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Effect of Microinjected *N*-Ethylmaleimide-modified Heavy Meromyosin on Cell Division in Amphibian Eggs

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ABSTRACT *N*-Ethylmaleimide-modified heavy meromyosin (NEM-HMM) microinjected into amphibian eggs inhibits cytokinesis and the cortical contractions associated with wound closure. Injection of NEM-HMM into two-cell *Rana pipiens* embryos produces a zone of cleavage inhibition around the point of injection. Early furrows followed by time-lapse microcinematography are seen to slow and stop as they enter the NEM-HMM-injected zone. Arrested furrows slowly regress, leaving a large region of cytoplasm uncleaved. Few nuclei are found in these regions of cleavage inhibition. Wound closure is often inhibited by NEM-HMM, especially when this inhibitor is injected just beneath the egg cortex. We observe that the surface of an unfertilized *Rana* egg is covered with microvilli that disappear during the course of development. The surfaces of NEM-HMM-inhibited zones remain covered with microvilli and resemble the unfertilized egg surface.

Cytokinesis in animal cells is accomplished by an equatorial constriction of the cortex that progressively pinches the cell in two, much like the action of a purse string. The discovery that a band of actin microfilaments, the contractile ring, forms in association with the developing cleavage furrow gave rise to a model of cytokinesis based on the role of actomyosin in the muscle sarcomere (1, 3, 26, 27, 29–32, 35, 38, 40).

Evidence supporting an actomyosin-based model for cytokinesis has come largely from studies at the light and electron microscope levels that demonstrate that both actin and myosin are present in the cleavage furrow (11, 17, 28). Microinjection of an antibody against myosin has been shown to prevent cytokinesis in starfish (18) and sea urchin blastomeres (15), and studies using cytochalasin B, although limited by questions regarding the drug's specificity, likewise suggest an actomyosin-based mechanism for cleavage (33).

We have previously demonstrated that rabbit skeletal muscle heavy meromyosin (HMM) treated with the sulfhydryl reagent *N*-ethylmaleimide (NEM) serves as a specific inhibitor of muscle-type force-generating systems (21). *N*-Ethylmaleimide-modified heavy meromyosin (NEM-HMM) binds tightly to actin and does not release in the presence of MgATP. After decoration with NEM-HMM, the actin becomes unavailable to native myosin. Inhibition of *in vitro* actomyosin interactions, the contraction of demembrated muscle myofibrils, and contractility of *Chaos* cytoplasm also result (21). It is not known

whether NEM-HMM interferes with actin polymerization and the organization of actin gels. However, NEM-HMM inhibits neither the beat of demembrated cilia nor *in vitro* microtubule polymerization (21).

We report here that microinjection of NEM-HMM into blastomeres of fertilized *Rana pipiens* eggs inhibits cleavage in a zone surrounding the injection point and can prevent wound closure. We also report that changes in microvilli distribution are inhibited by NEM-HMM.

Our findings confirm the involvement of an actomyosin force-generating mechanism in cleavage and are consistent with the premise that formation of the contractile ring results from a contractile event (27, 30, 32).

MATERIALS AND METHODS

Preparation of Proteins

HMM was prepared by chymotryptic digestion of myosin, as described by Weeds and Taylor (41), and was treated with NEM as reported previously (21). After treatment with NEM for 75 min, HMM's calcium ATPase was still activated; however, there was no detectable K⁺-EDTA ATPase activity, and the modified NEM-HMM inhibited glycerinated myofibril contraction (Table I). We found that in the presence of 1% (wt/vol) sucrose, freezing and lyophilization caused minimal loss of HMM's or NEM-HMM's ATPase activities (Table I). Aliquots of lyophilized HMM and NEM-HMM were stored desiccated at –20°C until needed for microinjection.

Actin was extracted from an acetone powder according to Etlinger et al. (7).

Conditions for ATPase assays were as described previously (21), and inorganic phosphate was determined by the procedure of Fiske and Subbarow (8).

Microinjection of Eggs

Sexually mature northern *Rana pipiens* were obtained from Arnold Nasco, Ltd., Fort Atkinson, Wis., in the fall and stored in the dark at -4°C . Frogs were induced to ovulate by the injection of one or two macerated pituitary glands into the body cavity and by intramuscular injection of progesterone, depending upon the season (43). Eggs were fertilized at 18°C with minced testis for 15 min, rinsed, and then allowed to develop in 10% Steinberg's solution (37) before injection. Eggs at the late two-cell stage were cooled to 10°C and microinjected through the jelly coat to avoid possible damage to the cortex during manual or chemical dejellying. The cells were then allowed to develop for 6–12 h before fixation. Microneedles were prepared according to Masui and Markert (19) on a microforge and had a tip diameter of 10–20 μm . The microneedles were calibrated by measuring the dimensions of drops of H_2O injected into a light oil with an ocular micrometer. Constant volumes (≤ 60 nl) but varying concentrations of proteins were injected into eggs by the method described by Masui and Markert (19).

