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Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity

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Contents

I. Introduction

Monensin, a Na^+ ionophore capable of collapsing Na^+ and H^+ gradients, has gained wide-spread acceptance as a biochemical and biological investigative tool to study Golgi apparatus function and to localize and identify the molecular pathways of subcellular vesicular traffic. Among its advantages are the low concentrations at which inhibitions are produced (0.01–1.0 μM), a minimum of troublesome side effects (e.g., little or no change of protein synthesis or ATP levels), and a reversible action [1]. The purpose of this review is to examine the mechanism of action and specificity of monensin in Na^+/H^+ exchange and to attempt to reconcile this to the large body of structural and biochemical information on monensin toxicity derived from animal studies

II. Background

In 1964, Pressman and co-workers [2] reported a class of antibiotics that induced alkali ion permeability in mitochondria and other membranous systems. These antibiotics functioned as ionophores (ion-carriers) to carry ions across lipid barriers as complexes soluble in the lipid phase of the membranes. The potential use of ionophores as probes of biological function, or as potential therapeutic agents, was recognized very early [2–5], but major economic importance was not forthcoming until the discovery of monensin in 1967 and the

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recognition of its potential in the poultry industry as a coccidiostat [6] Subsequently, it was discovered that ionophores also could improve feed conversion in ruminants such as cattle [7,8], thus adding further to their commercial value Of the more than 100 ionophores that have been reported [9], three, monensin, lasalocid and salinomysin, have widespread commercial use Of those licensed, monensin is probably used most widely.

Several monensins have been identified [6,10]. Monensin A, and specifically the sodium salt of monensin A (hereafter simply designated as monensin) is derived from *Streptomyces cinnamonensis* [11], and a crude mycelial preparation (Rumensin®) containing about 6.6% monensin is used commercially. The ion specificity of monensin is $Ag > Na \gg K > Rb > Cs > Li > Ca$ [6,12] with approximately a 10-fold selectivity for sodium over potassium [9] and little tendency to bind calcium [13]

III. Mode of action

One of the original interests in ionophores was their perceived potential for directly modifying intracellular ionic gradients, particularly Ca^{2+}, which would lead, hopefully, to the development of useful pharmacologic agents or, alternatively, provide a tool for studying cellular functions mediated by changes in Ca^{2+} [13,14]. Of particular interest were divalent ionophores such as X-537A (normally considered a Ca^{2+} ionophore – note, however, X-537A complexes Na^+ and K^+ almost as well as Ca^{2+}) which have been shown to induce contraction of aortic strips and increase the rate of contractility of isolated perfused rabbit heart [5,15], and release Ca^{2+} from energy-loaded vesicles derived from the sarcoplasmic reticulum of muscle [16–18] X-537A may also increase blood flow through coronary arteries and increase cardiac output [19]. The emphasis on heart physiology stemmed from this organ's strong dependence on calcium for proper functioning [20]. Indeed, subsequent studies of X-537A used as a feed additive for poultry and cattle has shown that the heart is a primary target for ionophore toxicity [21]. X-537A affects many other cellular functions such as release of biologically active agents and the induction of sperm acrosome reactions of several species [19,22].

It was soon realized, however, that many of the ionotropic effects of the divalent ionophores could be duplicated with even greater efficiency by monovalent ionophores such as monensin which complexes Na^+ but almost no Ca^{2+} This response apparently occurs because the movement of Na^+ into a cellular compartment by monensin facilitates the entry of Ca^{2+} by a Na^+-out/Ca^{2+}-in exchange [4,9,23,24]. Thus, a Ca^{2+} shift is still the primary factor mediating cellular responses although other factors may also play significant roles in monensin physiology For example, many ionophores, either directly or as a result of the ionic imbalance, may transport, promote uptake, or release effector substances such as serotonin, histamine, prostaglandin and catacholamine which, in turn, have profound effects on cellular function [4,19] Similarly, monensin, through alteration of the pH of intracellular compartments may inhibit the release and/or transport of numerous agents and, in so doing, perturbate cellular function In cardiac tissues, both positive and negative inotropism has been observed sequentially (the variable factor being time) in tissues following exposure to monensin [4] Concentration of monensin may, also, cause similar positive/negative inotropic responses [4,24]. Finally, some monensin ingested by an animal is metabolized to other ionophores, the properties of which are largely unknown

Thus, ionophores, in spite of many common characteristics, differ individually in their effects on cells Moreover, cells may respond to both direct ionophore interaction as well as secondary effects that develop from the initial ionophore reactions. The latter is particularly likely in the whole animal where metabolites with unknown properties are produced from ionophore breakdown and where changes in the products of one organ can affect the function of other organs.

