Fate of Secretory Proteins Trapped in Oocytes of *Xenopus laevis* by Disruption of the Cytoskeleton or by Imbalanced Subunit Synthesis

ALAN COLMAN, JOHN MORSER, CHARLES LANE, JAYNE BESLEY, CHRISTOPHER WYLIE, and GIORGIO VALLE

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, Laboratory of Developmental Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, and Department of Anatomy, St. George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, England

ABSTRACT The effects of imbalanced subunit synthesis, temperature, colchicine, and cytochalasin on the secretion from *Xenopus laevis* oocytes of a variety of avian and mammalian proteins were investigated; these proteins were encoded by microinjected messenger RNA. Cytochalasin and colchicine together severely reduced secretion in a temperature-independent manner, the exact reduction varying among the different proteins. In contrast cytochalasin alone had no effect, whereas colchicine alone caused a smaller, temperature-dependent reduction. The synthesis and subcellular compartmentation of these proteins were unaffected by the drug treatments; however, the proteins did not accumulate in the drug-treated oocytes but were degraded. The rate of degradation of each protein was similar to its rate of exocytosis from untreated oocytes. A similar result was obtained without recourse to drugs by studying the fate of immunoglobulin light chains trapped in oocytes by a deficiency in heavy chain synthesis. These results are discussed in terms of the disruptive effects, as revealed by electron microscopy, of the drug treatments on the cytoskeleton of the oocyte.

Microtubules and microfilaments have long been implicated in the secretion of proteins from cells. Much of this evidence has accrued through observation of the effects of the drugs colchicine and cytochalasin on cellular protein secretion. Colchicine, which binds to soluble tubulin and prevents its polymerization into microtubules (for reviews, see references 1–4), inhibits the secretion of many proteins including collagen (5), rat gonadotrophins (6), and insulin (7, 8). Cytochalasin affects the integrity of microfilaments by binding to the actin component (9, 10) and has also been shown to inhibit the secretion of a variety of proteins (for review see reference 11). While there are many examples where inhibition does not occur in the presence of these drugs (11), it is not clear to what extent this reflects intrinsic differences between the various secretory proteins or differences in the organization of secretory apparatus between the various cell types.

These difficulties in interpretation might be circumvented if a single cell type could be used to investigate the participation of the cytoskeleton in the secretion of proteins from heterologous sources. The oocytes of *Xenopus laevis* provide such a system. These cells contain microfilaments, microtubules (12), and large pools of tubulin and actin monomers (13–16) that presumably become available for recruitment into microtubules and microfilaments during early development. Recently these oocytes have been shown to export secretory proteins encoded by microinjected mRNAs (17). This process is highly specific since nonsecretory proteins are not exported. We have investigated the possibility that microtubules and/or microfilaments might be involved in the secretion from oocytes of heterologous proteins. In this report, we examine the effects of colchicine and cytochalasin on this secretion: both drugs disrupt the oocyte's cytoskeleton and we find that the secretion of all proteins examined, is inhibited by the application of colchicine and cytochalasin simultaneously or colchicine alone at low temperature. Surprisingly, each trapped protein is degraded at a rate similar to its rate of secretion, a result that we have also obtained for one protein without recourse to cytoskeletal disruption.

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MATERIALS AND METHODS

Animals

Adult *Xenopus laevis*, obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa), were kept at 19°C.

Chemicals

Except where otherwise mentioned, all chemicals were of analytical grade and were purchased from British Drug Houses Ltd., Poole, U. K. [^{35}S]Methionine (150–300 Ci/mmol), ^{3}H-cytochalasin B (20 Ci/mmol), and ring A-4-^{3}H-colchicine (15 Ci/mmol) were supplied by the Radiochemical Centre, Amersham, U. K. Colchicine, cytochalasin B, vinblastine, and griseofulvin obtained from Sigma Ltd., U. K. Nocadazole was from Aldrich Chemical Co., Dorset, U. K.

Preparation of Messenger RNAs

Total poly(A$^+$)-mRNAs were isolated by the methods of Morser et al. (18) from Namalwa cells (a human lymphoblastoid line) that had been induced to produce interferon with Sendai virus. Lactating guinea-pig mammary gland mRNAs were prepared as described by Craig et al. (19) and were a kind gift of Dr. R. Craig; the principal milk proteins encoded in this preparation were caseins A, B, and C and α-lactalbumin, which have molecular weights of 28,000, 25,000, 20,500, and 14,500 respectively (20). Chick oviduct mRNA prepared by the method of Palmiter (21) was a kind gift of Dr. M. Houghton. Immunoglobulin mRNA was prepared from P3-X63 Ag8 cells by the method of Cowan et al. (22).

Preparation and Microinjection of Oocytes

Large *Xenopus laevis* females were killed by the injection of barbiturates, and the ovary was removed, divided into small clumps of oocytes, and stored in modified Barths' solution (23) containing sodium penicillin and streptomycin sulphate, both at 10 μg/ml, gentamycin at 100 μg/ml, and mycostatin at 20 U/ml.

Microinjection of oocytes with ~30-nl aliquots of messenger RNAs in distilled water was performed as described by Gurdon (24). Unless otherwise indicated, injected oocytes were first cultured for 24 h in Barths' solution (50 oocytes/5 ml). Any damaged oocytes were then discarded and the remainder were cultured in small wells containing 30 μl of 0.5–1.0 mCi/ml [^{35}S]methionine in Barths' solution, with five oocytes per well. Incubation temperatures were varied and are reported in the Results section. At the end of the incubation period oocytes and incubation media were frozen separately at −70°C before further analysis. Incubation medium was only analyzed from those wells in which all the oocytes appeared morphologically healthy. Oocytes and incubation media were assayed for interferon activity, as described earlier (17).

Polyacrylamide Gel Electrophoresis

Oocyte samples for gel electrophoresis were homogenized at five oocytes/200 μl 1% (vol/vol) Nonidet P-40 (NP-40) in phosphate-buffered saline pH 7.6 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation for 5 min at room temperature in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) at 10,000 *g*, the supernatant was removed and diluted with an equal volume 20% (wt/vol) sucrose, 2% (wt/vol) sodium dodecyl sulfate, 1% 2-mercaptoethanol, 0.01% bromophenol blue 12.5 mM Tris/HCl, pH 6.8. Samples of incubation media were also diluted with an equal volume of the above sample buffer. 10-μl aliquots of oocyte samples (equivalent to 0.125 oocyte) and 20-μl aliquots of incubation media samples (equivalent to 1.67 oocytes) were electrophoresed overnight on either 10–22.5% exponential gradient or 12.5% polyacrylamide gels (25) at 8 mA and then fixed in 45% methanol, 10% acetic acid, before drying down and exposing to Fuji x-ray film. In some cases fixed gels were impregnated with 25-diphenyloxazole (PPO) and fluorographed (26).

