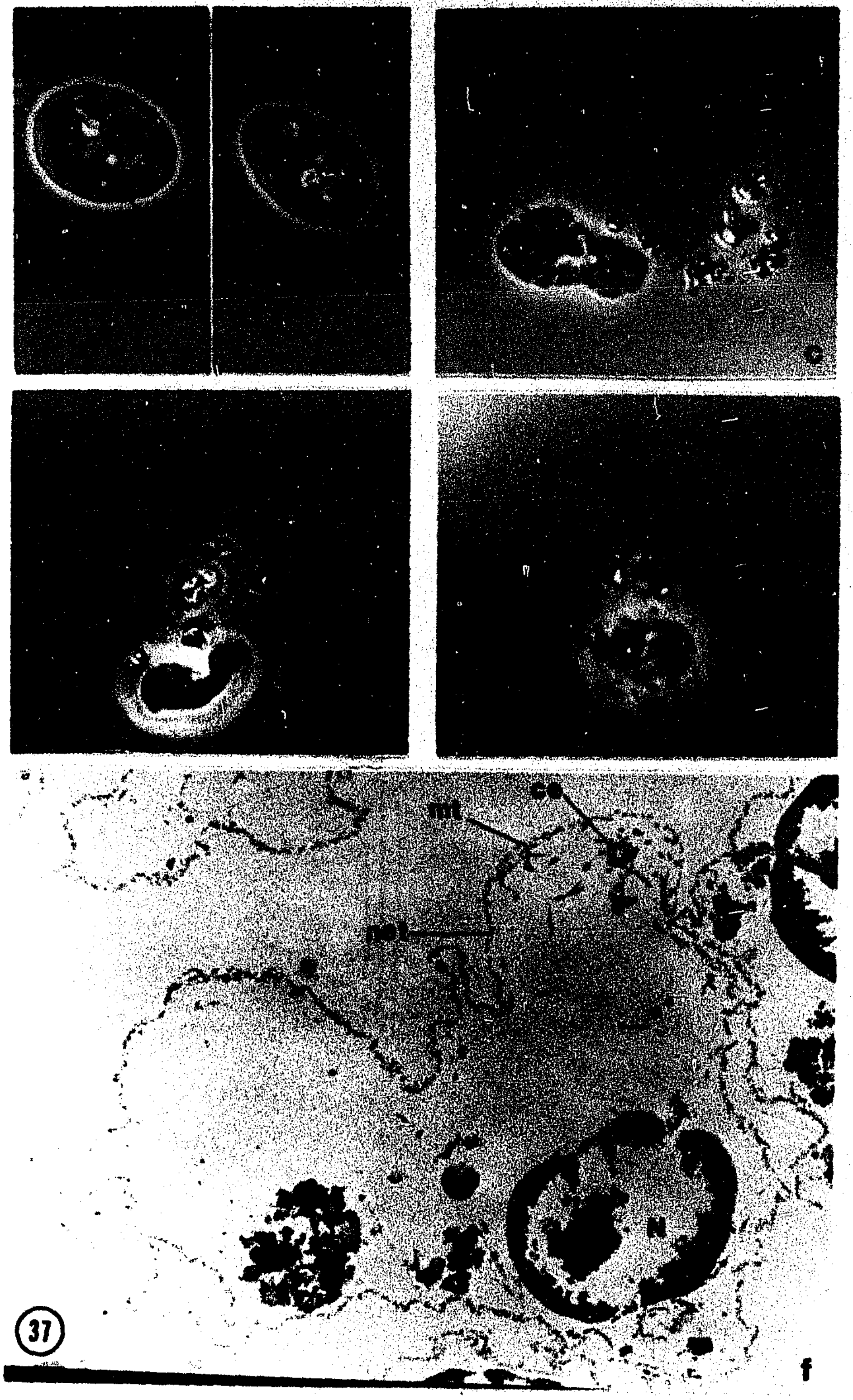

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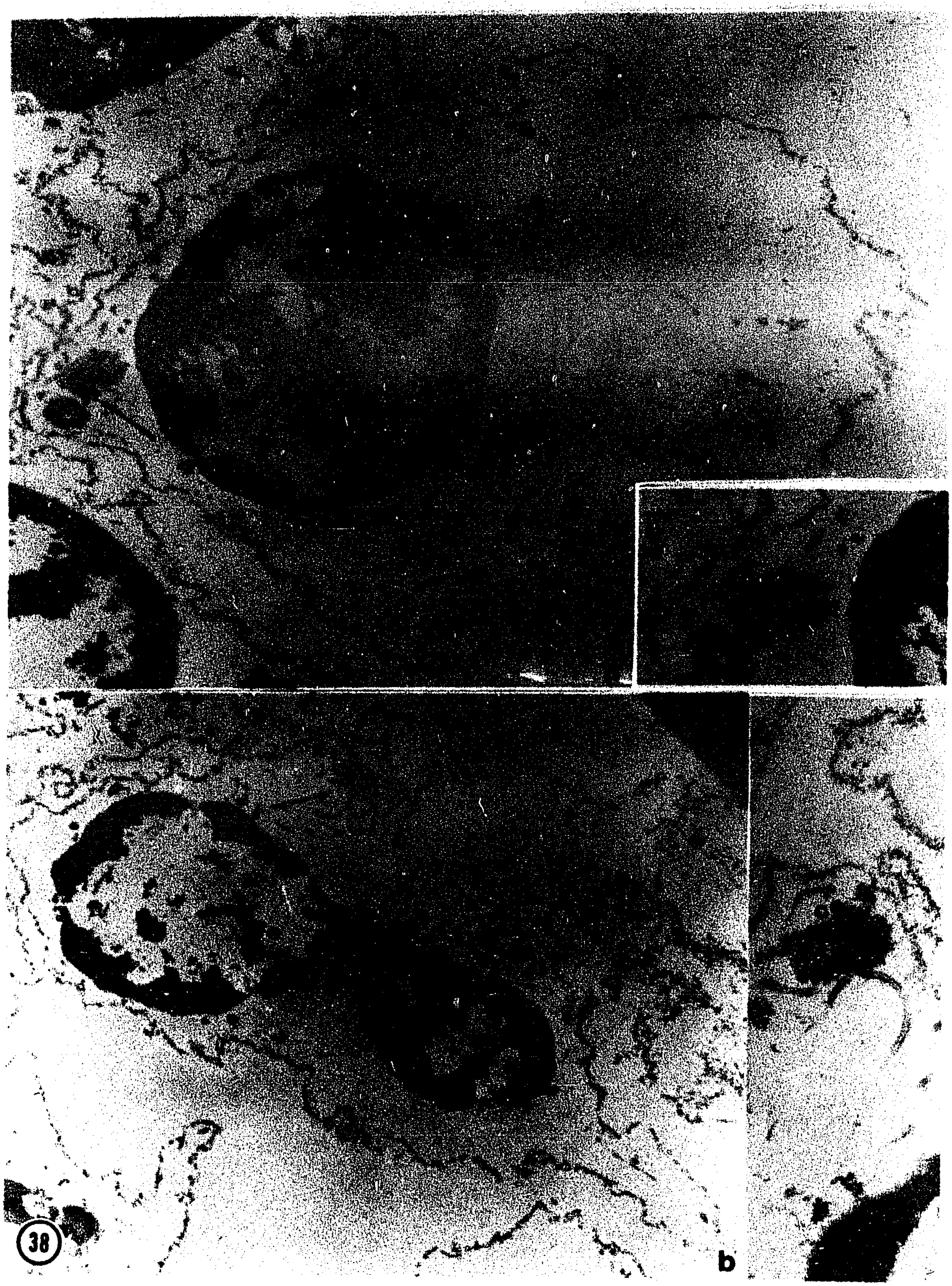
Figure 38. N. ponderosa. Erythrocytes Rewarmed for 2 Min. These thin sections show short lengths of MTs in the region of the centrioles. In *b* MTs appear to be inserted in the dense material around the centriole. Insets are higher magnification photographs of the centriolar regions. No MTs are observed at the ends of the cells opposite the centrioles. TEM. (*a*) x28,000, inset x45,400, (*b*) x19,600, inset x45,600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end of the ellipse and an opposite slightly wrinkled edge (Fig. 39a & b). In lysed cells viewed under the LM, continuous, albeit very thin, MBs could be seen which came to a point in the region of the centrioles (Fig. 39c-f). In panels d and e of the figure, the former photographed through an air bubble, threads could clearly be seen emanating from the centriolar area. Thin sections revealed the presence of MTs in all regions of the cells. Although the MTs do not appear to be organized into well-defined bundles (Fig. 40b), an emerging plane of flattening is defined by the disposition of the nascent MBs which can be seen in near cross section at opposite poles of many cells (Figs. 39g; 40a). MTs focussed on the centrioles in greater numbers than those seen in the 2 min samples (Fig. 41a). The regions containing the centrioles were often observed in protrusions of the network, giving the impression that the growing MTs were stretching the encompassing material (Fig. 41b & c). Figure 40(c), a tangential section of the nascent MB, illustrates its lack of tight organization (cf. Fig. 34c).