TABLE I
Properties of HMM and NEM-HMM Used in Microinjection Studies

	Calcium ATPase	EDTA ATPase	Inhibits myofibril contraction
HMM	0.78	7.28	No
NEM-HMM	1.16	0.00	Yes
HMM (lyophilized)	0.56	6.38	No
NEM-HMM (lyophilized)	0.76	0.00	Yes

ATPase activities expressed as molecules of ATP hydrolyzed per head s^{-1} . Rabbit skeletal muscle myofibrils were incubated in 3 mg/ml protein for 15 min, rinsed twice, and triggered to contract with 0.1 mM MgATP.

Time-lapse Microcinematography

Eggs to be monitored by time-lapse microcinematography were very carefully dejellied with watchmaker forceps before microinjection and were photographed on Kodak 2498 16-mm film at three frames per minute with an Opti-Quip time-lapse cine apparatus (Opti-Quip Inc., Highland Mills, N. Y.) mounted on a Wild dissecting microscope. Illumination was provided by a fiber optic-equipped halogen light source with an automatic shutter so that the eggs were only illuminated for ~ 3 s of each minute. The temperature of the egg chamber (20°C) was virtually unaffected by the light source, and egg development proceeded normally under these conditions.

Microscopy

For scanning electron microscopy, embryos were placed in Smith's fixative overnight (36), dehydrated in a graded ethanol series, and then critical-point dried in a CO_2 drier. They were observed in a Coates and Welter scanning electron microscope after being coated with gold-palladium. For light microscopy, eggs were fixed in Smith's fixative, dehydrated in ethanol, and embedded in paraffin. Sections were stained with Feulgen's stain and counterstained with fast green (5, 23). Estimates of volumes of sectioned eggs were made by cutting and weighing camera lucida sketches of serial sections through NEM-HMM-inhibited regions.

RESULTS

Inhibition of Cleavage by NEM-HMM

Injection of NEM-HMM at the two-cell stage inhibits subsequent cleavages of the injected blastomere in a zone surrounding the injection point (Fig. 1a). Eggs injected with native HMM, NEM-treated bovine serum albumin (NEM-BSA), dialysate from the NEM-HMM preparation, or buffer all cleave normally (Fig. 1b and c, and Table II). Cleavage was inhibited when as little as 165 ng of NEM-HMM was injected, but when more NEM-HMM was introduced a higher percent-