Subcellular Fractionation of Oocytes

Oocytes were subfractionated by a modified single-step procedure (27); thus groups of 20 oocytes were homogenized in 400 μl, 50 mM NaCl, 10 mM magnesium acetate, 20 mM Tris/HCl, pH 7.6 (T buffer) supplemented with 10% (wt/vol) sucrose, 100 mM NaCl, and 1 mM PMSF at 4°C. 20 μl of the homogenates (H) were spun for 2 min in an Eppendorf microcentrifuge and the resulting supernatants were retained for electrophoresis. The remaining homogenates were layered onto 1 ml of T buffer containing 20% (wt/vol) sucrose, 1 mM PMSF, in 5 ml M.S.E. (Crawley, U. K.) polyacarbonate tubes and spun in an M.S.E. 8 × 5 ml rotor at 17,000 g_{max} for 30 min at 4°C. The supernatants representing the oocyte cytosol (C) were removed and retained for electrophoresis. The pellets containing yolk and oocyte vesicular elements were further extracted with 200 μl of phosphate-buffered saline pH 7.6 containing 1 mM PMSF and 1% NP-40 followed by centrifugation at 10,000 rpm in an Eppendorf microcentrifuge at 4°C. The supernatant (V) containing extracted vesicles was retained for electrophoresis.

To H, C, and V, an equal volume of electrophoresis sample buffer was added. 5-μl aliquots of C and V samples and 10 μl of H samples were then electrophoresed as above.

Immunoprecipitation

50-μml aliquots of oocyte homogenates were diluted to 500 μl by addition of immunoprecipitation buffer containing 1% Triton X-100 (vol/vol), 1% sodium deoxycholate (wt/vol), 0.5% sodium dodecyl sulfate (wt/vol), 1 mM PMSF, 100 mM KCl, 5 mM $MgCl_2$, 100 mM Tris/HCl, pH 8.2 (Dr. A. Williamson, personal communication) 5 μl of mixed anticasein and anti-α-lactalbumin antibody (a kind gift of Dr. R. Craig) or antiovalbumin antibody (a kind gift of Dr. M. Houghton) or anti-mouse IgG (Miles Laboratories, Elkhart, Ind.) were added and the mixture left for 1 h at room temperature. 50 μl of a 10% suspension of heat-killed formalin-treated *Staphylococcus aureus* Cowen I bacteria (28) were added and the mixture kept overnight at 4°C. The bacteria were collected by centrifugation and washed three times in immunoprecipitation buffer, 50 μl of electrophoresis buffer was added to the washed pellets, and the protein was eluted by boiling for 2 min. 5-μl aliquots (equivalent to 0.125 oocyte) were electrophoresed as described above.

Microscopy

Oocytes were fixed in half-strength Karnovsky's fixative (29), postfixed in 1 % OsO_4 in 0.1 M sodium cacodylate buffer, dehydrated through a graded series of ethanol, and embedded in Araldite. Unstained 2 μm sections were examined by Nomarski optics under a Zeiss Universal microscope. Gold sections were stained in 1% uranyl acetate in ethanol and a mixture of lead salts (30) and examined in a Phillips 301 electron microscope.

RESULTS

Effects of Colchicine and Cytochalasin on Oocyte Morphology

The external appearance of treated oocytes is shown in Fig. 1 *a–d*. Both drugs individually have an effect on the pigment pattern of the animal pole. Cytochalasin (Fig. 1 *c*) has the more marked effect, causing mottling of the pigment. When both drugs are used, mottling is accompanied by the appearance of a pigment-free area in the animal pole, which appears at whichever area of the oocyte surface is uppermost.

Fig. 1 *e–h* shows the effects seen at light microscope level. Each micrograph shows a vertical section through the middle of the animal pole. The animal pole of the frog oocyte (Fig. 1 *e*) consists of a highly organized array of organelles. Beneath the surface pigment layer, the cytoplasm is divided into yolk platelet-rich areas, divided from each other by regions of yolk-free cytoplasm, which appear to radiate outward from the nucleus. The two drugs exert mutually distinct effects on this organization: Cytochalasin causes the pigment layer to bunch into pigment-rich zones, leaving some patches relatively pigment-free. It also alters the pattern of cytoplasm beneath the pigment layer. Colchicine treatment results in the sinking of pigment layer into the cytoplasm and the formation of large "lagoons" of yolk-free cytoplasm; in some experiments the nucleus is displaced towards the oocyte surface while retaining its spherical shape. Together the drugs acted synergistically: The nucleus loses its shape and rises to the surface. The pigment and subpigment layers of cytoplasm are completely disrupted. These characteristic morphological effects are seen when considerably (see Fig. 1 *b* and *c*) lower concentrations of cytochalasin and colchine are used and also when vinblastine is substituted for colchicine.

Further effects are seen at EM level (Fig. 2). The untreated oocytes (*a*) show an even distribution of microvilli, a micro-