IV. Effect of Colchicine on Reassembly of MBs: N. ponderosa

Samples of N. ponderosa erythrocytes suspended in I.O. and incubated for 1 hr at 0°C in the presence (C) or absence (NC) of 0.1 mM colchicine were observed, upon subsequent lysis to have lost their MBs (Fig. 42a & b). Additional samples of both C and NC were resuspended in I.O. without colchicine, and rewarmed at ambient temperature for 1 hr. In these, the MBs reassembled, but whereas those in the lysed NC cells appeared normal, the MBs in the C sample had points in the vicinity of the centrioles (Fig. 42c & d). The same results were obtained when the cells were kept at 0°C for 1-1/2 hr followed by 19-1/2 hr without colchicine at ambient temperature (approx. 20°C; Fig. 42e & f).

In another experiment, cells were incubated for 2 hr at 0°C at which time colchicine (to a final concentration of about 0.1 mM = C) or Instant Ocean (NC)

Figure 39. N. ponderosa. Erythrocytes Rewarmed for 5 Min. The intact cells have fairly smooth edges (a & b) typically with a slight bump at one end (b). The lysed cells (c-f) have MBs that extend all the way around the cell (small arrowhead in d) and come to a point in the vicinity of the centrioles. The fibrous nature of the MB is seen to advantage in the MB in d which was photographed through an air bubble. (g) In sectioned material at low magnification MT profiles are observed at opposite sides, or poles, of the cells (arrows). (a-f) Phase contrast (g) TEM. (a & b) x960, (c-f) x1,700, (g) 14,400

Figure 40. N. ponderosa. Erythrocytes Rewarmed for 5 Min. In (a) the nascent MB is seen in section at opposite poles of the cell (arrows). MT profiles, often considerably longer than those observed in the 2 min rewarmed samples, are seen throughout the cell in b. (c) The short profiles of the MTs in this tangential section indicate that organized bundles have not yet been formed. TEM. (a) x16,500, (b) x23,000, (c) x27,300

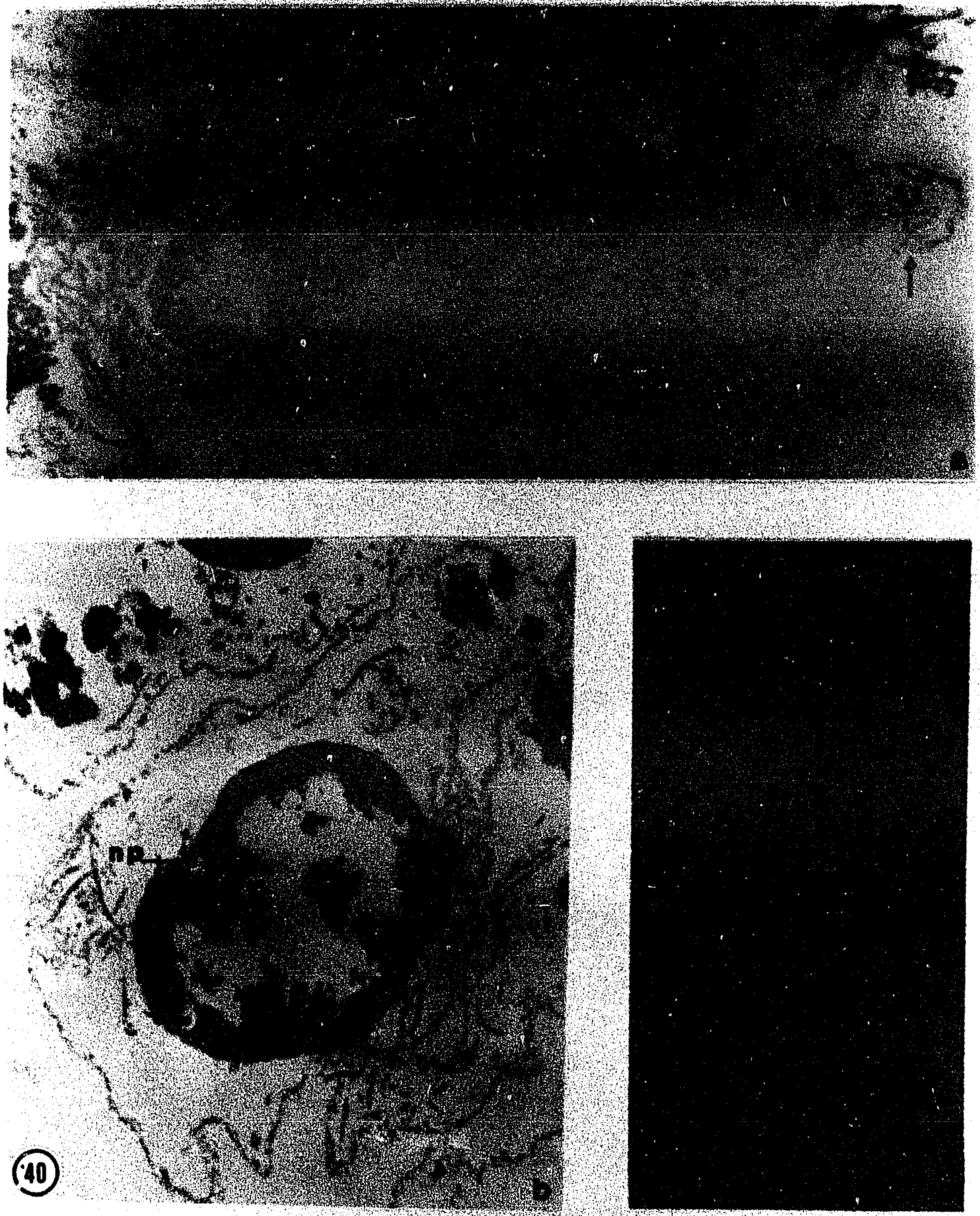
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Figure 41. N. ponderosa. Erythrocytes Rewarmed for 5 Min. Centrioles are associated with the reassembling MBs. In a MTs (arrow) are seen at the side of the cell opposite the centrioles. The centrioles in b and c, associated with relatively long MT profiles, are located in network protrusions. Long network filaments are seen in c (small arrow). TEM. (a) x15,700, (b) x45,600, (c) x45,700

