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Rapid Purification and the Immunological Specificity of Mammalian Microtubular Paracrystals Possessing an ATPase Activity*

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Abstract. A new procedure for the rapid purification of vinblastine-induced microtubular paracrystals is described. The paracrystals constitute about 2 per cent of the total protein in L cells. They contain a labile ATPase with a Michaelis constant of 1.0×10^{-4}. In the immunofluorescence test, sera prepared against paracrystals reacted positively with intracellular paracrystals and the mitotic apparatus. Antisera against paracrystals from a malignant murine cell also labeled the mitotic apparatus of HeLa and chicken embryonic cells, revealing the presence of a common or related antigen in the mitotic apparatus of these higher vertebrates.

Introduction. Microtubules or microtubular proteins from cilia, flagella, and neurons have been isolated and examined both chemically and morphologically.[1–4] The microtubules of the achromatic mitotic apparatus of eucaryotic cells have also been well characterized morphologically,[5] but their isolation as a homogeneous material has not yet been reported. It therefore seemed desirable to obtain such microtubules in a pure state for further analysis of their chemical and biological properties. For this purpose we undertook to isolate the microtubules from a continuous line of mammalian cells, the L strain mouse fibroblasts.[6] The method described in the present report is based upon a recent demonstration by Bensch and Malawista[7] that vinblastine and vincristine, alkaloids derived from *Vinca rosea*, elicit the regrouping of microtubules into characteristic paracrystalline aggregates. The rapid isolation procedure of such paracrystals, described in this report, has consistently yielded a homogeneous material with an ATPase activity. Antisera prepared against the paracrystals antigen from mouse cells showed an immunological identity with the mitotic apparatus from murine, human, and chicken cells.

Materials and Methods. Media employed: Nutrient medium A for culturing cells and eliciting formation of paracrystals was Eagle's[8] minimum essential Spinner medium supplemented with 10% fetal bovine serum and 10^{-5} *M* vinblastine (Velban, Eli Lilly). Medium B used during the isolation of paracrystals consisted of the Spinner medium supplemented with 10^{-5} *M* vinblastine, but lacking sodium bicarbonate. The pH was adjusted to 6.5 with NaOH.

Preparation and testing of antisera: Rabbit antisera were prepared by repeatedly immunizing albino rabbits with 50 μg aliquots of purified paracrystals. The first intramuscular injection consisted of paracrystals suspended in 0.1 ml each of phosphate-buffered saline (a)[9] and complete Freund's adjuvant. Subsequent intravenous in-

jections of paracrystals in 2.0 ml phosphate-buffered saline began 3 weeks later and were administered at weekly intervals three more times. Rabbits were bled 1 week after the final injection. Preliminary tests for presence of specific antibody were conducted by mixing suspensions of paracrystals with varying dilutions of antisera. Agglutination of paracrystals of the type illustrated in Figure 7 was observed at antiserum dilutions of 1:2 to 1:16, while none occurred with previously immune sera (see Fig. 6). Antiserum specificity was demonstrated by the Ouchterlony test.[10]

Fluorescein-antibody staining: Monolayer cultures of primary chick embryo fibroblast, L_2-strain and HeLa cells on cover glass were fixed in acetone at $-20°$. After hydration in phosphate-buffered saline, the specificity of antiserum was tested by the indirect binding method.[11]

ATPase assay: The assay was performed by using a modification[12] of the procedure in which ATP-γ-^{32}P (2 mCi/μmole in aqueous solution, Nuclear-Chicago) was used as substrate.[13] Unless specified otherwise, the assay was performed for 15 min at 37° in a mixture containing 10 mM $CaCl_2$, 50 mM Tris-maleate buffer at pH 7.0. In testing the optimum pH, in the range 5–7, the solution was buffered with Tris-maleate and at pH 7.5–9.0 with Tris-HCl. Activities were maximum when enzyme was assayed within 10 to 15 min after isolation of paracrystals.