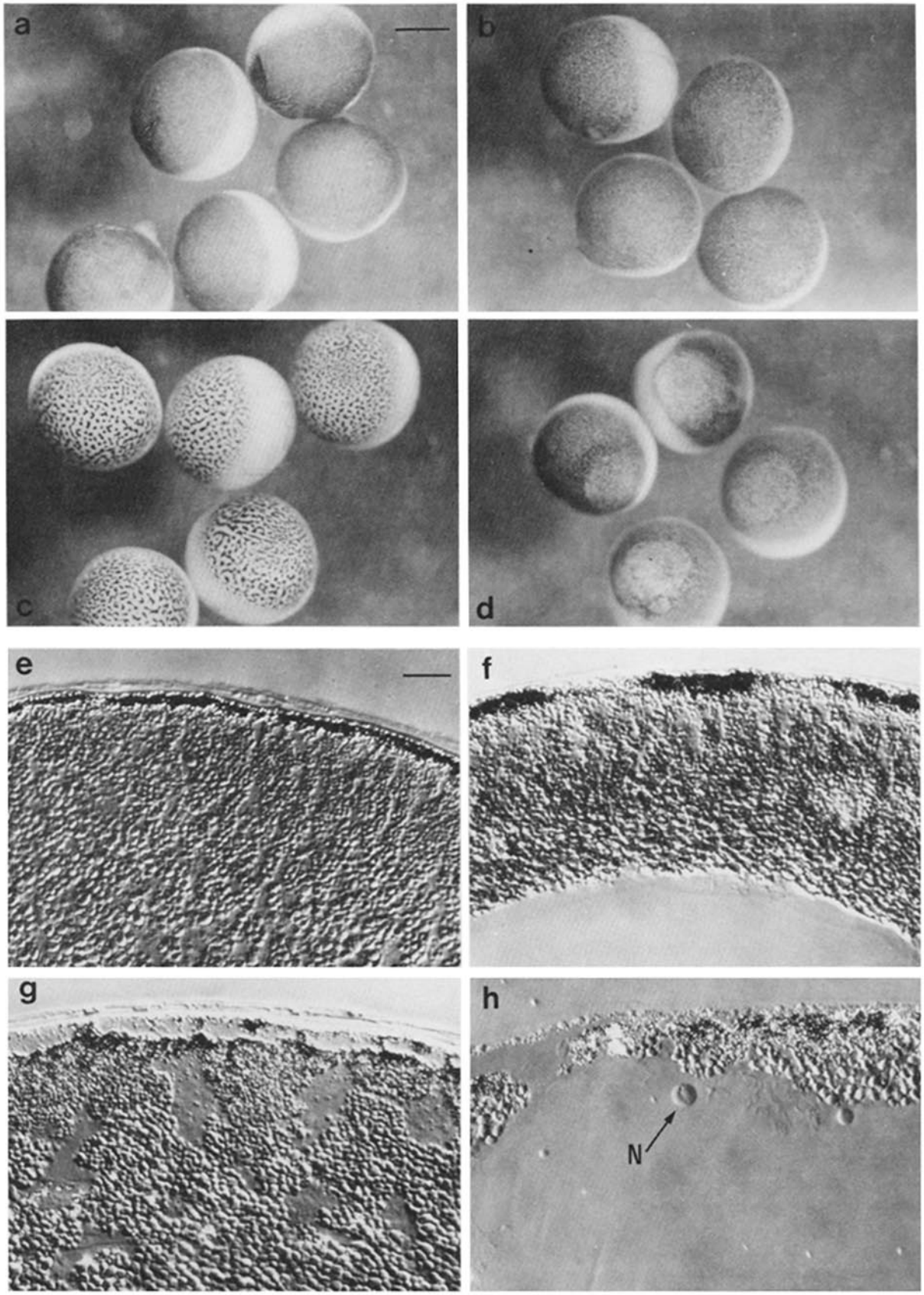

FIGURE 1 *a–d* are surface views of whole oocytes treated with (*a*) saline only, (*b*) 2 mM colchicine only, (*c*) 25 μg/ml cytochalasin only, and (*d*) both drugs. Bar, 0.5 mm. *e–h* are micrographs of unstained 2 μm sections through the middle of the animal pole, and photographed using Nomarski optics. (*e*) control, (*f*) 25 μg/ml cytochalasin only, (*g*) 2 mM colchicine only, (*h*) both drugs. Notice that each drug has a different effect on the organelle distribution, and that both drugs together act in a synergistic manner. *N*, nucleolus. Bar, 20 μm.

TABLE I

Protein Secretion in the Presence of Increasing Concentrations of Cytochalasin and Colchicine

(a) Assay by band excision

Inhibitor	Concentration	Relative secretion, %			
		Casein A	Casein B	Ovalbumin	Lysozyme
Colchicine	2 mM	53 ± 8	63 ± 6	70 ± 10	77 ± 4
	5 mM	63 ± 11	57 ± 4	66 ± 7	73 ± 10
	10 mM	58 ± 4	49 ± 7	63 ± 2	66 ± 12
Cytochalasin	25 μg/ml	87 ± 13	95 ± 12	110 ± 11	100 ± 3
	50 μg/ml	106 ± 8	98 ± 7	99 ± 4	100 ± 10
	125 μg/ml	92 ± 7	89 ± 5	86 ± 18	94 ± 9
Colchicine + cytochalasin	2 mM, 25 μg/ml	4 ± 0.5	3 ± 0.5	28 ± 5	43 ± 6
No inhibitor		100	100	100	100

(b) Assay by quantitative immunoprecipitation

Inhibitor	Concentration	Relative secretion of ovalbumin
		%
Exp. 1		
Colchicine	2 mM	40
Cytochalasin B	25 μg/ml	112
Colchicine + cytochalasin B	2 mM, 25 μg/ml	23
Colchicine + cytochalasin B	0.1 mM, 1.25 μg/ml	16
Vinblastine	1 μM	48
Vinblastine + cytochalasin B	1 μM, 25 μg/ml	25
No inhibitor		100
Exp. 2		
Colchicine	2 mM	56
Colchicine	10 μM	98
Colchicine + cytochalasin B	10 μM, 2.5 μg/ml	24
No inhibitor		100

(*a*) Oocytes from one animal were injected either with mammary gland or oviduct mRNA and cultured as described in Fig. 3. The amount of secretion from oocytes, of caseins A and B, ovalbumin, and lysozyme at 21°C in the presence of increasing doses of colchicine and cytochalasin, was estimated by the methods described in Fig. 3. The secretion of a given protein has been normalized with respect to its secretion in the absence of inhibitors. Each estimate represents the mean of the relevant gel bands excised from three independently run gels.

(*b*) Oocytes were injected with ovalbumin mRNA and cultured in the presence of the drugs above as described in Fig. 3. Oocyte homogenates or surrounding media were quantitatively immunoprecipitated with Miles anti-ovalbumin antibody (this antibody was completely specific for ovalbumin; cf. Fig. 5, legend). Immunoprecipitates were dissolved in electrophoresis sample buffer and precipitated with cold trichloroacetic acid, and the filtered precipitates were quantitated by liquid scintillation. Values obtained from control (no injection) oocyte homogenates and media were then subtracted and the amount of secretion is expressed in relation to that occurring from non-drug-treated oocytes. Exps. *1* and *2* utilized different frogs and were conducted at 19° and 21°C, respectively.

filamentous cortex, and beneath this an evenly distributed layer of cortical granules. Cytochalasin causes bunching of the cortex into electron-dense areas that project from the surface and from which sparse and stunted microvilli arise. The cortical granules are dispersed and no microfilaments are present. In contrast, colchicine (*c*) has had little effect on either the microvillus surface or the cortical granule layer. Moreover, the microfilamentous cortex is unaffected. Together the two drugs show synergistic effects. The cortex is drawn into extremely electron-dense areas, which alternate with clear areas. There is no distinguishable microfilamentous cortex outside the electron-dense areas. All cortical arrangements are disrupted. Particularly interesting is the fact that organelle types seem to aggregate, with large areas of granular endoplasmic reticulum and ribosomes alternating with large areas containing only mitochondria.

We conclude that cytochalasin and colchicine have both independent and synergistic effects on oocyte morphology. It is tempting to view the above-mentioned morphological disruption as a consequence of the disassembly of the microtubular-microfilamentous cortex. Certainly in the presence of cytochalasin, as expected, no microfilaments were seen. However, too few microtubules were seen even in control sections for their fate in the presence of colchicine to be clear; this is probably a consequence of the fixation procedures utilized for electron microscopy. Unfortunately we were unable to produce oocyte sections suitable for immunocytochemical observations of microtubules because of the yolky nature of oocyte cytoplasm.

Effect of Colchicine and Cytochalasin on Protein Secretion by Oocytes

Oocytes were microinjected with mRNAs from guinea pig mammary gland or chick oviduct and cultured in the presence of colchicine, cytochalasin, or both drugs for 24 h. Healthy oocytes were then cultured for a further 24 h in similar fresh media that had been supplemented with [^{35}S]methionine. Oocytes and incubation media were then processed for electrophoresis. The guinea pig caseins A and B and chick proteins