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Figure 42. *N. ponderosa*. Effect of Colchicine on MB Reassembly. The cells were incubated for one hr at $0^{\circ}C$ in the absence (a, c and e) or presence (b, d and f) of 0.1 mM colchicine. In c and d the cells were lysed after 1 hr of rewarming at ambient temperature, while in e and f the cells were rewarmed for 19-1/2 hr before lysis. The cells in a and b, which were not rewarmed, have no MBs. In the untreated rewarmed cells normal appearing MBs reappear, whereas the reassembled MBs in the colchicine treated cells are relatively less dense and come to a point in the region of the centrioles. Phase contrast. (a-d) x1,000, (e & f) x2,300

was added to the tubes. After 1 hr at ambient temperature, very thin MBs had formed in both preparations, but again the C bands had points at one end with the centrioles located at the tips (Fig. 43a & b). Successively longer rewarming periods resulted in progressively thicker and more elliptical MBs in the NC samples while the C MBs remained flimsy and pointed (Fig. 43c-i). The two types of experiments appear to demonstrate that colchicine binding to the tubulin subunits results in abnormal or incompletely reassembled MBs.

V. Phylum Arthropoda: Other Blood Cell Types

1) Homarus americanus. Coelomocytes (Clotting cells). The coelomocytes of H. americanus are flattened and elliptical, and can be preserved in their native state by dilution into crustacean Ringer's solution containing 0.1% glutaraldehyde (Fig. 44a). When the cells were diluted 1:10 (vol/vol) into Ringer's solution (without glutaraldehyde) followed by immediate lysis, the MBs were readily visualized (Fig. 44b & c). When freshly drawn cells were placed directly on a slide they rapidly assumed an amoeboid shape (Fig. 45a). At this time, if the cells were lysed by perfusion, the MBs seemed to have disappeared (Fig. 45b).

MB Disassembly

Indirect immunofluorescence, using antitubulin antibody and FITC-conjugated secondary antibody, was employed to follow the sequence of events during MB disassembly as the cells undergo morphological transition (Fig. 46). Five minutes after cellular transformation had begun, some MBs appeared to be undergoing disorganization (Fig. 46b) while others still remained intact (Fig. 46a). It should be noted that the cells were not synchronized with respect to their rate of transformation. After 10 min the MBs were barely recognizable (Fig. 46c), while at the end of the 20 min spreading period no

Figure 43. *N. ponderosa*. Effect of Colchicine on MB Reassembly. Erythrocytes were cooled for 2 hr at 0°C at which time 0.1 mM colchicine was added to the experimental tubes (b, e, g & i) and all tubes were rewarmed at ambient temperature for 1 (a & b), 2 (c, d, & e), 2-1/2 (f & g) and 3 hr (h & i). In the controls (a, c, d, f & h) elliptical, normal appearing MBS eventually re-formed. In the colchicine treated cells flimsy MBs were formed. These come to a point in the vicinity of the centrioles. Phase contrast. x1,000

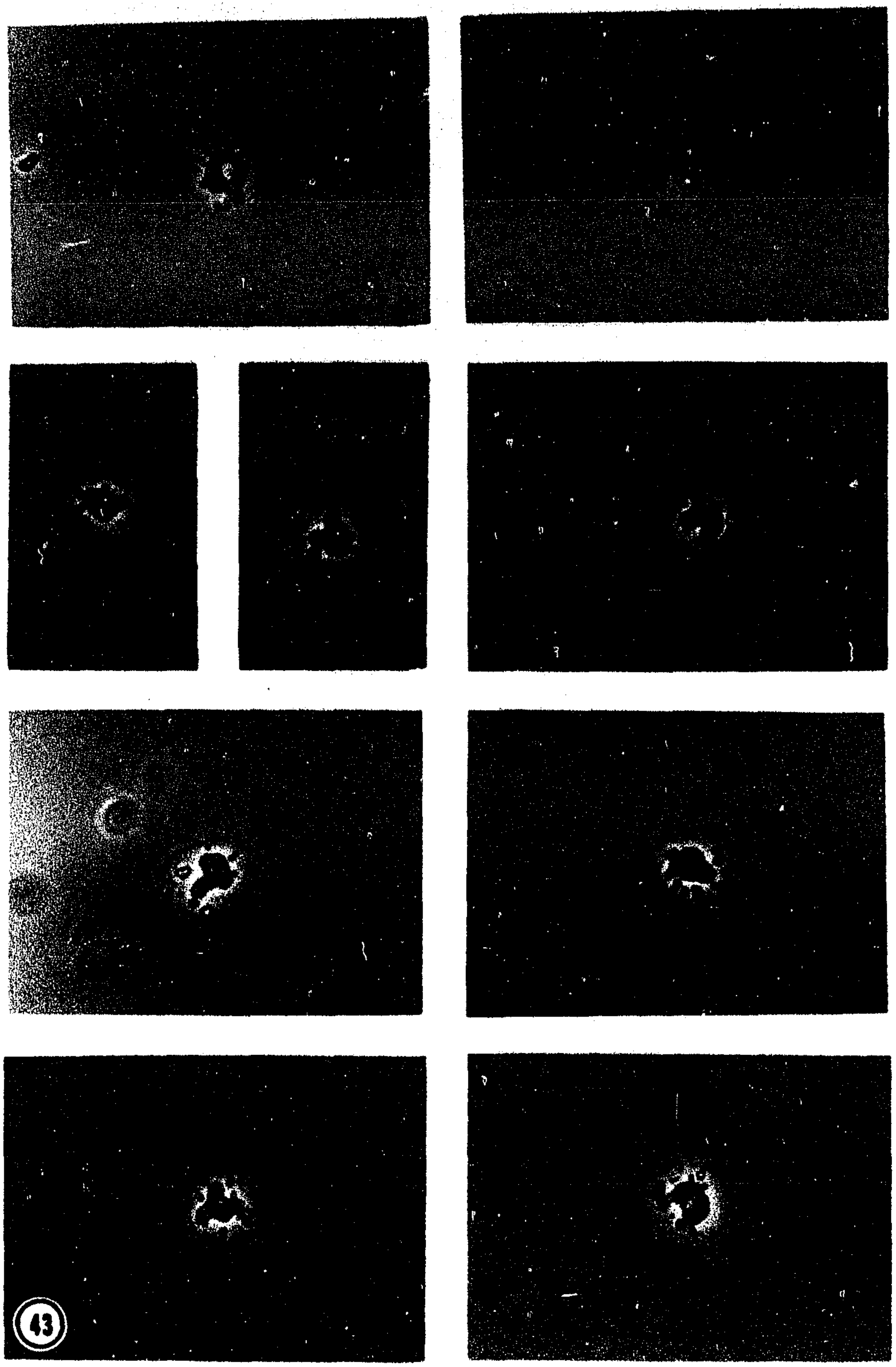
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Figure 44. Homarus americanus coelomocytes. The flattened elliptical shape of these granule-filled clotting cells is depicted in A. When cells, prevented from undergoing morphological transformation, are lysed (B and C) they are observed to contain MBs. c = coelomocyte. Phase contrast. x950