Results. Purification scheme and electron microscopic identification of paracrystals: (1) Confluent monolayer cultures were incubated for 16 hr in medium A to form paracrystals. (2) As starting material 3×10^8 cells in 150 ml of medium A were scraped from the plates and placed in centrifuge tubes. (All subsequent operations were performed at 0 to 4°.) (3) The cells were spun into pellets at 150 *g* mean for 5 min and resuspended in 15 ml of medium B (without vinblastine in medium B the paracrystals disappear rapidly). After 0.15 ml of Nonidet P_{40} (Shell) was added the mixture was agitated on a Votex mixer for 10 sec. (4) To the above were added 15 ml of Genesolv-D (Allied Chemical), and the aqueous and fluorocarbon phases were mixed on the Vortex. Following phase separation after centrifugation at 150 *g* mean for 3 min, the upper phase was transferred to another tube and the fluorocarbon, containing the bulk of cellular debris, was discarded. (5) After the aqueous phase has been spun at 2000 *g* mean for 15 min, the paracrystal pellets which had formed were resuspended in 10 ml of medium B and fluorocarbon extraction was repeated three times. Finally the aqueous phase was centrifuged for 15 min at 2000 *g*.

The results of an electron microscopic characterization of paracrystal pellets obtained in the final step of purification are illustrated in Figures 1–5. The length, width, and fine structure shown in Figures 4 and 5 corresponds closely to that of intracellular paracrystals.[7] Suspension of paracrystals, judged to be pure by examination in the light microscope (Fig. 6), proved to be free of contaminating cellular material when examined as thin sections of pellets (Figs. 3 and 5).

Amount of paracrystal protein within L cells: The yields of protein associated with the paracrystals obtained in three separate extractions are given in Table 1. The low recovery, about 7% on the average, was calculated on the basis of the total number of intracellular paracrystals in the starting material and that remaining in purified suspensions. This low yield of paracrystals can be ascribed to (*a*) incomplete release from cells, (*b*) losses during extraction, and (*c*) dispersion after extraction. From the number of paracrystals recovered, their protein content, and the amount of protein in L cells, it was estimated that

Figures 1–11 are electron micrographs (legends on facing pages).