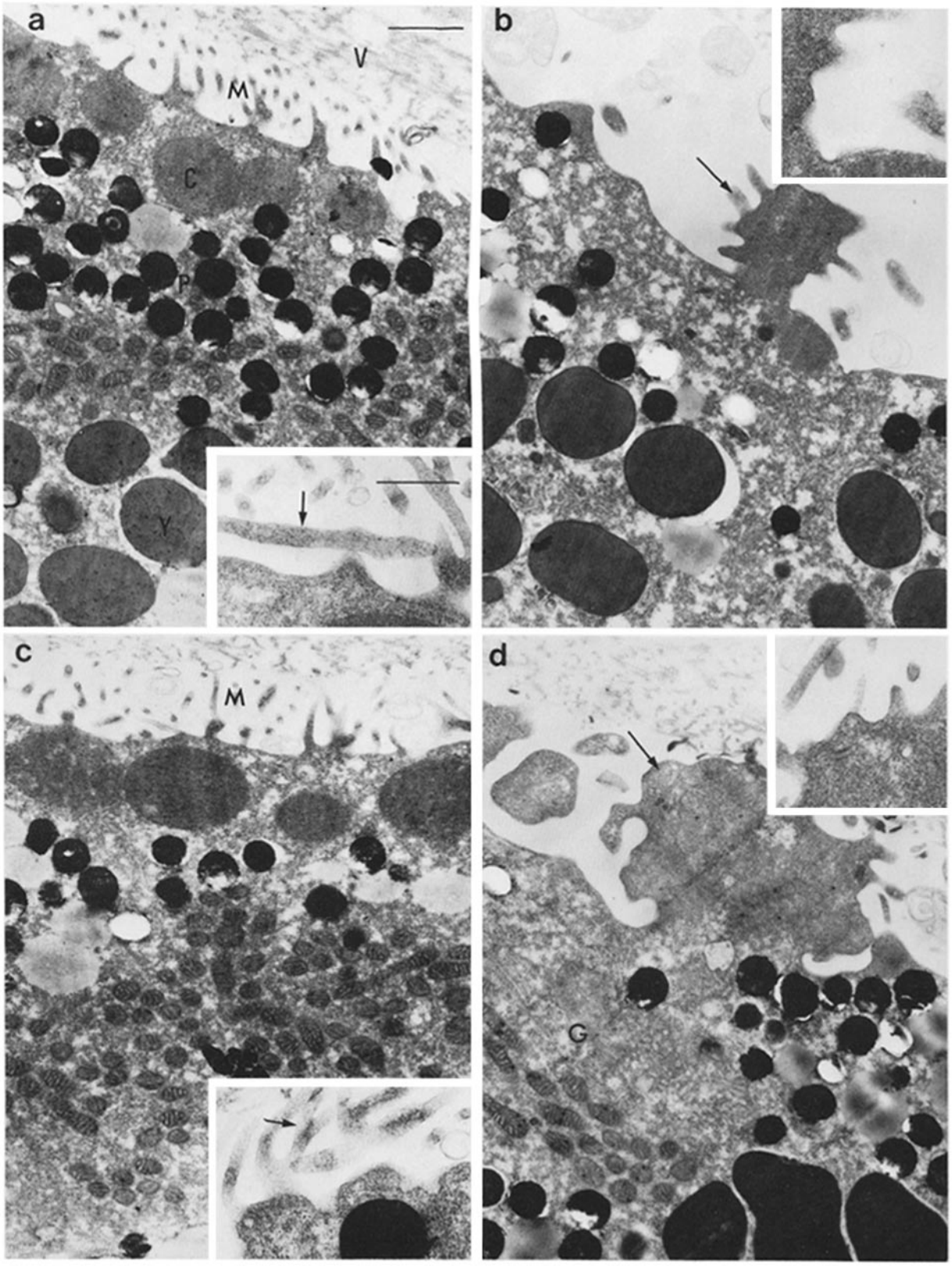

FIGURE 2 Low power electron micrographs through animal pole surfaces of oocytes treated with (*a*) saline, (*b*) 25 μg/ml cytochalasin (*c*) 2 mM colchicine, and (*d*) both drugs. *C*, cortical granules; *P*, pigment granules, *V*, vitelline membrane; *M*, microvilli (mostly out of the plane of the section); *G*, Golgi apparatus. Notice the dense accumulations of cortical material (arrows) and the virtual absence of microfilamentous microvilli in oocytes treated with cytochalasin only and both drugs. *Insets* show higher power views of the surface in each case; arrows in insets indicate the microfilamentous nature of the microvilli. Bar, 1 μm (0.5 μm for *insets*).

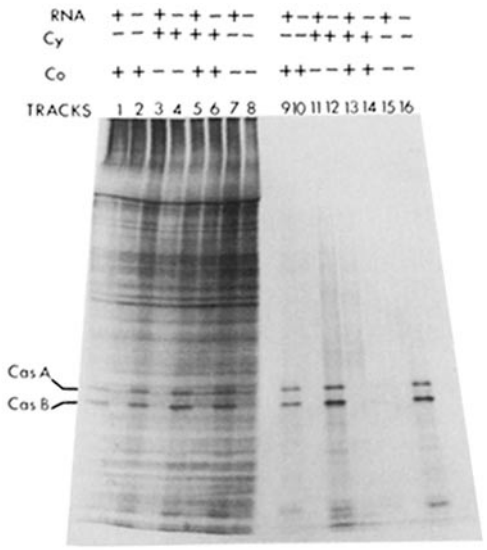

FIGURE 3 Effect of colchicine and cytochalasin on milk protein secretion. Oocytes were prepared, micro-injected with mammary gland mRNA, and cultured as described in Materials and Methods except that culture media were supplemented with either 2 mM colchicine or 25 μg/ml cytochalasin B or 2 mM colchicine and 25 μg/ml cytochalasin. After labeling for 24 h at 21°C with [^{35}S]methionine at 1 mCi/ml (500 Ci/mmol) incubation media and oocytes were prepared for electrophoresis. Samples were electrophoresed on 10–22.5% exponential gradient gels. The radioactivity in selected bands was quantitated by excising the bands and counting in an NCS (Amersham-Searle)/toluene-based scintillant; corrections were made for the radioactivity migrating in the corresponding regions of control tracks. Tracks *1–8* contain oocyte homogenates; tracks *9–16* contain incubation media. *Cy*, cytochalasin B; *Co*, colchicine; *Cas A* and *B* refer to the positions of immunoprecipitable caseins A and B.

ovalbumin and lysozyme had previously been shown to be secreted by oocytes after microinjection of their respective mRNAs (31). The results shown in Fig. 3 and Table I *a* reveal that the synthesis of both oocyte and heterologous proteins was unaffected by any of the drug treatments. However, significant differences are evident in the degree to which the secretory proteins appeared in the incubation media (cf. tracks *13* and *15*, Fig. 3; Table I). Quantitation of the secreted proteins (Table I *a*) shows that cytochalasin alone had no significant effect on secretion. In contrast colchicine alone reduced secretion of all the proteins by 30–50% and the combined use of cytochalasin and colchicine reduced the secretion of the milk proteins by >95%, chick ovalbumin by 70%, and lysozyme by 60%. These experiments have been repeated eight times with essentially the same results. Raising the concentration of either drug separately had no additional inhibitory effect (Table I *a*), and we conclude that the two drugs are acting synergistically, the synergism being more pronounced with the milk proteins. Table I *b* and *c* shows the results of experiments where the effect of cytoskeletal inhibitors on ovalbumin secretion has been monitored by quantitative immunoprecipitation. Significantly lower quantities of colchicine and cytochalasin effect a similar synergistic inhibition of secretion. Moreover colchicine can be substituted by low concentrations (1 μM) of vinblastine with the same resultant effect. We also used the drugs griseofulvin (100 μM) and nocadazole (10 μM) but found them to be highly toxic to oocytes. Griseofulvin treatment resulted in the curious extrusion from the oocyte of discrete "blebs" of cytoplasm.

Experiments involving the uptake by oocytes of radioactive colchicine or cytochalasin have shown that this synergism did not result from an enhanced permeability of the oocyte to either drug (data not shown). In fact it was clear that whereas colchicine entered the oocyte and was rapidly equilibrated with the external colchicine, cytochalasin was sequestered and concentrated by the oocyte. However, the uptake of neither drug was significantly increased by the presence of the other, and the observed cooperative effects of the drugs on protein secretion (and morphology) evidently require a different explanation (see Discussion).