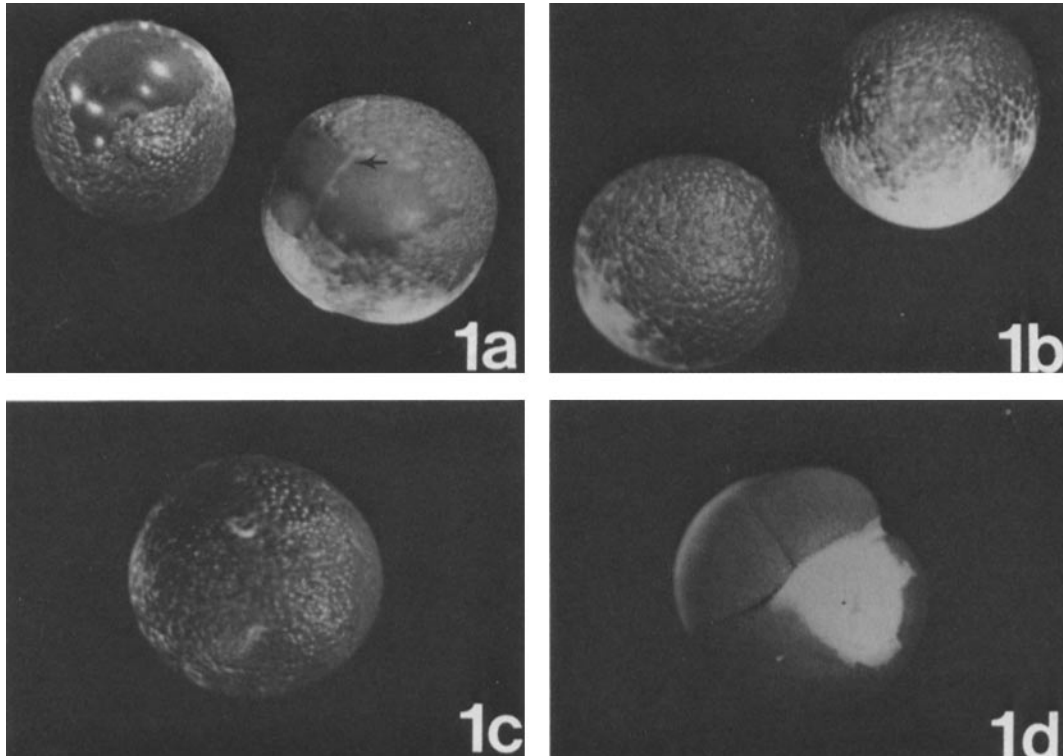


FIGURE 1 Effect of microinjected NEM-HMM on cleavage in *Rana* embryos. Injection of 400 ng of NEM-HMM at the two-cell stage inhibits subsequent cleavages of the injected blastomere in a region around the injection site (a). Occasionally an early furrow successfully crosses the injected zone (arrow; see text). Embryos injected with HMM (b) are indistinguishable from uninjected controls (c). Shallow injections of NEM-HMM often result in inhibition of wound closure (d). The embryo in d was fixed 20 min after injection. The embryos in a–c were fixed 16 h after injection. All figures shown at $\sim \times 30$.

age of injected eggs were inhibited (Fig. 2). Injections of 500 ng or more consistently produced cleavage inhibition in 80% of the eggs injected with NEM-HMM. No obvious relation between amount injected and surface area of the affected zone was observed.

In all experiments, embryos were injected shortly before second division, which was only rarely prevented. The site for injection was chosen out of the path of the second division furrow to avoid the complication of furrow formation in a region of the cortex that is already undergoing wound closure. When the zone of cleavage inhibition occasionally extends across the second division plane, this cleavage nevertheless occurs (Fig. 1a, arrow), suggesting that NEM-HMM must be injected >10 min before cleavage to inhibit.

Behavior of Furrows

Development of four eggs injected with NEM-HMM was followed by time-lapse microcinematography through the 10th division (Fig. 3). In all four eggs, second division proceeded normally, but furrows that arose during third, fourth, and fifth divisions entered the injected zone, slowed, stopped, and then slowly regressed (Fig. 3). These furrows arose synchronously with those in the control blastomere but did not propagate across the injected region. Furrows in this region usually remained arrested for one to one and one-half cell cycles (50–75 min) and then regressed slowly, requiring another one to two cell cycles to completely disappear. The same division

furrows in control eggs or uninjected regions required <4 min to propagate through a blastomere.

After regression of the early (third through fifth) division furrows, no further furrowing activity was observed in the inhibited zones. Meanwhile, the rest of the embryo continued to divide synchronously. No external signs of degeneration (uneven pigment distribution) were observed in the inhibited region.

Occasionally, a shallow furrow is seen to have successfully penetrated a noncleaving region (Fig. 1a, arrow). In the case in which this was observed, under time-lapse cinematography, this furrow was identified as arising during second division, which usually begins 5–10 min after NEM-HMM injection.

Effect on Nuclei

Embryos injected with NEM-HMM possess large uncleaved

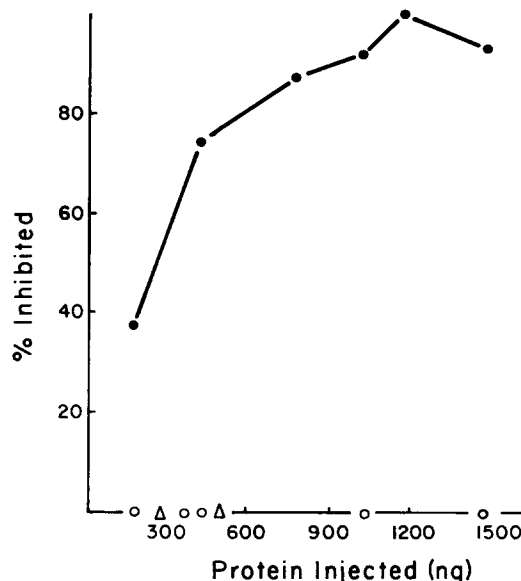


FIGURE 2 Percent of injected *Rana* eggs showing cleavage inhibition as a function of amount injected. Each point represents at least eight injections. Data include inhibition of wound response (see text). ●, NEM-HMM; ○, HMM; △, NEM-BSA.