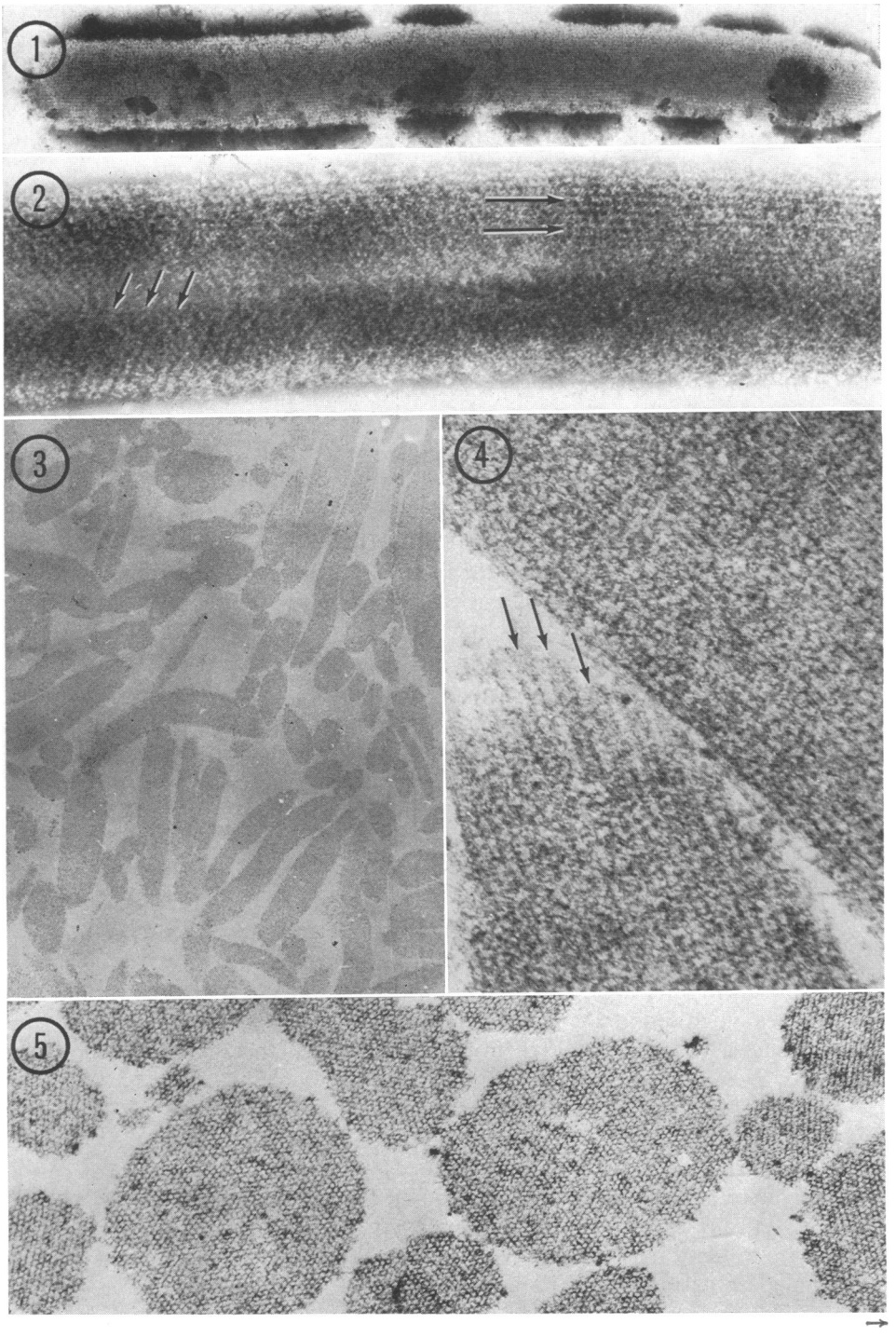


TABLE 1. *Amount of paracrystal protein within L cells and in purified preparations.*

Total paracrystal protein recovered* (μg)	Total no. of paracrystals recovered†	Average no. of paracrystals per cell‡	Paracrystals recovered (%)	Paracrystal protein per cell ($\mu\mu$g)	Total protein per cell* ($\mu\mu$g)	Paracrystal protein per cell (%)
125	7.7×10^7	3.46	5.6	5.5	260	2.1
140	1.1×10^8	2.60	8.7	3.4	220	1.6
145	9.2×10^7	2.90	7.0	4.6	250	1.8

* The protein content of purified paracrystals and L cells was determined by the procedure of Lowry *et al.*[16] (Caution: in the presence of vinblastine at 5×10^{-5} *M* or greater in the Lowry assay, the same color reaction as with protein was observed.)

† Paracrystals in suspension were enumerated by a Petroff-Hauser bacterial counting chamber.

‡ Intracellular paracrystals were enumerated by phase-contrast microscopy in monolayer cultures of cells, previously fixed with 100% cold acetone and prepared as wet mounts in 10% glycerol.

1.6–2.1% of the total cellular protein is contained in paracrystals. This percentage should be compared with a value of 9 to 12% given for the prominent mitotic apparatus in dividing sea urchin eggs.[17, 18] However, our estimate may be too low if some of the microtubule protein was not integrated into microscopically detectable paracrystals after vinblastine treatment.

Immunofluorescence of paracrystals and mitotic apparatus: Antigenic specificity of immune sera judged to be positive by the agglutination test (Fig. 7) was demonstrated by the Ouchterlony method.

Development of one precipitin line in agar indicated that a single antigenic determinant was involved (Fig. 12). Specific staining of both paracrystals and mitotic apparatus within L cells of murine origin was demonstrated by immunofluorescence indicating that an antigen is common to these structures containing microtubules. Presence of the same or of a closely related antigen among different orders of mammals and even vertebrate classes became apparent from the staining of paracrystals and mitotic apparatus in HeLa and chick embryo fibroblasts (Figs. 8–11). It has yet to be determined whether this antigen occurs universally.

ATPase activity associated with paracrystals: Circumstantial evidence linking microtubules with cellular mobility[5] prompted us to search for an ATPase in our paracrystal preparations. When such an activity could be demonstrated it was necessary to determine the optimum assay conditions with respect to pH optimum, substrate concentration, and cation requirement. The peak ATPase

FIG. 1.—Whole mount of a single paracrystal fixed in suspension with 1% glutaraldehyde, then negatively contrasted with neutral phosphotungstate.[14] Note the fine surface periodicity and the convex ends characteristic of isolated paracrystals. ×18,000.