Quantitation of the effects of colchicine and cytochalasin on the secretion of the guinea pig and chick proteins was based on comparison of radioactivity incorporated into the various polypeptide bands. Our conclusions would be questionable if drug treatment resulted in an increase in the specific activity of the radioactive methionine pool within the oocyte. While this was unlikely, since total [^{35}S]methionine incorporation into oocyte proteins was unchanged, we have circumvented this problem, at least for one protein, by analyzing the secretion of human lymphoblastoid interferon from oocytes injected with mRNA from induced lymphoblastoid cells; in these experiments interferon is quantitated by bioassay. The results displayed in Table II show that cytochalasin and colchicine together greatly inhibit interferon secretion both from oocytes and lymphoblastoid cells, and thus reinforce the result obtained using [^{35}S]methionine labeling. Because of inaccuracy of interferon bioassays (±50%) (32), we cannot deduce whether any inhibition resulted from the exposure of oocytes to either drug alone. In view of the known effects of colchicine on microtubule integrity (1–4), we conclude tentatively that efficient secretion from oocytes might require the mediation of intact microtubules.

Effect of Temperature on Secretion

Microtubule integrity is also very sensitive to temperature (33, 1), with net microtubule disaggregation occurring as the temperature falls. We have therefore tested the effect of temperature on secretion from oocytes. Lowering the temperature of incubation has a dramatic effect on the secretion of both chick and milk proteins from oocytes. Fig. 4 shows the results of several experiments where secretion of the caseins and chick proteins was monitored at different temperatures and in the presence of colchicine (Fig. 4 *A* and *C*) and cytochalasin (Fig. 4 *b*). It is clear that whilst protein synthesis was reduced at lower temperatures, the effect of temperature on secretion was far more dramatic. Lowering the temperature for 21° to 15°C caused a 55% reduction in protein synthesis but an 87% reduction in secretion of chick proteins. When colchicine was included in the incubation medium, protein secretion, though not synthesis, was further reduced in a temperature-dependent manner. In fact, compared with controls, ovalbumin secretion was reduced by colchicine by ~40% at 21°C but by 95% at 15°C. The same trend was observed for both chick lysozyme and guinea pig caseins A and B and was even more marked at lower temperatures. Significantly, when cytochalasin and colchicine were used together, casein secretion, though again not synthesis of protein, was severely reduced at every temperature used.

Subcellular Location and Fate of Nonsecreted Proteins

When oocytes are subjected to gentle subcellular fractionation, a vesicle-containing preparation can be isolated (34). These vesicles are thought to include disrupted elements from the oocyte's endoplasmic reticulum and have been shown to

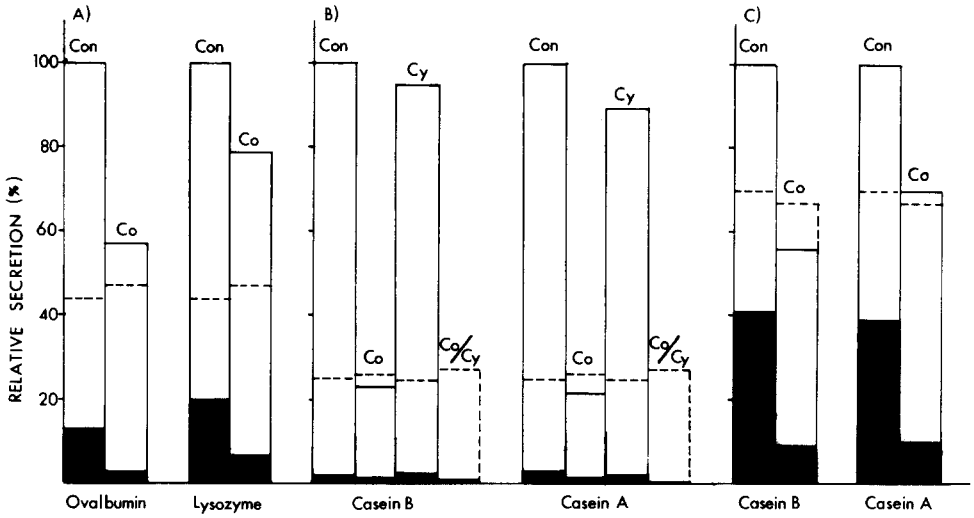

FIGURE 4 Effect of temperature, colchicine, and cytochalasin on secretion. Oocytes were injected with either mammary gland or oviduct mRNA and cultured at two different temperatures (see below) in the presence or absence of cytochalasin and/or colchicine as described in Materials and Methods. After 24 h in media containing 1 mCi/ml [^{35}S]methionine (50 Ci/mmol), oocytes and incubation media were processed for electrophoresis and electrophoresed on 12.5% polyacrylamide gels (see Figs. 3 and 5). The radioactivity in selected bands corresponding to secreted proteins was quantitated as described in Fig. 3. Incorporation into total oocyte protein was calculated by precipitation of aliquots with cold trichloroacetic acid on to Whatman No. 1 paper followed by scintillation counting. The relative secretion of each protein has been normalized with respect to the secretion of that protein at the higher temperature (unshaded area) and in the absence of inhibitors; secretion at the lower temperature is shown by the shaded areas. The relative incorporation of radioactivity into oocyte protein at the lower temperatures is normalized with respect to the incorporation at the higher temperature and is shown by the broken line. In no case was incorporation at any temperature effected by more than ±5% by the presence of the inhibitor. Incubation temperatures were: (*A*) 21° and 15°C; (*B*) 18° and 10°C; (*C*) 21° and 18°C. *Con*, control, no inhibitors; *Co*, colchicine (2 mM); *Cy*, cytochalasin (25 μg/ml).

contain most of the secretory proteins synthesized in oocytes after the injection of milk mRNAs. We have recently shown that a secretory protein miscompartmentalized in the oocyte cytosol was not secreted (31). It seemed possible therefore that the failure of drug-treated oocytes to secrete guinea-pig chick protein resulted from the miscompartmentation of these proteins as a consequence of their leakage into the cytosol from the lumen of the endoplasmic reticulum. However the experiment shown in Fig. 5 rules out this explanation. In this experiment mRNA-injected oocytes were incubated in cytochalasin and colchicine and then fractionated. The chick proteins were still found predominantly in the vesicle fraction thus indicating that drug treatment does not facilitate leakage from the endoplasmic reticulum.

Since during drug treatment protein secretion is considerably reduced, it is pertinent to ask whether the unsecreted protein accumulates inside the oocyte. Where interferon secretion was studied (Table II), it was clear that oocytes (or cells) treated with colchicine and cytochalasin did not accumulate interferon. A similar observation was made with lysozyme: since this protein is rapidly exported from oocytes, we might expect that drug-induced inhibition of this secretion would result in a marked increase in the intracellular pool of this protein. However, no significant accumulation of lysozyme was observed (cf. Fig. 5 *a* and *b*). We therefore conclude that some intracellular degradation of lysozyme and interferon occurs when secretion is inhibited. However, the type of analysis applied to lysozyme could not usefully be extended to the milk proteins or ovalbumin since in most experiments the secreted proteins represent only 10–20% of the respective amounts within the oocyte; consequently an anticipated increase of each protein within the drug-treated oocyte by this amount was difficult to verify because of problems in accurate quantitation. Accordingly, a different approach to this question was adopted: oocytes were allowed to accumulate radioactive guinea pig and chick proteins in the presence or absence of colchicine and cytochalasin. The oocytes were then incubated in unlabeled 10 mM methionine for 2–3 d. At the end of what constituted a "methionine chase" experiment, oocytes and incubation media were processed for electrophoresis. The results shown in Table III indicate that in the presence of drugs very little of the milk protein and 36% of the chick ovalbumin lost from the oocyte during the chase were subsequently recovered in the incubation media. These results contrast with those of the controls where >70% of caseins A and B and α-lactalbumin, 85% of the ovalbumin, and 20% of the casein C lost from the oocyte during the chase, were recovered. The low recovery of casein C is probably due to its proteolytic cleavage during or just after secretion since extra milk-specific lower molecular weight peptides, not present in the oocyte, accumulate in the medium (data not shown).