TABLE II

Effects of Microinjections on *Rana* Egg Cleavages

Sample injected	Number of recipients*	Cleavage inhibition	Wound closure inhibition
		%	%
NEM-HMM	110	73	13
HMM	70	0	0
NEM-BSA	26	0	0
Dialysate	17	0	0
Buffer	33	0	0

* Total of nine separate experiments in which various amounts of protein or buffer were injected into eggs from 11 different frogs.

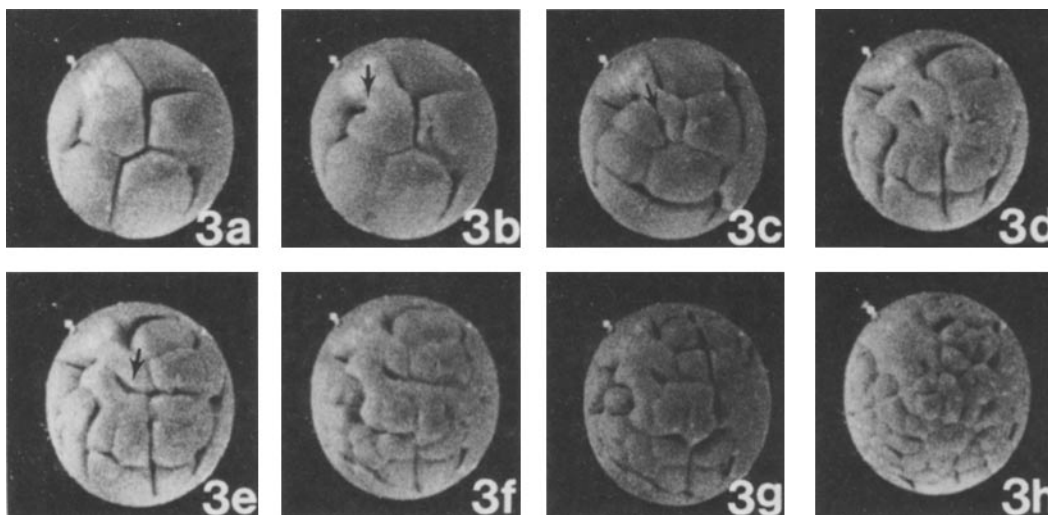


FIGURE 3 Behavior of cleavage furrows in NEM-HMM-injected zone. Eggs were injected with 1,000 ng of NEM-HMM. Small white yolk plug marks site of injection on upper left of egg. (a) Third division begins. (b) 20 min after third division. Furrow in injected zone is arrested (arrow). (c) Fourth division has occurred and again furrow entering injected zone arrests (arrow). (d and e) Fifth division. A third furrow enters zone and stops (arrow). (f–h) Early furrows regress as sixth through eighth divisions proceed. $\times 20$.

regions around the injection site not seen in sectioned control eggs (Fig. 4 *a* and *b*). These uncleaved regions of cytoplasm are variable in size and contain few nuclei. The three embryos analyzed in detail (Table III) are typical of other NEM-HMM-injected embryos examined. Inhibited regions usually contain from one to 10 nuclei and comprise from about one-sixth to

one-third the volume of the injected blastomere. Equivalent volumes of cytoplasm from the uninjected blastomere or from blastomeres injected with control proteins contain hundreds of nuclei. However, both interphase nuclei and mitotic figures are found in the NEM-HMM inhibited regions. These are similar in morphology and staining properties to nuclei or mitotic