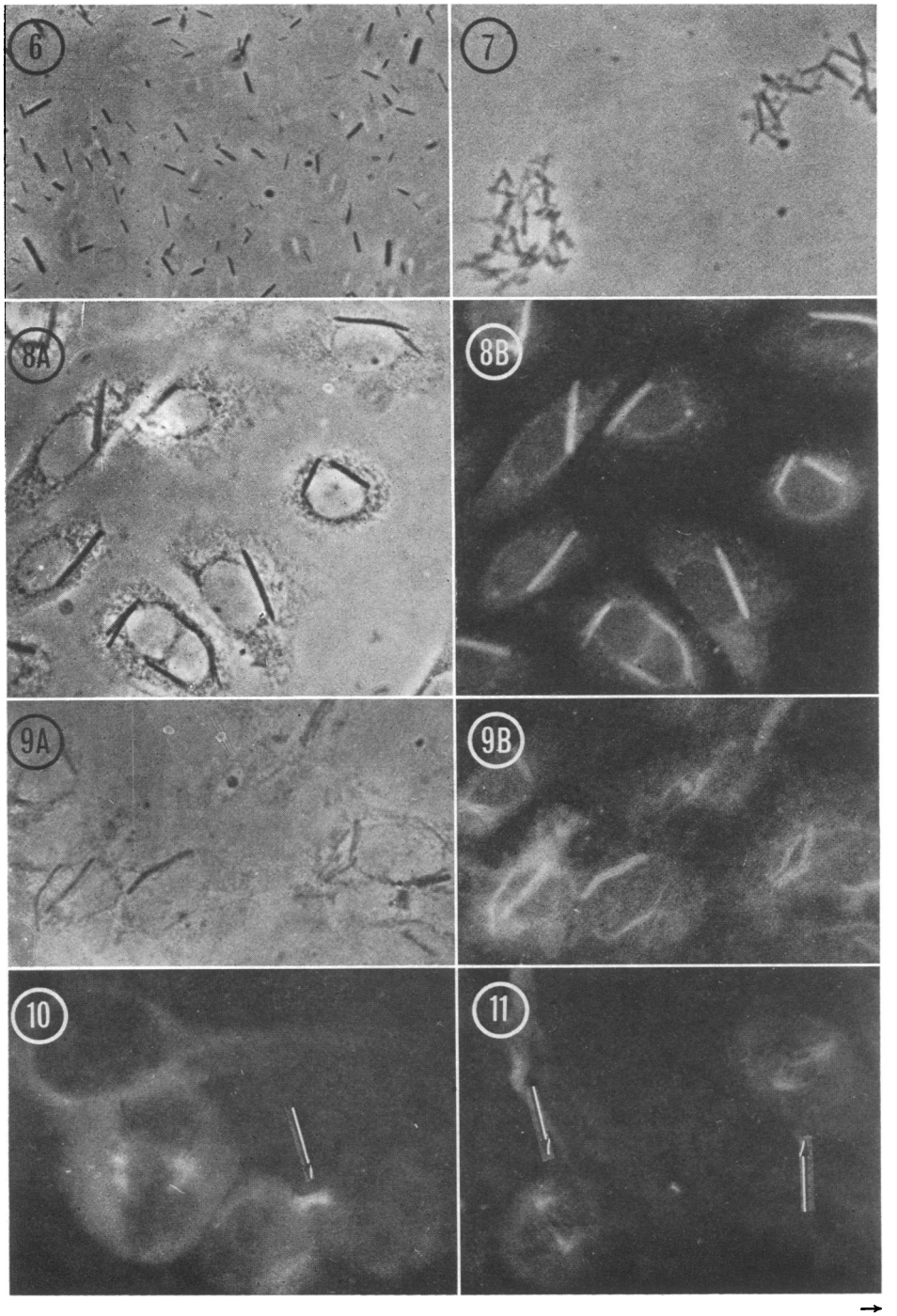
FIG. 2.—Another paracrystal, prepared as in Fig. 1, possesses alternating dense and lucent periods (*a*) running parallel to the long axis (*long arrows*), delineating the "walls" and hollow centers of 300 Å wide microtubules and (*b*) disposed at a 65° angle to the long axis (*short arrows*), indicating the presence of a helical organization in the microtubules. ×51,000.