When the corresponding amounts of each protein within the postchase oocytes were examined, it was found that all detectable casein had disappeared from oocytes incubated in the presence or absence of drugs. In contrast, a considerable proportion of the prechase ovalbumin (50%) and α-lactalbumin (50%) remained in oocytes chased in the presence or absence of drugs. Significantly, although the intracellular pools of milk protein and ovalbumin decreased during the chase, the endogenous oocyte proteins remained both quantitatively and qualitatively unchanged (Fig. 5, data not shown). We conclude that in the presence of the drugs, nonsecreted protein is not accumulated within the oocyte but degraded.

If in the above experiments <10% casein had remained within the oocyte after 48 h, the method of quantitation used would not have detected this against the high background of endogenous protein. In two further experiments in which intracellular casein was assayed electrophoretically after immu-

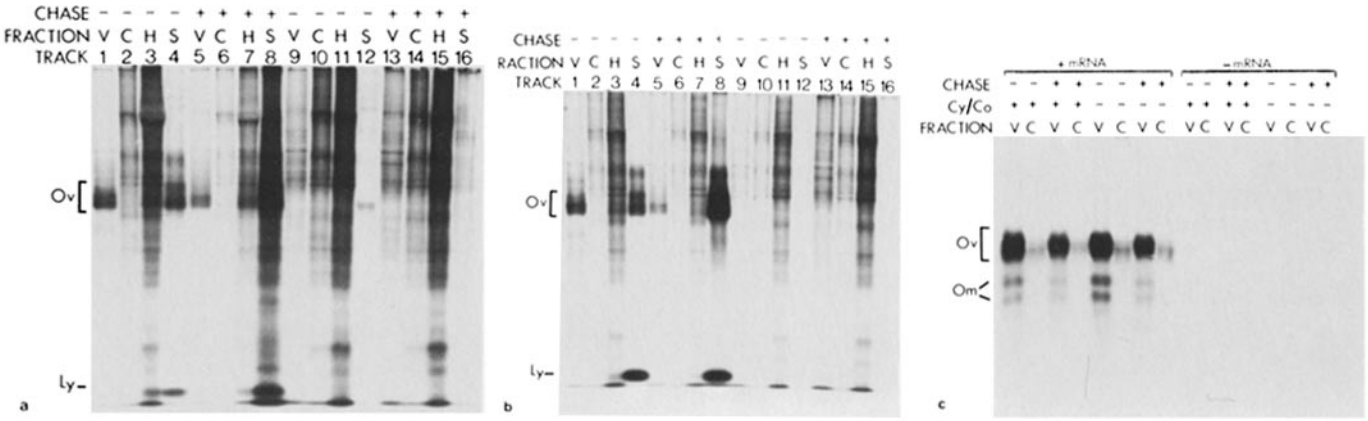

FIGURE 5 Subcellular compartmentation of secretory proteins. Oocytes were injected with oviduct mRNA and cultured in the presence (panel *a*) or absence (panel *b*) of colchicine (2 mM) and cytochalasin (25 μg/ml). After 24 h oocytes were transferred to media containing 1 mCi/ml [^{35}S]methionine (300 Ci/mmol). Incubation was continued for a further 24 h before the incubation media were collected and groups of 8–15 oocytes were removed for immediate subcellular fractionation. The remaining oocytes in groups of 8–15 were transferred to 400 μl of unlabeled media with or without cytochalasin and colchicine, and containing 10 mM methionine. Culture was continued for a further 72 h (chase). The incubation media were then retained and the oocytes removed for immediate subcellular fractionation. Homogenates (*H*), vesicle (*V*), or cytosol (*C*) fractions were either immunoprecipitated (panel *c*) or prepared directly (panels *a* and *b*) for electrophoresis on 12.5% gels. Aliquots of the 0–24 h incubation media (*S*) were diluted with an equal volume of electrophoresis sample buffer and 20-μl samples were electrophoresed. The 24–96 h incubation media (*S*) were precipitated with cold 10% trichloroacetic acid after the addition of 10 μg of bovine serum albumin. Precipitates were washed with acetone, air-dried, and resuspended in sample buffer at 12 μl per oocyte; 20-μl aliquots were electrophoresed. The fixed gels were fluorographed for 3 d (panels *a* and *c*) or 1 d (panel *b*). Tracks *1–8*, mRNA; tracks *9–16*, controls. *Cy*, cytochalasin; *Co*, colchicine; *V*, vesicles; *C*, cytosol; *H*, homogenate; *S*, incubation media; *Ov* and *Ly* refer to the positions of immunoprecipitable ovalbumin and marker lysozyme respectively. The antibody used contained some anti-ovomucoid activity hence the coprecipitation of the two extra polypeptides that correspond to two species of glycosylated ovomucoid (*Om*). When pure anti-ovomucoid antibody was used only these polypeptides precipitated (31).

TABLE II

Effect of Colchicine and Cytochalasin on Interferon Secretion

Cell type	Inhibitor	Interferon (U/oocyte or U/10^6 cells) in: Incubation media	Oocytes or cells
Oocyte	None	251	68
	Colchicine (2 mM)	158	68
	Cytochalasin (25 μg/ml)	200	68
	Colchicine + cytochalasin	32	68
Namalwa cells	None	62,500	62.5
	Colchicine	10,000	80
	Cytochalasin	31,500	80
	Colchicine + cytochalasin	150	50

Oocytes were injected with interferon mRNA and cultured at 21°C in the presence or absence of inhibitors as shown above. Incubation media were collected between 24–48 h of culture, whereas oocytes were collected after 48 h. Homogenized oocytes and media after exhaustive dialysis were then assayed for interferon activity as described by Colman and Morser (17). Namalwa cells were induced as described by Morser et al. (18) and then incubated at 2 × 10^6 cells/ml in the presence of the above inhibitors from 3.5 to 10 h after induction. Media and cells were then assayed for interferon activity as above.

noprecipitation, we found that some casein was left in both drug-treated and control oocytes after a 48-h chase (Fig. 6). Moreover, similar amounts of each casein remain in both drug-treated and control oocytes.

The possibility that the residual secretory protein seen within oocytes after a "chase" period had become miscompartmentalized and was therefore unavailable for secretion was excluded by the data presented in Fig. 5. This clearly shows that irrespective of drug treatment ovalbumin was predominantly localized in the vesicle fractions both before and after a 72-h "chase." The small proportion (~10%) of ovalbumin seen in the cytosol fraction (Fig. 5 *c*) probably results from some vesicle breakage during preparation.

From these results we conclude that nonsecreted caseins A, B, and C, α-lactalbumin, ovalbumin, lysozyme, and interferon are not stored but are degraded within drug-treated oocytes. Furthermore, it is clear that for those proteins where the relevant data were available (caseins, α-lactalbumin, and ovalbumin), this rate of degradation is similar to the rate of exocytosis seen in the absence of drugs.