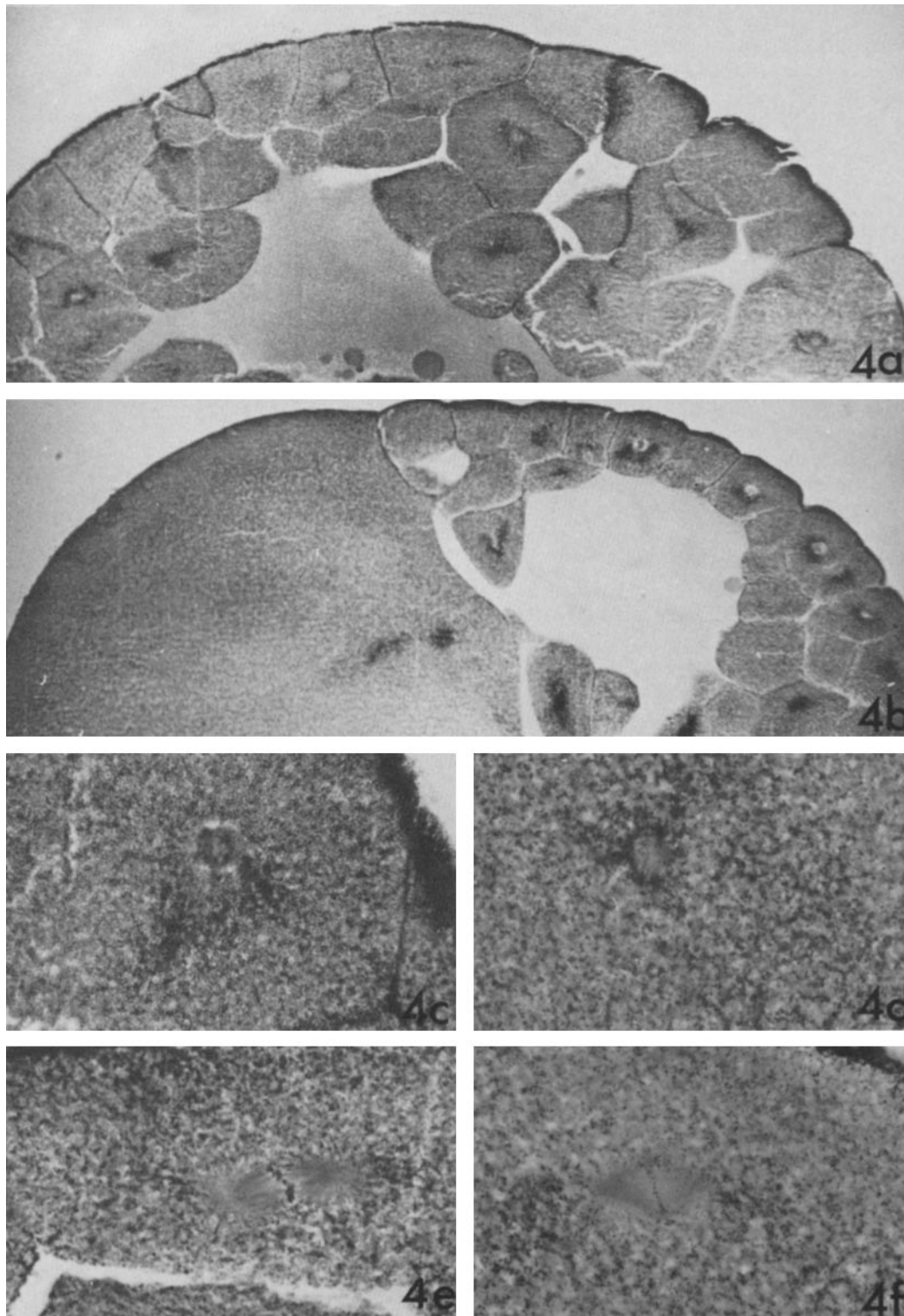


FIGURE 4 Sectioned *Rana* eggs injected with 1,000 ng HMM (*a*) and NEM-HMM (*b*) $\times 100$. Interphase (*d*) and metaphase (*f*) figures from NEM-HMM-inhibited region are similar to those in normally cleaving eggs injected with HMM (*c* and *e*). The metaphase figure in *e* is not aligned parallel to the plane of sectioning and the asters are located in adjacent sections (not shown).

figures of normally dividing blastomeres (Fig. 4 *c-f*). In one egg, an anaphase figure was found in an uncleaved zone. Because the spindle was obliquely sectioned, no micrographs are included here of the mitotic figures. Cytoplasm inside the zone of cleavage inhibition is indistinguishable from that outside.

Effect on Wound Response

In amphibian eggs, the wound caused by microinjection is

TABLE III
Numbers of Nuclei in NEM-HMM-Inhibited and Control Regions

Embryo	Sections examined*	Blastomere	Number of nuclei	Density of nuclei <i>per mm</i> ³
		Volume inhibited‡		
1	Inhibited zone	16	8	57
	Control zone	—	325	2,166
2	Inhibited zone	33	3	10
	Control zone	—	242	1,152
3	Inhibited zone	13	8	73
	Control zone	—	143	477

* Complete serial sections were available for analysis of embryo one which was embedded in plastic and sectioned at 5 μ m on a glass knife. Embryos two and three were sectioned at 10 μ m in paraffin. More than 90% of the sections were available for examination. Embryos were injected with 1,040, 1,160, and 1,160 ng NEM-HMM at the two-cell stage, respectively.