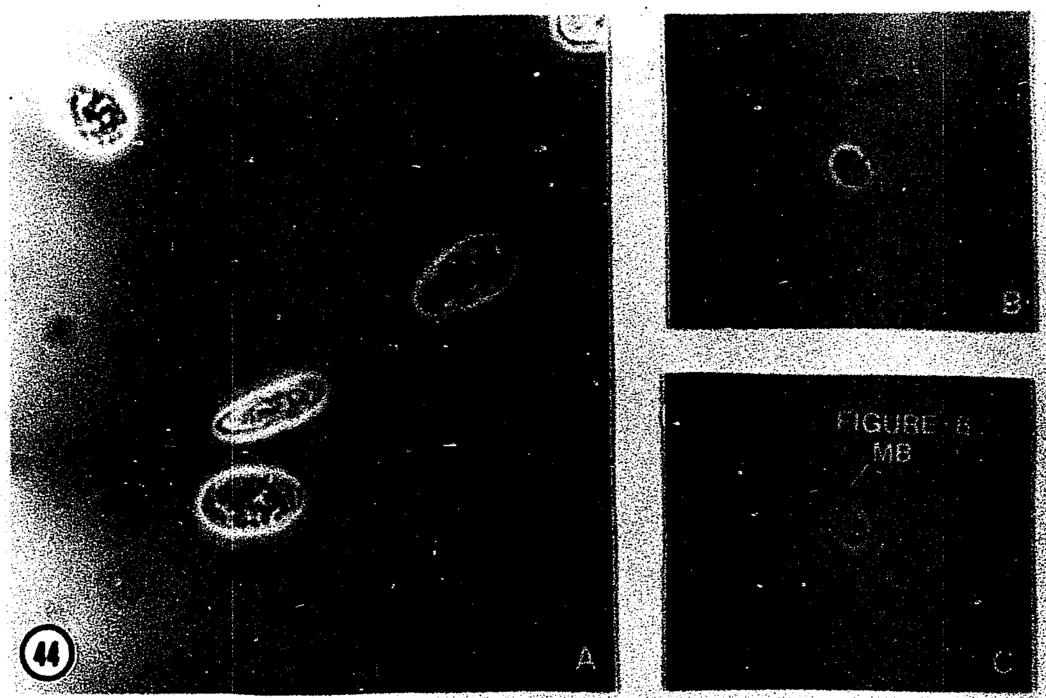
44
A
B
FIGURE 6
MB
C

Figure 45. H. americanus. Transformed Amebocytes. When the spread cells (A) are lysed by perfusion (B) no MBs are seen. Phase contrast. x425

Figure 46. H. americanus. MB Disassembly in Transforming Cells. With indirect immunofluorescence using antitubulin antibody, stages in natural MB disassembly can be followed. The cells were lysed by perfusion after intervals of spreading. After 5 min of spreading on a coverslip (a & b) the bands begin to become disorganized, but are still recognizable as MBs. (c) the MBs are almost totally disorganized after the cells have been allowed to spread for 10 min. In d, after 20 min, no remnants of the MBs are visible. x1,660

remnant of the band was visible (Fig. 46d).

2) Limulus polyphemus. Amebocytes (Clotting cells). Limulus amebocytes, prevented from undergoing amoeboid transition by treatment with propranolol, appeared as flat ellipses filled with numerous granules that contain clotting substances (Fig. 47a). Lysed cells contained MBs and nuclei embedded in a coagulum, presumably released by the clotting granules (Fig. 47b). In thin sections, the MBs of lysed cells were observed to consist of closely apposed, sometimes cross-linked MTs 22.5 nm diameter (Fig. 47c & d).

3) Crab Species: Amebocytes. The hemolymphs of the crab species examined contain elliptical, flattened cells that rapidly transform into amoeboid shapes upon contact with a glass slide (Fig. 48a, d & e). Following morphological transformation the cells could be observed, with phase-contrast microscopy, to undergo amoeboid locomotion (Fig. 48d & e). Dilution into crustacean Ringer's slowed the transition to the amoeboid state but did not prevent it.

When cells were lysed immediately after dilution into Ringer's solution, many of the nuclei seen were surrounded by MBs (Figs. 48b & c; 49). The proportion of nuclei having MBs seemed to be directly related to the rate of morphological transformation of the amoebocytes. For example, the cells of Cancer borealis transformed very rapidly even when in Ringer's solution, and few of the lysed cells appeared to have MBs, whereas in the relatively slowly transforming cells of Carcinus maenas there were few nuclei that, when examined under phase contrast, did not have MBs. These results imply a relationship between cellular morphology and MBs suggesting that the bands are necesary for cell shape maintenance, and/or must be disassembled in order for amoeboid transformation to occur.

The remaining invertebrate species examined did not appear to have MBs in lysed blood cells examined with phase-contrast microscopy. This does not

Figure 47. *Limulus polyphemus*: *Amebocytes*. (*a*) These clotting cells were prevented from undergoing transition to the amoeboid state by treatment with 2 mM propranolol. When such cells are lysed, they are observed to contain MBs (*b*). The arrows indicate an MB constricting the coagulum resulting from secretions of the clotting granules (g). In *c* and *d* cross and longitudinal thin sections show that the MBs consist of microtubule bundles. a = amebocyte. (*a* & *b*) Phase contrast. (*c* & *d*) TEM. (*a*) x1,060, (*b*) x825, (*c*) x40,000, (*d*) x46,000

Figure 48. Carcinus maenas: Amebocytes. When morphological transformation is slowed by dilution of hemolymph into crustacean Ringer's (a) MBs are observed when the cells are subsequently lysed (b & c). In d and e two transformed cells were photographed during amoeboid motion. Phase contrast. (a, c, d & e) x1,630, (b) x825

Figure 49. Cancer borealis (a), C. irroratus (b) and Libinia emarginata (c). Lysed Amebocytes. MBs are seen in the cells of all three species. Phase contrast. (a & c) x1,060, (b) x960

necessarily mean that there are no marginal bands in these species, but that with the methods employed, none were detected.

DISCUSSION

I. Phylogenetic Distribution of the Marginal Band System

The present work establishes that MBs are broadly distributed in blood cells of invertebrates. Table III summarizes the distribution of MB-containing blood cells in both vertebrates and invertebrates. The presence of MBs in members of all the vertebrate classes, as well as in four invertebrate phyla (including six classes) indicates that mature mammalian erythrocytes are quite exceptional. Mammalian blood cells have retained the ability to form MBs, as seen in platelets and primitive erythrocytes, but in these organisms bands are apparently not required in the mature, definitive erythrocytes.

II. Invertebrate Erythrocytes

MB Function

1) Cell shape: With regard to invertebrate erythrocytes, this work has established the presence of MBs in *P. gouldii*, members of the Arcidae and two species of sea cucumbers. The phylogenetic distribution of these structures suggests a number of different, but not mutually exclusive functions for them. Observations in this dissertation that the MBs appear to exert a stretching force on the encompassing submembranous network, indicate a role for the MB in the morphogenesis of a flattened cell. Flatness in an erythrocyte offers an obvious advantage for efficient gas exchange (Maser and Phillpott, 1964), and is considered an adaptation to flow as well (Schmid-Schönbein, 1975).