Monensin is an open chain molecule that is capable of ion complexation through a cyclic form stabilized by hydrogen bonding between the carboxyl and hydroxyl groups Charge transfer bonding within the cavity formed is responsible for ion binding (Fig 1). Because the affinity of monensin for Na^+ is 10-times that for K^+, its nearest competitor in biological systems, monensin mediates primarily a Na^+/H^+ exchange

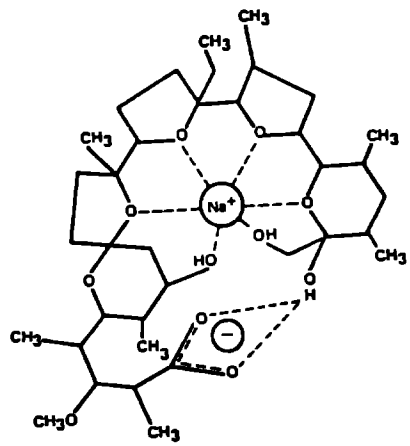

Fig 1 Monensin is a monovalent polyether antibiotic in which the oxygen functions are concentrated at the center of the structure where they are available for the complexation of a suitable cation (in this case sodium) The alkyl groups are spread over the outer surface rendering the complex lipid soluble thus allowing the antibiotic to enter and diffuse through biological membranes (From Calbiochem-Behring Corp Bulletin on Monensin [12])

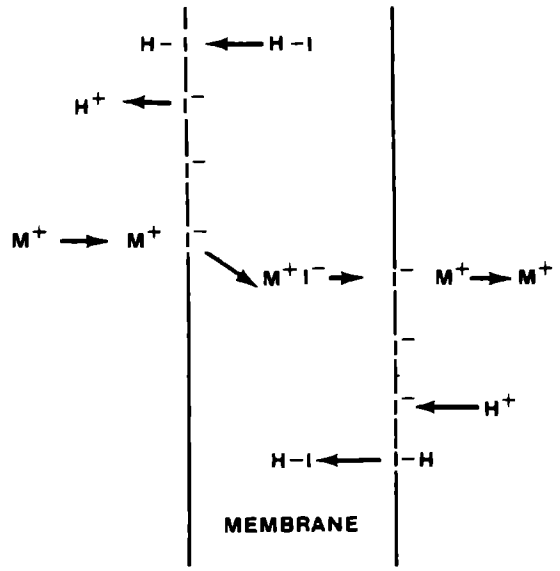

Fig 2. Diagrammatic interpretation of carboxylic ionophore-mediated cation transfer across a bimolecular lipid membrane. M^+, metal cation, I, ionophore, H^+, proton, H-I, protonated ionophore, M^+I^-, zwitterion of metal cation and anionic form of ionophore (From: Bergan and Bates [26]) The anionic form of the ionophore is stabilized by the polar environment characteristic to the surface of a membrane. The ionophore is capable of ion pairing with a metal cation either at the terminal carboxylic acid moiety or at other internal sites The binding of a cation initiates the formation of a lipophilic, cyclic cation-ionophore complex that can diffuse through the interior of the bimolecular membrane structure. After traversing the membrane, the complex is again subjected to a polar environment where the electrostatic forces that had stabilized the complex are no longer greater than the unfavorable Gibbs free energy change of cyclization The ionophore then releases its enclosed cation and reverts to the low energy acyclic conformation

Monensin, like other carboxylic ionophores, binds metal ions through liganding sites such that the ions become centrally oriented (Fig. 1) and masked from the extracellular environment [10,12,19]. The outer surface of the ionophore-ion complex is composed largely of nonpolar hydrocarbon, which imparts a high solubility to the complexes in nonpolar solvents [10,12,19] In biological systems, these complexes are freely soluble in the lipid components of membranes and, presumably, diffuse through the membranes from one aqueous membrane interface to the other [10,25]. Once the ion traverses the membrane as a monensin-ion complex, the ion is released, and the monensin molecule picks up a proton to form an undissociated molecule which then retraverses the membrane to release the proton to the outside of the cell, vesicle, organelle, or other subcellular compartment [19] (Fig. 2). Thus, the net effect for monensin is a trans-membrane exchange of monovalent ions for protons. The ion transfer rates may be very high and can approach, or even exceed, normal enzyme diffusion rates [19], although the actual rate may be markedly altered depending on factors such as the concentration of K^+ in the external medium and the type and concentration of permeant ion that accompanies the accumulated K^+ [27]. However, effects of ionophores on cation transport and their distribution among different membrane-bounded compartments within the cell, will vary depending upon the physical and chemical properties of the different membranes. Membrane fluidity, thickness, curvature, charge and orientation of polar head groups of phospholipids, cholesterol content, and protein content, all influence solubility, penetration, and expression of the ionophore [28]. Moreover, in asymmetric membranes (i.e., biological membranes), ionophores generally exhibit asymmetric transport properties [29]. For example, Kovac et al [28] showed that both valinomycin and nigericin (an ionophore similar to monensin) crossed the plasma membrane of *Saccharomyces cerevisiae* at a rather low rate but then were preferentially located, and active as ionophores, in the inner mitochondrial membrane. Thus, the physiological effects of monensin will depend on the membrane composition and functional characteristics of the different compartments involved.