Trapped Immunoglobulin Chains Are Also Degraded

It is possible that the above relationship between degradation and secretion is a pathological effect resulting from drug treatment. However similar data have been obtained where secretory proteins were trapped inside oocytes without recourse to the use of drugs: the mouse myeloma cell line P3 × 63-Ag 8 secretes the MOPC 21 immunoglobulin (IgG) which consists of two heavy (γ1) and two light (κ) chains; free heavy or light chains are never secreted (35). When mRNA purified from these cells was injected into oocytes both heavy and light immunoglobulin chains were made (Fig. 7). However, when allowance was made for the numbers of methionine residues present in each chain (four in the light chain, nine in the heavy [36, 37]), it was clear that excess light chains were synthesized; in fact the intracellular ratio of light to heavy chain was 7:1. In contrast the secreted immunoglobulin consisted of equimolar amounts of light and heavy chain and was in the tetrameric form (38). Thus since the light chains can only be secreted in conjunction with equal numbers of heavy chains, the oocyte

TABLE III

Fate of Nonsecreted Milk and Chicken Proteins

Chase time	Inhibitors	Counts of [^{35}S]methionine incorporated into				
		Casein A	Casein B	Casein C	α-Lactalbumin	Ovalbumin*
Incubation media						
Total accumulated from 0–48 h	A −	2,160	2,410	671	4,010	46,873
	A +	101	56	ND	211	10,400
Oocyte						
Total lost from 0–48 h	A −	2,942	3,412	3,097	5,485	55,145
	A +	3,268	3,246	2,982	5,042	28,890
% Remaining in oocytes	B −	0	0	0	54	47
	B +	0	0	0	54	54
% Recovery‡ ($A/B \times 100$)	−	73	71	22	73	85
	+	3.1	1.7	[0]	4.2	36

Oocytes were injected with mammary gland mRNA or oviduct mRNA and labeled for 24 h in 1 mCi/ml [^{35}S]methionine in the presence (+) or absence (−) of both cytochalasin (25 μg/ml) and colchicine (2 mM). Batches of 25 oocytes were then removed from the radioactive media and cultured for the periods indicated above in unlabeled medium containing 10 mM methionine; these are effective chase conditions (27; Colman, unpublished observations). At the end of each period, incubation medium was removed for electrophoresis. After 48 h, oocytes were also processed for electrophoresis. Electrophoresis and the subsequent quantitation of selected proteins were performed as described in Fig. 3. Excised bands were counted for 5 min. The figures have been corrected for the counts obtained from bands excised from analogous regions of control (−mRNA) gel tracks. *ND*, none detected.

* 0- to 72-h chase was used in this experiment. Incubation media were collected after 72 h of continuous exposure to oocytes.

‡ Refers to the total counts found in incubation media as a percentage of the total counts lost from the oocyte.

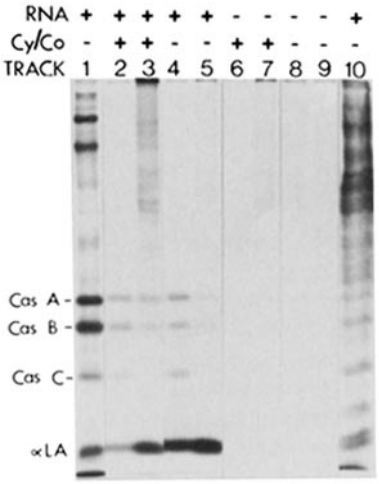

FIGURE 6 Immunoprecipitation of milk proteins in "chased" oocytes. Oocytes were injected with mammary gland mRNA and cultured in the presence or absence of colchicine and cytochalasin as described in Fig. 5. However oocytes were not fractionated but were homogenized before (tracks *2*, *4*, *6*, and *8*) or after (tracks *3*, *5*, *7*, *9*, and *10*) a 48-h chase and the intracellular milk protein immunoprecipitated using mixed anticasein and anti-α-lactalbumin antibody as described in Materials and Methods. Immunoprecipitates were dissolved in sample buffer and electrophoresed on a 12.5% polyacrylamide gel. The fixed gel was impregnated with PPO and fluorographed for 2 d (tracks *1* and *10*) and 21 d (remaining tracks). Tracks *2*, *4*, *6*, and *8* each received 5 μl aliquots whereas tracks *3*, *5*, *7*, and *9* received 25-μl aliquots. Tracks *1* and *10* received the unprecipitated incubation medium and homogenate from 2 and 0.125 oocytes respectively. *Cas A*, *B*, and *C*: caseins A, B, and C; *α-LA*: α-lactalbumin.

contains a pool of trapped light chains. If these trapped light chains are degraded at the same rate as assembled light chains are secreted, we would expect that during a "chase" the ratio of heavy to light chain inside the oocyte would remain constant. Quantitation of the results shown in Fig. 7 confirmed this expectation; the chain ratio of both intracellular and secreted immunoglobulin remained constant. We conclude that the similarity in the rates of secretion and degradation of a given protein in oocytes is a general phenomenon that is not of necessity related to the use of the cytoskeletal inhibitors.

DISCUSSION

Inhibition of Secretion by Cytochalasin and Colchicine

The pharmacological drugs colchicine, which causes disaggregation of the microtubules (39), and the cytochalasins, which impair the function of microfilaments (40, 41), have been widely used to study the role of microtubules and microfilaments in protein secretion (3, 11). Depending on the cell type and protein studied both these drugs have been shown to inhibit secretion, although cases where inhibition does not occur have been cited (11). The reasons for this lack of consensus are unclear although cell-type specificities in the organization of the secretory apparatus or secondary effects of these drugs (42–44) cannot be excluded. Malaisse and his co-workers (44), using drugs affecting the cytoskeleton, have postulated the involvement of a microtubular-microfilamentous network in protein secretion. Surprisingly, the simultaneous effect of cytochalasin and colchicine on secretion is not routinely studied. In this report we have examined the effects on protein secretion of both drugs used separately or together. The unexpected synergism revealed that the action of the two drugs on the inhibition of protein secretion suggests that there is an interaction between microtubules and microfilaments.

Secretion from oocytes of guinea-pig, chick, and human proteins at 21°C was unaffected by cytochalasin concentrations from 25–125 μg/ml. In contrast, colchicine alone at 2 mM decreased the secretion, at 21°C, of the guinea-pig and chick proteins by 20–50% depending on the protein (the inaccuracy of the interferon bioassay precludes a similar conclusion for this protein); however, this inhibition was not enhanced by raising the colchicine concentration fivefold. When cytochalasin and colchicine were used together at 21°C, this inhibition of secretion was almost total for caseins and significantly increased for the chick proteins (Figs. 3 and 5, and Table I). This synergistic inhibition was also observed when colchicine