Experiments on vertebrate erythrocytes suggest that MBs are required for cellular transformation from spherical to flattened elliptical shape. Correlation of MB appearance and morphogenesis has been observed in the developing

Table III

Phylogenetic Distribution of the Marginal Band System

Phylum and species[a]	Common name	Cell type and morphology[b]		References[c]
Phylum Sipuncula				
Golfingia gouldi	Peanut worm	Erythrocyte	F,C or E,N	Cohen *et al.* (1977)
Phylum Mollusca				
Anadara transversa	Blood clam	Erythrocyte	F,C or E,N.	Nemhauser *et al.* (1979)
Noetia ponderosa		Erythrocyte	F,E,N	Cohen & Nemhauser (1980)
Phylum Arthropoda				
Xiphosura				
Limulus polyphemus	Horseshoe Crab	amebocyte	F,E,N	Nemhauser *et al.* (1980)
Crustacea				
Homarus americanus	Lobster	coelomocyte	F,E,N	Cohen *et al.* (1978)
Cancer borealis	Rock crab	amebocyte	F,E,N	Cohen *et al.* (1978)
Insecta				
Periplaneta americana	Cockroach	hemocyte	F,?,N	Baerwold & Boush (1970)
Phylum Echinodermata				
Cucumaria, miniata	Sea cucumber	hemocyte	F,E,N	Fontaine and Lambert (1972)
Molpadia arenicola		hemocyte	F,E,N	Clifford (1969)
Phylum Chordata (Vertebrate classes)				
Agnatha				
Eptatretus stoutii	Pacific hagfish	Erythrocyte	F,E,N	Sekhon & Maxwell (1970)
Chondrichthyes				
Mustelus canis	Smooth dogfish	Erythrocyte	F,E,N	Cohen *et al.* (1977)
Raja erinacea	Little skate	Erythrocyte	F,E,N	Cohen *et al.* (1977)
Osteichthyes				
Opsanus tau	Toadfish	Erythrocyte	F,E,N	Fawcett & Witebsky (1964)
Carassius auratus	Goldfish	Erythrocyte	F,E,N	Weinreb & Weinreb (1965)
Amphibia				
Rana pipiens	Leopard frog	Erythrocyte	F,E,N	Maser (1963)
Amphiuma tridactylum	Giant salamander	Erythrocyte	F,E,A	Maser & Philpott (1964)
Batrachoseps attenuata	Slender salamander	Erythrocyte	F,E,A (majority are anucleate)	Fawcett & Witebsky (1964)
Reptila				
Dipsosaurus dorsalis	Desert iguana	Erythrocyte	F,E,N	Maser (1963)
Aves				
Gallus domesticus	Chicken	Erythrocyte	F,E,N	Behnke (1970)
Mammalia				
Homo sapiens	Human	Platelet	F,C (E?),A	Behnke (1965)
Homo sapiens	Human	Primitive erythrocyte	F,E?,N	van Deurs & Behnke (1965)
Lepus	Rabbit	Fetal liver erythroblast	?,?,N	Grasso (1966)
Camelus dromedarius	Camel	Reticulocytes	F,E,A	Barclay (1966)
Llama glama	Llama	Late erythroblasts of red bone marrow	F,E,A	Goniakowska-Witalińska & Witaliński (1977)

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[a]: Representative species. [b]: Key: F, flattened; C, circular, E, elliptical; N, nucleated; A, anucleate; ?, data lacking. [c] See Nemhauser *et al.* (1980) for additional references

erythrocytes of ducks (Barclay, 1966), trout (Sekhon and Beams, 1969; Yamamoto and Iuchi, 1975) and chickens (Barrett and Dawson, 1974). Barrett and Dawson demonstrated that the microtubule inhibitors colchicine and vincristine sulfate prevented the "sphere to disc" transformation, and that the number of MTs in the mature erythrocyte decreased after the MB had formed. They therefore concluded that the MB was necessary for formation of the flattened disc shape but not for its maintenance. This opinion was concurred in by Behnke (1970), who observed that cold-induced disassembly of MBs in mature chicken erythrocytes did not result in loss of cell shape, and by Small (1972) whose results were similar to those of Barrett and Dawson. Yamamoto and Iuchi (1975) also reported a decrease in MT number in mature as opposed to developing erythrocytes. Circumstantial evidence for a similar role of marginal bands in mammals derives from the report of MBs in camel erythrocytes by Barclay (1966), and their reported appearance during erythropoiesis in the Llama as the cells assume an elliptical shape (Goniakowska-Witalińska and Witaliński, 1977). Further work (Cohen and Terwilliger, 1979) indicates that MBs, though possibly involved in morphogenesis, are not required for shape maintenance in the camel red blood cell, since they are not seen in the mature erythrocytes. There is also the possibility that MBs observed in circulating primitive erythrocytes of rats, mice and man, by van Deurs and Behnke (1965), could be involved in the formation of these elliptical cells (Jones, 1963). It may even be that MBs, as seen in fetal rabbit erythroblasts (Grasso, 1966), might serve a similar purpose in erythropoiesis of mammalian definitive erythroblasts (other than the Camelidae), only to disappear once they have served their purpose.

There remains the possibility, of course, that in many species the continuing presence of an MB is necessary for cell shape maintenance or resilience and indeed, a positive correlation has been found in vertebrates between the number of MTs in the marginal band and erythrocyte size (Goniakowska-Witalińska and Witaliński, 1976), suggesting that the larger cells are in need of a relatively thick MB for retention of shape or resiliency. This relationship does not hold true for the relatively large (30 μm) erythrocytes of the sipunculan worm P. gouldii and their thin (up to 13 MT) MBs reported in the present work. The opposite situation obtains in Arcidae erythrocytes which, though 10-15 μm in diameter along their long axes, contain on the average 50 MTs in cross sections of their MBs. This is a greater number than expected when compared with vertebrate erythrocytes of comparable size. Of the invertebrate cells examined ultrastructurally, only Limulus amebocytes, measuring 20-25 μm in length and containing 20-40 MTs in cross section (Nemhauser et al., 1980), conform to the conclusions of Goniakowska-Witalińska and Witaliński. Thus, the available data on invertebrate blood cells are not consistent with the theory of proportionality between cell size and MB diameter. The functional implications of these findings and the question of whether or not they are typical for invertebrates remain to be determined.

The characteristic flattened, elliptical shape shared by the MB-containing erythrocytes may be the result of interaction of the MB and network, the latter exerting a countervailing force unevenly applied across the cell axes. Thus an ellipse could result if tension were exerted by the network mainly along one axis (Cohen, 1978). In this way the opposing forces exerted by the components of the marginal band system (MB and network) would produce a flattened elliptical shape. Although the results presented here do not elucidate the chemical nature of the network observed under the EM, a network, containing actin and spectrin, has been demonstrated biochemically and ultrastructurally in mammalian erythrocytes (Tilney and Detmars, 1975; Puszkin et al., 1978; Ralston, 1978; Sheetz and Sawyer, 1978; Sheetz, 1979; Fowler and Taylor, 1980; and Tsukita et al., 1980). Actin and spectrin, acting in concert with other extrinsic membrane proteins appear to be involved in shear stress resistance (Fischer et al., 1978; Cohen and Korsgren, 1980), endocytosis (Hardy et al., 1979) and generalized cellular deformability (Nakashima and Beutler, 1978; Palek et al., 1978; Sheetz et al., 1978; Johnson et al., 1980). Furthermore, a membrane-associated protein with an apparent molecular weight of 2.7×10^5, immunologically cross-reactive with anti-human spectrin, has been identified in the erythrocytes of Terebella lapidaria, a marine annelid (Pinder et al., 1978). The presence of spectrin and actin has not been demonstrated in the erythrocytes described in the present work, and further experiments are required to substantiate the hypothesis proposed above.