Although mechanisms for ion transfer through a bimolecular leaflet (membrane) have been proposed, questions still remain as to how this action is related to known effects of monensin on cell function and what relationships these may have, in turn, on biochemical mechanisms leading to animal toxicity. While any consideration of ionophore action must focus on the mechanism of ionophore interaction with biological membranes [26], the complexity of the process and the multiplicity of potential pathways involved suggests that a single causal mechanism cannot explain both the cellular responses and the clinical expressions of toxicity in animals

III-A System effects of monensin

Monensin is cost-effective in increasing the yield of meat from both fowl and ruminants [8]. In fowl, this increase in productivity is derived almost directly through the control of coccidia that, if present, would adversely affect animal health [8] In ruminants, increased productivity appears to result from several factors, the most obvious being an increase in the effectiveness of feed utilization [26,30,31]. These whole-animal effects (i e, systems effects) are well documented [26,30–32] and will be paraphrased only briefly here.

The beneficial effects of monensin in cattle accrue, in part, through shifts in rumen microflora population. For example, gram-positive bacteria (that are primarily acetate, butyrate, H_2, and formate producers) are inhibited by monensin; whereas, gram-negative bacteria (many of which produce succinate) are less sensitive to monensin [26]. The outer layer of the multilayered well of the gram-negative bacterium may contribute to this resistance by acting as a barrier to the penetration of

TABLE I

Examples of cellular effects of monensin

Adapted from Ledger and Tanzer [1]

In secretion

Reduced secretion procollagen [34–37], fibronectin [34–36,38], proteoglycans [37,39,40], prolactin [41], albumin [42], transferrin [42], proinsulin polypeptides [43], laminin [44], α-amylase isoenzymes [45,46], newly synthesized proteins [47], secretory proteins[48], proteins for fast axonal transport [49,50], thyroxine-binding globulin [51], acetylcholinesterase [52,53], chorionic gonadotropin [54], phytohemagglutinin [55], very-low-density lipoprotein [56], maize rootcap polysaccharides [57] (see however, Sticher and Jones [58] for lack of monensin effect), vesicular stomatitis virus glycoprotein [59], extracellular matrix [60], type II collagen [37,39], reviews [19,25,61]

Increased secretion catecholamine [62], cathepsin D [63].

Defective processing Pro-albumin to serum albumin [64], receptors for insulin and somatatomedin C [65], pro-opiomelanocortin [66]

Incomplete processing of oligosaccharides (*N*-linked and/or *O*-linked) myeloperoxidase [67], PrENV glycoprotein [68]; fibronectin [69], hCG subunits [54], blocked formation of complex oligosaccharides [63], Herpes simplex glycoproteins [70], HLA-DR-associated invariant chain [71], coronavirus glycoprotein [72], review [73]

Undersulphation proteoglycans [37], glycosaminoglycan chains [39], β-D-xyloside glycosaminoglycans [74,75]

In endocytosis and endosome acidification

Inhibition of internalization arylsulfatase [63], immunoglobulin [76], α-2-macroglobulin [77], semliki forest virus [78], horseradish peroxidase [79]

Inhibition of dissociation of internalized ligand asialoglycoproteins [80], asialo-orosomucoid [81]

Inhibition of ligand transfer sinbis virus nuclear capsids to cytoplasm [82], epidermal growth factor, β-hexasaminidase, low-density lipoprotein, immunoglobulin, and proteoglycans to lysosomes [40,76,83]

Inhibition of acidification endocytic vesicles [84–86], lysosomal and prelysosomal compartments [63], interference with semliki forest virus genome penetration [78], expression of diphtheria toxin [87]; recycling of LDL receptors [88], release of diphtheria toxin from endocytic vesicles [40,87]