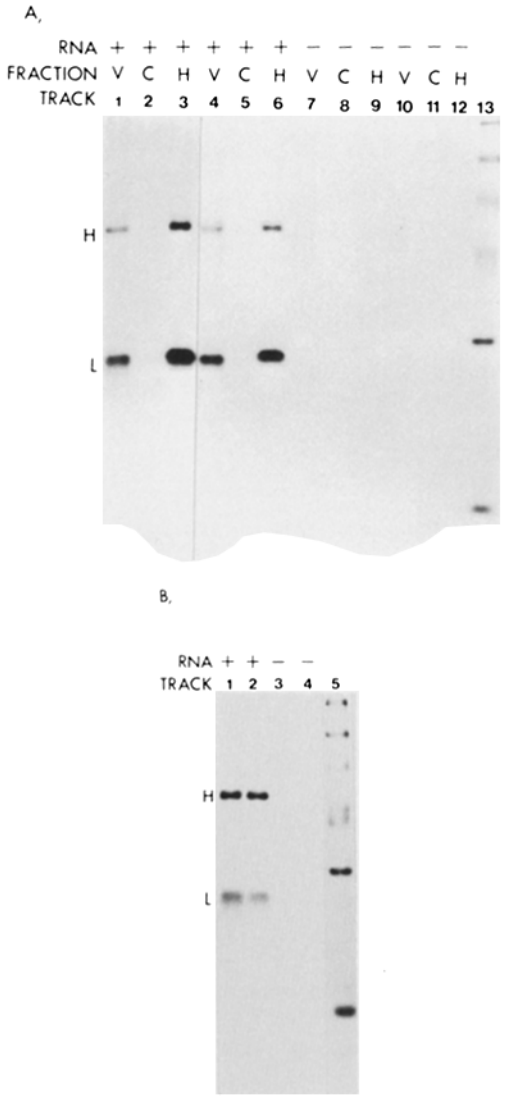

FIGURE 7 Fate of trapped immunoglobulin chains. Oocytes were injected with mouse immunoglobulin mRNA and cultured as described in Fig. 6 except that no drugs were present and oocytes were fractionated. Immunoprecipitates of fractions (panel *A*) and incubation media (panel *B*) were electrophoresed on 12.5% gels. Gels were fixed, and then fluorographed for 3 d. Prechase samples are in tracks *1–3*, *7–9* in panel *A* and tracks *1* and *3* in panel *B*; the remaining tracks contain postchase samples except tracks *13* (panel *A*) and *5* (panel *B*), which contain ^{14}C labeled marker proteins (Radiochemical Centre, U. K.). *V*, vesicle fraction; *C*, cytosol fractions; *H*, homogenates; *H*, heavy chain; *L*, light chain.

and cytochalasin were used together at concentrations of 10 μM and 2.5 μg/ml, respectively, and even when vinblastine (1 μM) was substituted for colchicine; we have recently extended these observations to the two major, low molecular weight, rat seminal vesicle proteins (Colman and Besley, unpublished observations). This synergism could not be explained by alterations in oocyte permeability to the drugs. The almost complete inhibition of secretion seen with both drugs at 21°C was also effected by colchicine alone when the incubation temperature was lowered (Fig. 4). These observations can be reconciled with the known dynamic properties of microtubules: microtubules in vivo are thought to coexist in equilibrium with a pool of tubulin subunits (33, 45, 46). Colchicine binds only to the tubulin in its subunit form and thereby shifts the equilibrium toward microtubule disassembly. Since low temperature also promotes such disassembly, colchicine binding to tubulin should be facilitated by lowering the incubation temperature, while this increased binding might be offset to some degree by the slower rate of binding of colchicine to tubulin at lower temperatures (1). We should emphasize that in all experiments oocytes were preincubated with the drugs for at least 24 h. If, as we suggest, microtubules are involved in secretion of the chick and guinea-pig proteins then these properties could explain the observed temperature-sensitivity of oocyte secretion to colchicine. Furthermore since this colchicine-mediated inhibition was also magnified by the presence of cytochalasin at all temperatures, we further speculate that microfilaments, in some way, stabilize the microtubules. Certainly the disruptive morphological effects of the drugs on the various oocyte organelles, especially the nucleus, reinforce this view: colchicine and cytochalasin together cause a rapid, random relocation of the nucleus upward to the oocyte surface; colchicine alone sometimes caused a similar effect but always more slowly. All cell nuclei so far examined have been shown to be enmeshed in, and presumably anchored by, a cytoskeletal network of actin and tubulin (47, 48). Although to our knowledge there are no reports describing the existence of a similar network in oocytes, we speculate that it is the disruption of this mesh that allows the nucleus to float upward.

We have consistently observed differences between the drug sensitivities of chick and guinea-pig protein secretion (see Table I). Furthermore, the slow export of endogenous vesicularized oocyte proteins is virtually unaffected by cytochalasin and colchicine (Lane, unpublished observations). Until recently it was believed that within a given cell all secretory proteins were cotranslocated (49, 50). However, Smilowitz (51) has presented evidence indicating the existence within cultured myotubes of two independent secretory routes. A possible explanation for our findings may be the existence in oocytes of an alternative, drug-insensitive mode of translocation to which the various proteins have differential access.

Stability of Secretory Proteins

In the presence of cytochalasin and colchicine, protein secretion is considerably reduced, yet we show that the synthesis and compartmentation of secretory proteins is unaffected by the drug treatment. What happens to the unsecreted protein? If unsecreted protein is completely stable we would have expected to have seen a compensatory accumulation of protein inside the oocyte. For the reasons stated earlier the relevant quantitation was often unobtainable; however where such estimates were possible it was clear that increased intracellular accumulation of secretory proteins in no way compensated for the shortfall in the amount of that protein secreted. However, such observations do not in themselves prove that the unsecreted proteins were unstable since these experiments do not exclude a compensatory reduction in the production of sequestered polypeptides as a consequence of their nonsecretion; such a reduction could result from decreased translation (52) or increased degradation of the nascent secretory protein (see reference 27). These explanations are unlikely as a result of the "chase" experiments shown in Table III and Figs. 5–7. These experiments clearly show that unsecreted protein is specifically degraded within the oocyte, while total endogenous protein remains stable. In the experiments shown in Figs. 5 and 6, immunoprecipitation of oocyte homogenates has shown that milk protein and ovalbumin can be detected at the end of a 48-

h chase. Moreover, similar amounts of the caseins and ovalbumin were present in both drug-treated and untreated oocytes. Since only in untreated oocytes were the intracellular losses of casein A, B, α-lactalbumin, and ovalbumin accounted for by their appearance in the medium (Table III), it would seem that for each of these proteins, the rate of intracellular loss to degradative processes is similar to the rate of intracellular loss through exocytosis, even though the rate of exocytosis of the caseins is significantly higher than that of ovalbumin and α-lactalbumin. Moreover by exploiting our observation that excess immunoglobulin light chains were trapped in the oocyte (Fig. 7), we have been able to establish that this relationship between degradation and secretion prevails in the absence of drugs. Similar results have been reported for mouse immunoglobulins by Cowan et al. (22) and Mosmann et al. (53), who compared the fates of intracellular light chains synthesized in secretory and nonsecretory myeloma cell lines.

The above behaviour is consistent with a view that secretory proteins are normally protected from degradation but that at some point in the secretory pathway the proteins undergo either exocytosis or intracellular degradation; in the presence of a reduced (by temperature) or disrupted (by cytochalasin and colchine) cytoskeleton, the latter fate would predominate. Although it is premature to speculate on the subcellular mechanisms involved in the stabilization or degradation of the secretory proteins, it is significant that lysosomal enzymes are thought to reach this organelle via the cell surface (54, 55). It is therefore conceivable that a block or delay at the final secretory step, that of exocytosis, consigns a secretory protein to lysosomal degradation.

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