‡ The blastomere volume is $\sim 1 \mu$ l.

sealed through a localized cortical contraction thought to involve actomyosin (4, 12). In 14 of the NEM-HMM injected eggs this process was inhibited (Table II). The small wound caused by the micropipette does not seal but begins to expand, revealing the densely packed white yolk platelets within the egg. Within 5–10 min a gaping hole is present in the cortex beneath the vitelline membrane (Fig. 1 *d*). This effect is not seen in control eggs injected with HMM, NEM-BSA, dialysate, or buffer and is distinctly different from the gradual leakage of cytoplasm that occurs when an injected egg has been damaged by a dull or overly large micropipette. Inhibition of wound closure occurs most frequently with shallow injections, (i.e., with the micropipette tip just below the cortex) and is seldom seen if care is taken to inject deeper into the cytoplasm.

Effect on Microvilli Distribution

The zone of cleavage inhibition resulting from NEM-HMM injection is readily apparent in the scanning electron microscope (Fig. 5 *a*). At higher magnification the uncleaved zone is seen to be densely covered with 0.3–0.5- μ m-long microvilli that stop abruptly at the boundary between the injected zone and the uninhibited, cleaving blastomeres (Fig. 5 *b*). After 12 h, normally dividing blastomeres display relatively few microvilli on their surface (Fig. 5 *c*). Furrows between uninhibited blastomeres contain a row of microvilli between smooth surfaces, whereas the shallow furrows occasionally seen to successfully penetrate an NEM-HMM inhibited zone are covered with microvilli and are almost indistinguishable from the rest of the zone's surface (Fig. 5 *d*). Eggs immediately after fertilization possess many microvilli (Fig. 6) that are similar in size and

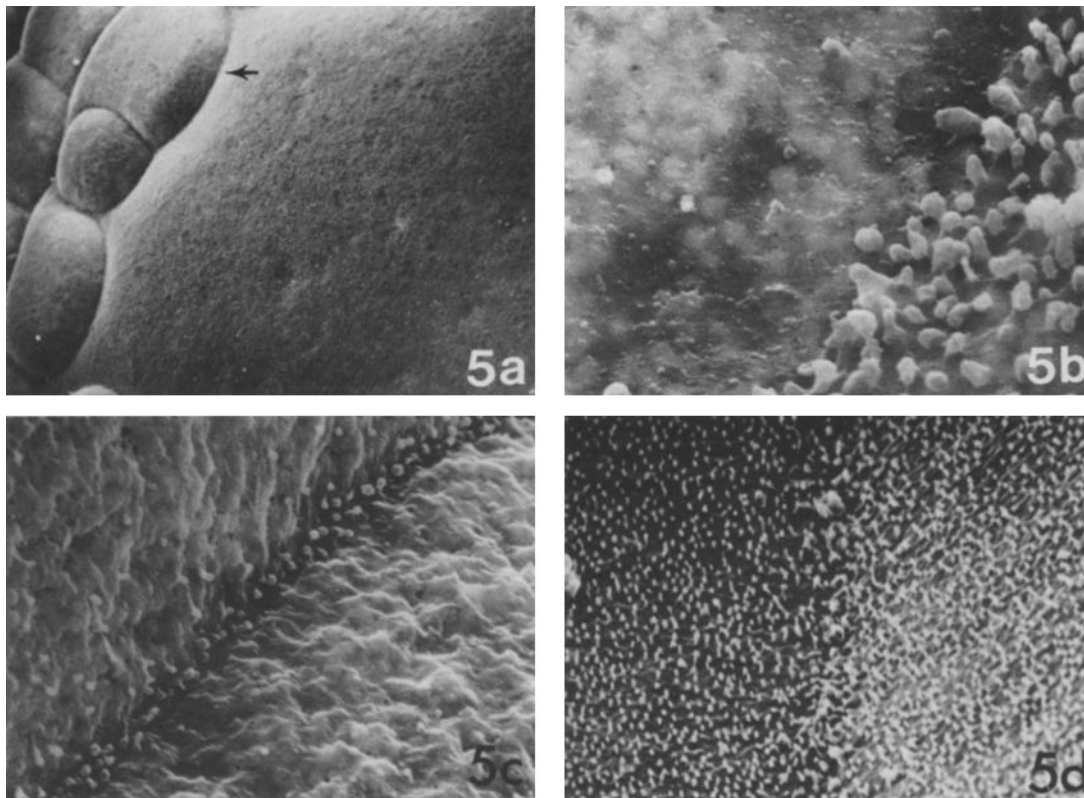


FIGURE 5 Effect of NEM-HMM on retraction of microvilli. Egg injected with 400 ng of NEM-HMM possesses a large region of uncleaved surface (*a*). $\times 370$. Higher magnification of border between cleaving and inhibited zones (arrow in *a*) shows many microvilli on surface of uncleaved region (lower right in *b*). $\times 10,000$. (*c*) Furrow between normally dividing blastomeres of egg in *a*. (*d*) An early furrow occasionally seen to penetrate the inhibited zone. $\times 2,800$ and $\times 5,000$.