Inhibition of intracellular degradation proteoglycans [40], insulin [89], lysosomal (methylamine-sensitive) protein degradation [90]

Inhibition of contraction of contractile vacuoles *Paramecium aurelia* [91]

Recycling of LDL receptors human skin fibroblasts [88]

Surface formation and growth

Altered secretion of cell surface molecules proteoglycan [37,39,40], type II collagen and/or procollagen [36,37,39]; fibronectin [36,38,69], laminin [44], incorporation of sulfatides into myelin [92], incorporation of Po protein and myelin basic proteins into myelin [93]

Inhibition of scale morphogenesis scales of the green alga *Pyramimonas inconstans* [94]

Inhibition of cell spreading cultured fibroblasts [95]; mesoderm cells [60]

TABLE I (continued)

Stimulation of receptor capping mouse T-lymphoma cells [96]

Inhibition of growth rye seedlings [97], *Pellia* [98]

Transport of molecules

Recognition of independent secretory pathways acetylcholine receptor and acetylcholinesterase [53], membrane glycoproteins/assembly of Uukuniemi virus [99], Ca^{2+}-dependent and Ca^{2+}-independent secretion of α-amylase [100], proteoglycan and hyaluronate [101], prolactin [41]; galactosyl receptor [102]

Maturation and/or transport of viral coat proteins vesicular stomatitis virus [42,103], herpes simplex virus [70,104], semliki forest virus [105], Uukuniemi virus [99], alphavirus [106], coronavirus glycoprotein [72], bovine herpes virus type 1 glycoproteins [107]

Stimulation of sugar and sugar nucleotide transport avian erythrocytes, isolated rat and mouse diaphragm muscle, and red cells [108,109,110]; mouse thymocytes [111]

Redirection of secretory product plasmalemma to tonoplast [112]

Inhibition of intracellular transport: protein to rod outer segments [113], myeloperoxidase [67], hCG subunits [54], accumulation of laminin [44]; gp70 glycoprotein [68], procollagen [34], fibronectin [34]

Interactions with other toxins

Enhancement of toxicity tiamulin in swine [114], disulfide-linked methotrexate-anti-transferrin receptor conjugate [115], specific cytotoxicity of a breast cancer-associated antigen immunotoxin in humans [116]

Reduction of toxicity selenium and vitamin E [114]

monensin; although, a more direct influence involving differenes in membrane energetics also has been implicated [26]

Monensin also may decrease the degradation of dietary protein in the rumen and, thus, increase the amount of protein available for digestion and uptake in the small intestine [26]. Both a reduction in overall cell numbers in the rumen and a direct effect of monensin on bacterial proteinase and deaminase activity have been suggested as contributing to this effect [26,33].

III-B. Cellular effects of monensin

One of the first subcellular effects observed in relation to the topical application of monensin was vacuolation of Golgi apparatus cisternae [34]. Subsequently, in vitro studies clearly demonstrated that monensin altered or inhibited numerous membrane-located phenomena (Table I). Among these were the transfer of α_2-macroglobulin from coated pits to receptosomes [77], recycling of low-density lipoprotein receptors [88], pinocytosis [79], transfer of product from endoplasmic reticulum to Golgi apparatus [34], maturation and/or transport of viral coat proteins [42,70,72,99,104,105,117,118], inhibition of transport of membrane proteins to rod outer segments [113], inhibition of cholesterol transport from

the Golgi apparatus to the mitochondrial site of steroidogenesis [119], blockage of phytohemagglutinin transport out of Golgi apparatus and into protein bodies [55], inhibition of procollagen and fibronectin secretion from cultured human fibroblasts [35], inhibition of carbohydrate processing in cultured human fibroblasts [63], and inhibition of processing and cellular secretion [34,37,41,120–122].

Additionally, cellular effects of monensin vary markedly depending upon the organism and the route of administration. Cultured cells, cells of tissue slices or explants, and plant organs that have received a topical exposure to monensin sufficient to inhibit growth or some cellular processes, usually show deviations in Golgi apparatus structure and function. In contrast, cells from animals poisoned by ingested monensin often exhibit gross mitochondrial lesions without the corresponding Golgi apparatus modifications. The reasons for these fundamental differences in cellular responses to monensin provide one focus for the present review and illustrate the many complexities surrounding the use of monensin as a probe specific to a single metabolic process.