In connection with the aforementioned depletion of MTs from the marginal bands of chicken erythrocytes, it should be noted that the amount of spectrin associated with the plasma membrane increases during cellular maturation (Chan, 1977), a situation which may be considered additional evidence for the cooperation of the MB system components in shape generation and maintenance. In fact, this finding along with the above observations on P. gouldii and N. ponderosa, and on those vertebrates in which the number of MTs diminish or the MB disappears after cell shape has been established, also suggest a mechanism for erythrocyte shape maintenance. It may be that in the erythrocytes of at least some species, subsequent to development of flattened, elliptical shape as the result of MB-network interaction, the latter is stabilized, perhaps by an increase in the amount of spectrin, and therefore able to perpetuate the cell shape alone or as the result of interaction with the respiratory pigment. Such a mechanism could account for the fact that at 0°C, N. ponderosa erythrocytes retain their general elliptical morphology, yet when the cells are lysed, causing release of the hemoglobin, the networks assume irregular contours.

Elliptically shaped erythrocytes may be an adaptation to flow requirements, for even mammalian (non-Camelidae) red blood cells, which have relatively more fluid plasma membranes than those of nonmammalian vertebrates, assume an elliptical shape under flow conditions (Schmid-Schönbein, 1975). The theory has been put forward that the MB system may be an alternative evolutionary mechanism for guaranteeing elliptical shape, or at least one more efficient for flow, in blood cells (Cohen, 1978b).

2) Osmoregulation: A second possible function for the components of the MB system may involve osmoregulation. Trotter (1956) demonstrated the resistance of the erythrocytes of a salamander to hypotonic solutions. Under conditions that cause a disc-to-sphere transformation in mammalian erythrocytes, the amphibian red blood cells were prevented from doing so, apparently by the presence of their MBs. The erythrocytes of *P. gouldii* are osmotically resistant to the extent that in 40% sea water up to 40% of the cells do not lyse (Cohen and Nemhauser, unpublished results). The necessity for osmotically resistant erythrocytes in *P. gouldii* may be related to the fact that their habitat, an estuarine mud flat,. is daily exposed to low tides. Heavy rainfall, at low tide, could further increase the osmotic stress to which the organisms might be exposed. The ability of the organism as a whole to withstand large fluctuations in environmental salinity has been reported (Adolph, 1936; Robertson, 1953), and similar findings have been made for other sipunculan species occupying comparable habitats (Virkar, 1971). It is noteworthy with regard to these findings that erythrocytes of another sipunculan studied in the present work apparently do not have MBs. The species, *Themiste*, inhabits the low intertidal zone of the protected outer coast of California, a region uncovered by the tides for only a few hours each month. These circumstances may obviate the necessity for osmotically resistant erythrocytes, and by

inference, MB systems.

Similarly, the erythrocytes of the mollusc A. transversa have been described as resistant to hypotonic solutions (L. Amende, personal communication). This species is found at the low water mark to subtidal, as is A. ovalis (Smith, 1964), while N. ponderosa occupies shallow water (Gosner, 1971). In addition, there are species of the Arcidae such as A. trapezia, found in estuaries and lagoons (Sullivan, 1961), and A. senilis, that inhabits estuarine mud flats (Yoloye, 1975), that are possibly subjected to osmotic stress; however, it remains to be demonstrated that these species have MBs. The available data on osmoregulation and habitat for the above-mentioned species lend themselves to speculation, but do not permit firm conclusions at this time; further experimentation, including investigation of deep-water relatives of these species, would be required to substantiate the proposed hypothesis.

III. Non-Erythrocyte Blood Cells of Invertebrates

The blood clotting cells (Dumont et al., 1968; Madaras et al., 1979) of the arthropods studied in the present work can be compared to mammalian platelets, in which activation has been shown by Behnke (1970) to involve shape change concomitant with MB disorganization. In fact, platelets, which lose their elliptical shape and their MBs upon cooling, can regain both upon rewarming (Behnke, 1967). The arthropod clotting cells, which are larger, and more readily obtainable in quantity than platelets, could be employed, using techniques like indirect immunofluorescence as described in this thesis, to study the role of MBs in the morphological shape changes associated with clotting cell activation. Investigation of a possible MB role in normal clotting cell function could serve as a model for the part MBs might play during platelet mediated hemostasis. However, Hovig (1969) reports that platelets deprived of MBs by the action of colchicine have unimpaired aggregating ability and form normal hemostatic plugs, while mean clotting times in colchicine-treated animals is increased over that in controls. It appears likely therefore, that MBs are needed for shape maintenance during flow in inactivated platelets, while during activation their disorganization is necessary in order that shape transformation may occur.

The present observations on the amebocytes of L. polyphemus and the various crab species, as well as on the coelomocytes of H. americanus, also lead to the conclusion that MBs are involved in maintenance of elliptical morphology in these cells and must be disassembled in order for the cells to undergo the characteristic changes involved in transition to the amoeboid state. With the activated cells thus "committing suicide," there would thus be no need for a mechanism of MB reassembly. The proposed functions of clotting cell MBs would therefore be similar to those mentioned above for erythrocytes, with the exception of the respiratory requirement. An osmoregulatory role is also suggested since Limulus can be found in the same mud flats where the P. gouldii specimens were collected.

IV. MB Structure: P. gouldii

In P. gouldii erythrocyte MBs isolated by lysis in the presence of ATP, it is possible to observe single MTs running virtually all the way around the circumference of the cell, leading to the rejection of one proposed model of three-dimensional MB organization; the concept of short, possibly overlapping, segments of MTs circumscribing the cell beneath the plasmalemma. The results do not, however, aid in deciding which of two other possible models, if either, is correct. One depicts the MB as composed of numerous MT hoops, the other envisions it as formed by one or more MTs encircling the cell a number of times. There are no examples of continuous MT hoops known to me, however the observations of Behnke and Zelander (1967) and Nachmias *et al.* (1977) on negatively stained platelet MBs in which they show one or two straight MTs emerging from otherwise intact bands, led them to conclude that these structures are composed of microtubules that are wound around the cells numerous times. The latter model thus seems reasonable for *P. gouldii* erythrocytes as well. The removal of the network by the action of ATP, seen also in dogfish (W.D. Cohen, personal communication) and frogs (Cohen, 1978c), indicates the possible similarity of this structure in these diverse species.

V. Centrioles and MB Assembly: A Model for MB Formation in the Arcidae

In the present work, centrioles have been unequivocally located in association with the MBs of Arcidae erythrocytes. This finding prompted a literature search to determine whether or not MB-centriole associations had been observed in other species. An investigation of the literature unearthed only one instance in which centrioles had been described in conjunction with MBs, and that was in the erythrocytes of the sea cucumber *C. miniata* (Fontaine and Lambert, 1972). Their presence in the red blood cells of *P. gouldii* and another sea cucumber species *M. arenicola* is inferred from examination of lysed erythrocytes under phase-contrast microscopy and comparison with similarly treated cells known to have MB-associated centrioles. The finding that centrioles were functional during blood clam MB assembly stimulated a preliminary search for them in vertebrate erythrocytes. Centriole-like structures, visualized by indirect immunofluorescence using antitubulin antibody, have been observed (in a minority of cells) only in the dogfish (Nemhauser *et al.*, 1979). It remains to be seen whether or not further investigation will reveal centrioles to be universally associated with marginal bands, at least during MB formation.