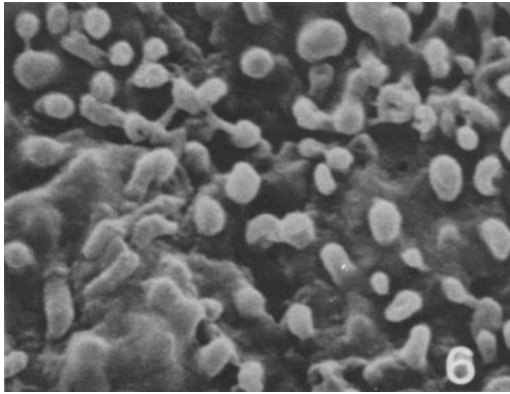


FIGURE 6 Behavior of microvilli during early development of *Rana* embryos. An unfertilized *Rana* egg is covered with microvilli. $\times 10,000$.

distribution to the microvilli seen in the NEM-HMM-treated zones (Fig. 5b).

DISCUSSION

We have previously demonstrated that NEM-modified HMM specifically inhibits actomyosin-dependent motile events *in vitro* and in cell models but does not interfere with the beat of demembrated cilia or with *in vitro* microtubule polymerization (21). We report here that NEM-HMM introduced into fertilized amphibian eggs by microinjection inhibits cytokinesis and wound closure, profoundly affects the distribution of surface microvilli, and produces large uncleaved regions of cytoplasm that contain few nuclei.

NEM-HMM binds tightly to actin in ATP-insensitive complexes (21). Whereas microinjected HMM should undergo cycles of binding and release from cytoplasmic actin and eventually diffuse throughout the cell, NEM-HMM would be expected to bind tightly to actin in a zone around the site of injection. This should produce an "inhibited" zone in the cytoplasm in which actomyosin force production is reduced or prevented because binding sites for myosin on the actin filaments would be occupied by NEM-HMM. The inhibition of cleavage that we observe after NEM-HMM injection is not caused by contamination by unreacted NEM because cleavage continues after injection of NEM-treated BSA or dialysate from the NEM-HMM preparation. The low ATPase activity of NEM-HMM under physiological conditions, $\sim 1/20$ that of HMM (Table I and reference 21), makes it unlikely that internal ATP pools are seriously affected.

We believe NEM-HMM inhibits these processes by physically occupying myosin binding sites on cytoplasmic actin filaments, thereby preventing myosin from interacting with actin to generate force. We cannot exclude the possibility that when NEM-HMM binds actin it also perturbs actin's association with other proteins, perhaps affecting actin-based cytoplasmic "gels" (39). However, microinjected HMM, which solates actin gels *in vitro*, does not disrupt cleavage (42).

If the concentration of actin in *Rana pipiens* eggs is similar to that reported for *Xenopus laevis* oocytes (~ 4 mg/ml, cf. reference 22), enough NEM-HMM can be injected to fully titrate $\sim 5\%$ of the blastomere's actin. If both "heads" of the NEM-HMM molecule bind actin, this figure doubles. However, predictions of the volume of cytoplasm expected to be inhibited by a given amount of NEM-HMM are complicated by the fact that actin is not uniformly distributed throughout

the cytoplasm (10). We find that typically one-third to one-sixth of the blastomere's volume is included within an uncleaved zone. If actin were uniformly distributed throughout the blastomere, we would expect that 15–30% of the actin in the inhibited zone would be complexed with NEM-HMM.

Cleavage

Inhibition of cleavage by NEM-HMM constitutes a direct demonstration of the involvement of actomyosin in cytokinesis. It confirms the findings of Kiehart et al. (15) and Mabuchi and Okuno (18), who demonstrated a role for myosin in cytokinesis by blocking cleavage of sea urchin and starfish blastomeres with microinjected antibodies against myosin.

The cine observations of microinjected eggs demonstrate that third and subsequent division furrows that would normally pass near the injection point are unable to do so (Fig. 3). Furrows arise on the periphery of the injected region, propagate into the zone, and stop. Regression is extremely slow and probably represents gradual dissolution of the furrow's contractile structures. After regression of these early furrows, no further furrows arise in the inhibited region. Because furrows can originate outside the injection zone during these early divisions, NEM-HMM does not block the events of furrow initiation but must interfere with furrow propagation and constriction within the affected zone.