FIG. 3.—Thin section through a pellet of purified paracrystal prepared as in ref. 15. Notice that profiles reveal flexing of the paracrystals. ×6300.

FIG. 4.—Higher resolution of a portion of two paracrystals from a preparation as in Fig. 3. Note the well-defined longitudinal periodicity and evidence of 300 Å wide microtubules (*arrows*) at the periphery. ×70,000.

FIG. 5.—Close packing of microtubules within each paracrystal is evident in predominantly cross sections from a selected area of the pellet in Fig. 3. ×27,000.

activity occurred at pH 6.5–7.0 and fell off on either side of this pH range. When 7–25 μg of paracrystal protein were added to the assay mixture, the



saturating [ATP] was 0.1 mM. Among the cations tested, 10 mM Ca^{++} enhanced the reaction slightly more than 5 mM Ca^{++} and appreciably more than Mg^{++} at the concentrations tested (Table 2). Addition of 150 mM K^+ or Na^+ was inhibitory but 20 mM added alone (Table 2) or together with ouabain at

TABLE 2. *Effect of modifying the reaction mixture on ATPase activity of paracrystal material.*

Modification of reaction mixture	Percentage of $^{32}P_i$ release compared with control	Modification of reaction mixture	Percentage of $^{32}P_i$ release compared with control
Ca^{++} 1 mM	72	ATP-γ-^{32}P 5 μM	100
Ca^{++} 5 mM	98	Unlabeled nucleotides present	
Ca^{++} 10 mM (control)	100	ATP 0.10 mM	12
Mg^{++} 1 mM	82	ATP 0.25 mM	4.8
Mg^{++} 5 mM	64	CTP 0.10 mM	60
Mg^{++} 10 mM	45	CTP 0.25 mM	48
Na^+ 20 mM, Ca^{++} 10 mM	98	GTP 0.10 mM	90.5
K^+ 20 mM, Ca^{++} 10 mM	95	GTP 0.25 mM	86
Rapid freezing and thawing	3–15	UTP 0.10 mM	85
Paracrystals isolated from HeLa cells	100	UTP 0.25 mM	69

Reaction mixture (0.1 ml) contained 7–20 μg protein and, if not otherwise specified, 0.1 mM ATP. In every experimental assay the controls were compared with samples containing identical amounts of paracrystal protein and ATP-γ-^{32}P.

10^{-3} *M* or rutamycin at 10 μg/ml had virtually no effect on the reaction, discounting the possibility that a contamination by cell membrane or mitochondrial ATPase was involved. Heating to 85° for 5 min destroyed all activity and rapid freezing, and thawing of paracrystal suspensions reduced P_i release 85–97% (Table 2). The specific ATPase activity of paracrystals from HeLa and L cells was the same (Table 2).

Time course of the ATPase activity was measured under conditions deemed to be optimum for the reaction (Fig. 13). The P_i release began without a lag and continued at a linear rate for about 15 min but declined thereafter. From

FIG. 6.—Suspension of purified paracrystals: phase contrast. ×500.

FIG. 7.—Agglutination of paracrystals after incubation at 37° for 15′ with a 1:8 dilution of immune serum: phase-contrast. ×500.

FIG. 8.—(*A*) Selected area from a culture of HeLa cells treated with vinblastine, containing dense paracrystals: phase-contrast. ×800.

(*B*) The same area viewed under ultraviolet light. Note the specific fluorescence of the paracrystals. ×800.

FIG. 9.—(*A*) Culture of chick embryo fibroblast cells treated with vinblastine containing paracrystals: phase contrast. ×800.

(*B*) The same area viewed under ultraviolet light showing specific staining of the paracrystals ×800.

FIG. 10.—HeLa cells not treated with vinblastine showing specific fluorescence under ultraviolet light in the mitotic apparatus of a cell at anaphase, in the center, and in a midbody of Flemming (*arrow*) of a cell in late telophase of mitosis. ×1600.