N. ponderosa MBs, which are cold-labile, were shown to reassemble, evidently under the influence of the centrioles and their associated amorphous material. This was the first demonstration of MTOC-nucleated MB assembly; this work and that of Cohen *et al.* (1980) on dogfish erythrocyte MBs are also the first descriptions of cold-labile MBs in poikilothermic organisms. The presence of cold-sensitive MBs in these organisms is probably made possible by their oceanic habitat in which it is doubtful that they encounter temperatures near 0°C within their respective ranges.

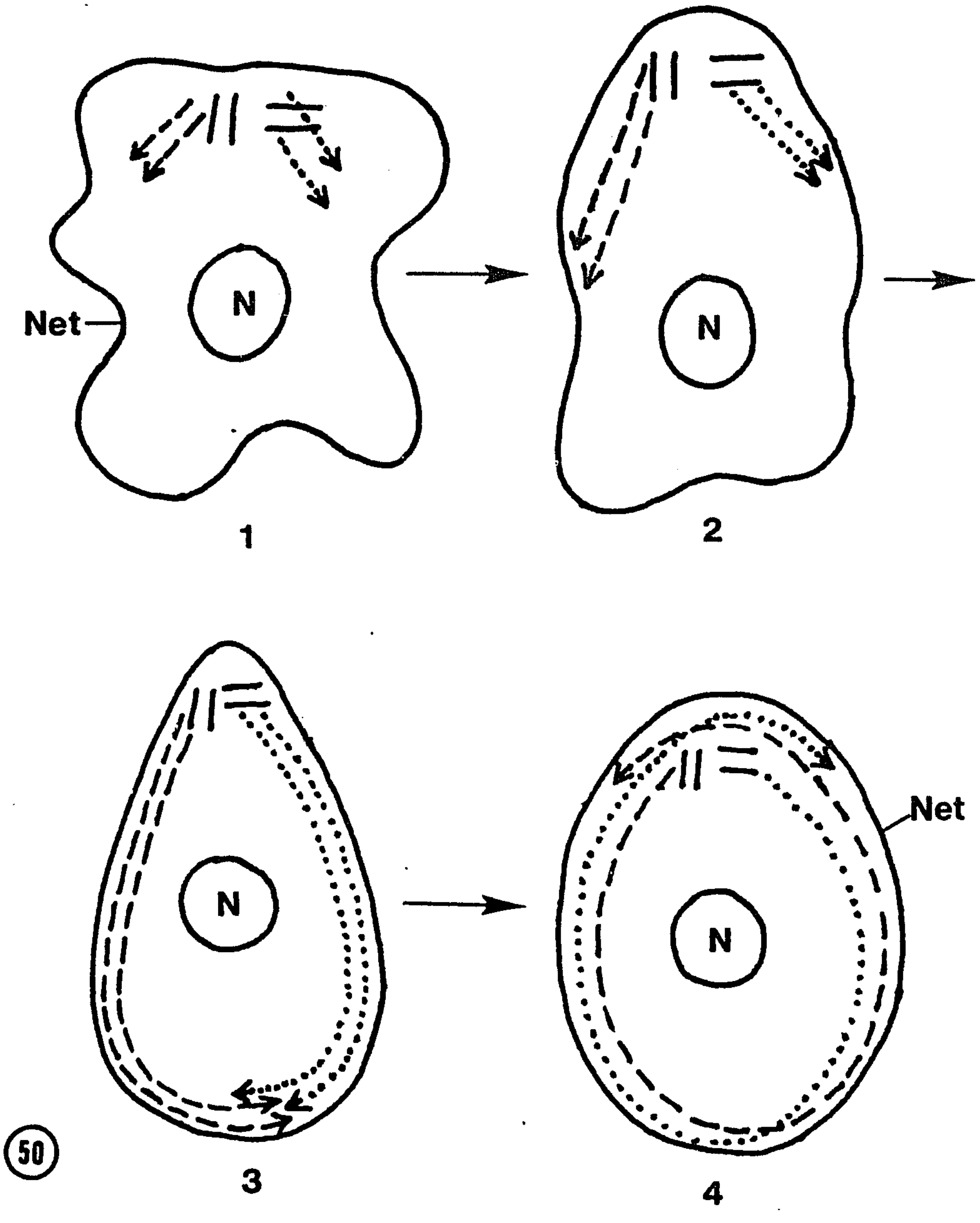
Although the nature of the amorphous material has not been determined, Rieder (1979) has provided evidence that the electron dense material around mitotic spindle centrioles contains ribonucleoprotein. In addition, Heideman *et al.* (1977) have shown that basal bodies treated with RNAase or proteolytic enzymes lose their *in situ* aster forming activity. They speculate that the RNA may contain the information required for assembling the pericentriolar material. The results of the present study, using indirect immunofluorescence on lysed *P. gouldii* erythrocytes, in which brightly fluorescent bodies are seen adjacent to the nuclei, suggest that the pericentriolar material may also contain unassembled tubulin. In fact, there are a number of reports in the literature describing the accretion of amorphous electron dense material around centrioles or other MTOCs which have no assembled MTs in their vicinity, and disappearance of this material concurrent with microtubule assembly (Aist and Williams, 1972; Sakai and Shigenaga, 1972; McGill and Brinkley, 1975; Byers and Porter, 1977). The dense flocculent material seen around the centrioles in *N. ponderosa* cells at 0°C may, therefore, be material (possibly tubulin) that is incorporated into assembling MBs.

Cold-disassembled lysed erythrocytes of Arcidae species might be utilized to study *in vitro* the MTOC competence of the centrioles. The ability of the centrioles and/or their associated material to nucleate MT assembly could be determined in the presence of exogenous purified tubulin dimers as was done by Pepper and Brinkley (1979) and Telzer and Rosenbaum (1979).

The results obtained from the serial sections, as well as indirect immunofluorescence studies and phase-contrast microscopy, suggest a model for MB biogenesis in N. ponderosa, and possibly in other organisms that have MB-associated centrioles as well (Fig. 50). The observations indicate that the MB microtubules originate in the centriolar area and circumscribe the cell, passing peripheral to the centrioles when they reach the area of their origin (Fig. 50, 4). As this occurs, the developing MB confers a flattened shape on the submembranous network and, therefore, on the cell as a whole. At the same time, if the growing MTs stretch an elastic network in one direction (along the axis from the centrioles to the opposite side of the cell), the network might, as a result, exert a constricting force on the MTs in a direction perpendicular to that axis. This nascent MB-network interaction would then impose an ovoid shape on the cell. In addition, by passing peripheral to the MB points at which the centrioles are located, the MTs would gradually compress that region so that the pointiness would be attenuated and finally disappear (Fig. 50, 3 & 4) and the final shape would be an ellipse (Fig. 50,4). The model also predicts that the MBs are made of MT segments long enough to encircle the cell at least once, and is thus in agreement with the findings on P. gouldii isolated bands described above.