Cortical Contractility

Amphibian embryos undergo a variety of contractile events during early development that are thought to involve cortical actomyosin (13, 34). Cortical contractions play a role in sperm penetration, grey crescent formation, wound closure, and other processes (4, 6, 12). Microinjection of the actomyosin inhibitor NEM-HMM not only prevents cleavage but also blocks the cortical contractions responsible for wound closure. In addition, preliminary experiments performed in collaboration with R. M. Ezzell indicate that NEM-HMM injected into oocytes of *Xenopus laevis* inhibits the massive cortical contractions induced by the calcium ionophore A23187 (Ezzell and Meeusen, unpublished data).

Scarcity of Nuclei in NEM-HMM-Inhibited Regions

Regions of uncleaved cytoplasm resulting from NEM-HMM injection contain as little as 1% of the number of nuclei per unit volume that control regions contain (Table III). One possible explanation is that NEM-HMM inhibits nuclear migration. Because of the *Rana* egg's great size, nuclei must migrate considerable distances after division. If this process involves actomyosin, NEM-HMM would be expected to delay entry of the nuclei into the injected zone, resulting in fewer nuclei per unit volume in this region of the blastomere. These nuclei instead would be found in cells surrounding the affected zone. It is unlikely that we could detect this event.

Alternatively, NEM-HMM may act on some other actomyosin-dependent event that is required for continuation of the cell cycle or for initiation of karyokinesis. For example, we observe that after fifth cleavage no other furrows arise near or propagate into the inhibited zone. If the cell cycle is arrested after fifth cleavage in the inhibited zone, we would expect to find only $2^3 (=8)$ nuclei in this region. This is approximately the number of nuclei we normally observe (Table III).

A third possibility is that NEM-HMM directly affects mitosis. It has been suggested that actomyosin plays a role in generating the forces necessary for anaphase chromosome movement (9, 14, 20, 25). However, we have found normal metaphase figures and one anaphase spindle in inhibited zones of eggs. In addition, Kiehart et al. (15) and Mabuchi and Okuno (18) have shown that a myosin antibody that inhibits cleavage when microinjected into marine eggs does not inhibit mitosis. Work under way in our laboratory demonstrates that anaphase chromosome movement in permeabilized PtK₁ cells is insensitive to NEM-modified myosin subfragment 1 and phalloidin (Cande and Meeusen, manuscript in preparation). These results, in toto, are inconsistent with a direct actomyosin involvement in chromosome movement.

Microvilli

The surface of an unfertilized *Rana* egg is densely covered with microvilli (Fig. 6). During early development these microvilli disappear from the embryo surface but not from the cleavage furrow region itself (W. Cande, unpublished data). The surfaces of zones inhibited by NEM-HMM remain covered with microvilli and resemble the surfaces of unfertilized eggs. This suggests that the microvillar distribution changes observed after fertilization may involve an actomyosin-based contraction. Alternatively, NEM-HMM may prevent organizational changes within the microvilli that are necessary for retraction to occur. We cannot exclude the possibility that cleavage and microvillar retraction are independent processes related only by a common dependence upon cortical actomyosin. However, when cytokinesis in *Rana* blastomeres is inhibited by injection of a cytoplasmic factor (CSF) from unfertilized eggs (23), the mitotic spindle is arrested at metaphase, and the microvilli also fail to retract (Peter Meyerhoff, personal communication). The failure of microvilli to retract when cleavage is prevented by either NEM-HMM or CSF is certainly consistent with an involvement of microvilli in cytokinesis.

The idea that microvilli play a role in cytokinesis is attractive because microvilli represent a potential source of actin, myosin, and sites of membrane attachment, three necessary (and perhaps sufficient) elements for organizing a cleavage furrow. Observations reported previously lend credence to this idea. In the amphibian egg, large numbers of microvilli arise immediately ahead of the advancing furrow (2), the zone in which preparation for furrow formation has been shown to be occurring (16). Contractile ring microfilaments have also been reported to insert into microvilli (38).

One possibility is that microvilli are directly incorporated into the developing furrow, acting as precursors to the cleavage furrow as recently suggested by David Begg (personal communication) for organization of the cleavage furrow in sea urchin eggs. Another possibility is that microvilli serve primarily as membrane attachment points, anchoring cortical actomyosin and transmitting the tension developed during cleavage to the cell surface. Microvilli would seem to be well suited to such a role because the actin filaments in the core of each microvillus are connected to the membrane both terminally and by means of lateral cross-links (24).

Experiments are in progress using permeabilized cell models and microinjected amphibian eggs to study the physiology of cleavage and the role of cortical actomyosin in these events.

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