FIG. 11.—Chick embryo fibroblast cells prepared as in Fig. 10 showing fluorescence of the mitotic apparatus (*arrows*). ×900.

these data it was calculated that 1 mg of paracrystals would hydrolyze 100 mμ-moles of ATP in 15 min. Specific activities of 90 to 350, with an average of 180 mμmoles, were recorded in repeated assays. In another series of experiments determination was made of P_i release in the presence of a constant amount of paracrystals and varying [ATP] in the reaction mixture. From the Lineweaver-Burk plot of these data, the calculated Michaelis constant (K_m) was 0.8–1.2 $\times$ 10^{-4}, with an average of 1.0 $\times$ 10^{-4}. Specificity of the enzyme for ATP was checked by adding 20–50 times more of unlabeled triphosphates (ATP, UTP, GTP, CTP; Schwarz BioResearch, Inc.) to the reaction. Only unlabeled ATP decreased proportionally the release of $^{32}P_i$ from ATP-γ-^{32}P (Table 2). Among the other triphosphates tested CTP caused some decrease of ATP hydrolysis (Table 2).

Discussion. The technique for isolating paracrystals described in this article yields a homogeneous material judged to be pure to the limit of detection employing our morphological and immunological criteria (Figs. 3, 5, 6, and 12).

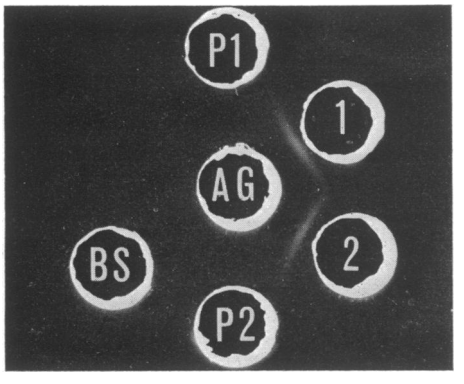


FIG. 12.—Ouchterlony test: center well contained 7 μg paracrystals protein; *BS* contained normal bovine serum; *P1* and *P2* preimmune sera and *1* and *2* the corresponding immune sera.

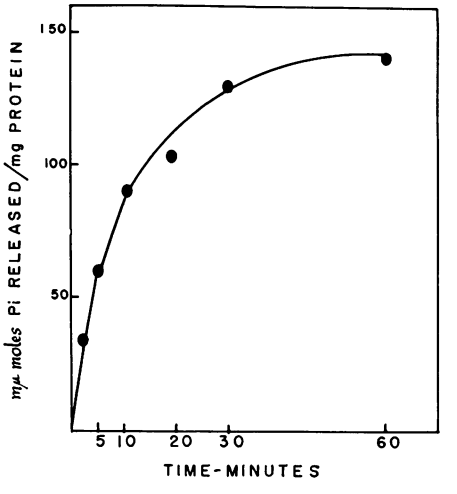


FIG. 13.—Release of $^{32}P_i$ from ATP-γ-^{32}P by purified paracrystals material. Each sample in the reaction mixture (0.1 ml) contained 7 μg of protein, 0.10 mM ATP.

With an alternative procedure the *in vitro* polymerization of microtubular protein by vinblastine has also been achieved,[4, 19] although the homogeneity of "crystals" derived by the latter method remains uncertain (J. Rosenbaum, personal communication). Our rapid purification provided us with fresh material required to demonstrate a labile ATPase associated with paracrystals. Among the proteins related to microtubules that possess ATPase activity similar to the paracrystal ATPase are bull sperm spermosin-p[20] and mitotic apparatus from the sea urchin.[18] The K_m 2.1 $\times$ 10^{-4} of spermosin-p approximates the K_m 1.0 $\times$ 10^{-4} established for the paracrystal ATPase. Specific activity of an ATPase in the dyenin fraction isolated from cilia[21] is, however,

much greater. The role of the paracrystal ATPase in the function of microtubules remains to be elucidated.

Specific intracellular immunofluorescence of paracrystals and mitotic apparatus shows that the same antigenic determinant occurs in both structures containing microtubules. The capacity of sera prepared against paracrystals from malignant cells of murine origin to label specifically the mitotic apparatus of human and chicken cells shows that a common or related antigen occurs in the mitotic apparatus of these higher vertebrates. It remains to be determined whether this common antigenic determinant is the microtubular protein.

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