The MTs originating from the two centrioles would be expected to be of opposite polarity (Bergen et al., Heidemann et al., 1980, McIntosh, 1980) as illustrated in the figure, and individual MTs could interdigitate as they passed one another (Fig. 50, 3) acquiring inter-MT bridges (McDonald et al., 1979) that might stabilize the MB structure. High molecular weight microtubule-associated proteins (MAPS) co-purify with tubulin that has been prepared by cycles of

Figure 50. A Model for MB Assembly in the Arcidae. Based on the experimental evidence, it is proposed that the MB MTs are nucleated by the centrioles (1) and that they stretch the network (Net) along the future long axis of the cell as they grow (2). Upon reaching the opposite end of the cell the MTs of opposite polarity could interdigitate, as depicted in the figure, or pass as discrete bundles (3). The network, if elastic in nature, would respond to the stretching by compressing the band across the perpendicular axis, resulting in an egg-shaped profile (or an ellipse with one pointed end). Further growth of the MTs around their point of origin and peripheral to the centrioles would result in compression of the point and formation of an ellipse (4).



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polymerization-depolymerization (Sloboda _et_ _al_., 1975), and Sloboda and Dickersen (1979) have demonstrated the presence of one of them (MAP 2) in the cytoskeletons of vertebrate erythrocytes. These then might constitute the inter-MT bridges seen in the control and, to a lesser extent, in reformed MBs.

_In_ _vitro_ assembled microtubules decorated with MAPS, which can be separated from tubulin by phosphocellulose chromatography, have been shown to be relatively stable to cold (Sloboda and Rosenbaum, 1979) and colchicine (Haga and Kurokawa, 1975). Furthermore, microtubule associated proteins stimulate _in vitro_ MT assembly (Dentler _et_ _al_., 1975; Sloboda _et_ _al_., 1976). The paucity of inter-MT cross bridges in the reassembled MBs in the present work may therefore have been a causative factor in the failure to re-form completely normal (with regard to MT number) bands. The diminution in MAPs may also have resulted in a lack of stability, causing the re-formed MBs in some cases to have a degraded appearance.

Based on the proposed model of MB assembly described above, the atypical or pointed, MBs found to some extent in all species of Arcidae (and at a relatively high frequency in one species) could be explained as the result of faulty construction or as an early stage in MB formation, caused by failure of the MTs to assemble all the way around the cell. This could be the result of an insufficient pool of tubulin in the cell, resulting in incomplete MT assembly. Evidence for this hypothesis comes from the experiments in which pointed MBs were seen in colchicine-treated cells. The results can be interpreted as a diminution in available tubulin dimers as the result of colchicine binding to the pool of tubulin made available upon MB disassembly. Such a decrease in the available tubulin could conceivably result in pointed, fragile MBs if the above model of MB assembly is valid. Based on these results, one would expect to be able, using a large enough concentration of colchicine, to completely prevent MB reassembly. The "tennis rackets" may have formed, according to the model, because of failure of the MTs to pass peripherally to their origin as they grew, passing centripetally to the centrioles instead.

In the present work phase-dense dots have not been observed on or near the MBs of invertebrate clotting cells. It would thus appear that the proposed model for MB assembly may be applicable only to the MBs of invertebrate erythrocytes. Nor have vertebrate erythrocytes, aside from the dogfish, been reported to be associated with centrioles. It may be that centrioles are present in vertebrate red blood cells, but are obscured by the hemoglobin, or somehow sequestered once they have fulfilled their function. A similar situation may exist in clotting cells. Behnke (1970) states that centrioles in platelets are a rarity, although he shows an electron micrograph of one adjacent to an MB. The observations on invertebrate clotting cells described in this thesis were largely restricted to light microscopy in which phase-dense dots could easily be hidden by the released contents of clotting granules.

VI. Iron in *P. gouldii* Erythrocytes

Plasmalemmal electron-dense granules and vacuolar inclusions in *P. gouldii* erythrocytes probably represent a storage form of Fe, possibly ferritin, since they were shown by X-ray microprobe analysis to contain that element. Both structures were also seen by Stang-Voss (1970).

VII. Summary: Major Findings

The phylogenetic distribution of MBs in invertebrate blood cells has been explored and described in the present work. MBs have been observed in four phyla, including representatives of six classes, of invertebrates. The results imply that the MB may be a nearly universal mechanism for generating flattened, usually elliptical, shape in cells that must be adapted to flow. Definitive mammalian (non-Camelidae) erythrocytes are thus even more exceptional in the animal Kingdom than formerly believed. The extensive distribution of MBs, and their similar (MT) subunit structure having common antigenic determinants, suggests that there may have been a single evolutionary source for this microtubule system in both the respiratory and clotting cells of such diverse forms.

The ultrastructure of the MB was studied in representative invertebrate species as well. The technique of simultaneous lysis and fixation, developed in the course of the work, made it possible to obtain unobstructed views of the relevant organelles and their interrelationships. The MBs were shown to resemble, in form and disposition, those previously seen in nonmammalian vertebrate erythrocytes, Camelidae reticulocytes and mammalian platelets. Their disposition within the cells was compatible with their proposed function in cell shape morphogenesis. Moreover, a filamentous network, presumably underlying the plasma membrane of the intact cell, was clearly seen enclosing the MB as well as the nucleus and remnants of other organelles. The presence of this network in invertebrate red blood cells is consistent with the hypothesis that erythrocyte MBs function universally by interaction with such a structure.

The cell types mentioned above were exploited in studies designed to test the theory that MBs are somehow responsible for the flattened, elliptical shape of blood cells bearing them. In arthropod clotting cells undergoing morphogenetic transition, it was demonstrated that MB disassembly is coincident with transition of these cells to amoeboid shape. These studies thus demonstrated a striking connection between MB presence and cell shape.

MB assembly was studied at the LM and EM levels in the erythrocytes of a member of the Arcidae, N. ponderosa. It was shown that the MBs are cold-labile in this poikilothermic organism, and that upon rewarming, the MBs can be made to reassemble. Furthermore, MBs of Arcidae erythrocytes were found to be associated with centrioles. Disassembly/reassembly experiments, taking advantage of the above mentioned characteristics of N. ponderosa erythrocytes, illustrated the MB nucleating capability of the centrioles and/or their associated electron-dense material. This appears to be the first demonstration of such a control mechanism in MB formation.

Based on the findings, a model for MB assembly in these organisms has been developed. The model in turn suggests a mechanism whereby interaction of the growing MB and the enclosed network would result in the flattened, elliptical cell shape. Colchicine experiments, in which the drug appeared to interfere with normal MB formation, served to confirm aspects of the model as did discovery of an Ark species with a high percentage of seemingly aberrant pointed MBs.

The lyse-fix technique, developed during the course of this work, could be utilized in future studies on MB structure and assembly. For example, the relationship between MBs and centrioles might be further clarified through combination of the lyse-fix method with high-voltage electron microscopy. In addition, the use of MT inhibitors other than colchicine, together with the above technique might provide further information on the mechanism of MB formation.

Future experiments with fluorescent antibodies, like those described herein, may help answer interesting questions about the molecular composition of invertebrate MBs. For example, do they also contain MAP 2? Are there differences in MB-associated MAPs that might account for the stability of erythrocyte MBs as compared with the naturally-occurring lability of clotting cell bands? The MB-containing cell types described in this work should be extremely useful tools for experiments designed to answer such questions.

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