III-B.1. Monensin impairment of Golgi apparatus function and exocytosis

The primary functional unit of the Golgi apparatus is a stack of membranous compartments (i e., the cisternae) each of which differs chemically, structurally, and functionally from the others [123–126]. The number of cisternae per stack varies widely; although, in most animal cells and higher plant cells, there are about 5–10 cisternae per stack. Each stack is polarized in the sense that product and membrane maturation appear to occur sequentially from a cis (forming) face on one side to a trans (maturing) face on the other side [127]. For simplicity, the stack may be divided into units (measured from cis to trans faces) each of which represents a known set of functions. Currently, there appear to be some 2–3 such units that make up each stack [128,129]. In reality, however, it seems more likely that these changes in function occur gradually across the stack of cisternae rather than in discrete steps.

There is, also, one or more membraneous structures (e.g., the trans Golgi network [130] or TGN and the partially coated reticulum [131] or PCR) that lie just off the trans poles of the stacks In plant cells, these structures appear to be derived from sloughed trans cisternae [132]. TGN and PCR participate in the separation (i.e., sorting) of both secretion and endocytic products [61,133–136] and regulate the release of endocytosed substances through a pH-sensitive mechanism [84]. The functions of these post-Golgi apparatus structures are rapidly affected by monensin.

Several mechanisms for the movement of membrane and product through the Golgi apparatus have been postulated. For example, movement may occur by sequential maturation of Golgi apparatus elements (i.e., formation, maturation and loss of cisternae) through the Golgi apparatus stack [127]. This would require the formation of new cisternae on one face of a Golgi apparatus sack and commensurate loss of cisternae from the opposite face of the stack The source of these new cisternae is a special region of endoplasmic reticulum which gives rise to transition vesicles that move and condense on the forming (cis) face of the stack where they fuse together to form the new cisternae [137–139] Product movement may also occur by shuttle vesicles at the peripheries of the cisternae that move proteins from one cisterna to the next [140]. However, both direct (i e., nonvesicular) movement of substances into Golgi apparatus cisternae and an endoplasmic reticulum-mediated movement of product through the peripheral tubules of the cisternae must also be considered as viable options for the delivery and transfer of substances in and out of the Golgi apparatus [141]. The post-Golgi apparatus structures appear to move membrane and product almost entirely via shuttle vesicles, many of which are coated [142–144]

Monensin exerts its most profound effects on the trans cisternae of the Golgi apparatus stacks in those regions of the apparatus primarily associated with the final stages of secretory vesicle maturation and in post-Golgi apparatus structures primarily associated with endocytosis and membrane/product sorting. Because of its relative specificity, biologists have used monensin extensively as an inhibitor of trans Golgi apparatus function.

Incorporation of radiolabeled [^{35}S]methionine into secreted immunoglobulin M molecules in monensin-treated cells was reduced as was sialylation of immunoglobulin M and lymphoid cell surface glycoproteins [145]. These latter findings showed that the intracellular processing of *N*-asparagine-linked oligosaccharides is altered in the presence of monensin with an effect primarily on those sugars (e.g., sialic acid, galactose, fucose) added late in the processing continuum [73] Fibronectin, secreted in human fibroblasts, was incompletely processed in the presence of monensin and exhibited a greater incorporation of mannose than did control protein molecules [69] Inhibition of fibronectin secretion in human melanoma also has been reported [38]. Similarly, when treated with monensin, rat astrocytes in primary culture accumulated laminin, another matrix glycoprotein involved in cell adhesion [44].

Not only do the monovalent ionophores block transport and surface expression of several secretory glycoproteins in normal cell functioning [34,35,42] and the transport of membrane glycoproteins or enveloped viruses [70,72,99,103,104,106,117,118], they inhibit formation of cell surfaces including assembly of peripheral myelin [92,99]. In mouse thymocytes, monensin leads to

stimulated incorporation of labeled sialyl-, galactosyl-, and *N*-acetyl glycosaminyl residues [111]. This enhanced accumulation was not due to a direct effect of monensin on glycosyltransferase activities but, rather, as a consequence of a greater entry and accumulation of labeled sugar nucleotides in the swollen cisternae. Galactosyl transferase itself was translocated through the Golgi apparatus at a slower rate with monensin. However, the sialylation of the *O*-linked oligosaccharides of the enzyme was unaffected by monensin treatment [59] Effects of monensin on glycosyltransferases also may be indirect. Monensin has been reported to decrease galactosyltransferase activity in Golgi apparatus of rat embryo fibroblasts [79] although it had no effect on this activity in baby hamster kidney cells [146]

Monensin is an especially useful inhibitor, since it blocks intracellular transport of protein at the level of the Golgi apparatus without directly affecting protein synthesis [35,52] The effect of monensin is considered to be on transport rather than on processing per se [121,147] One argument is that oligosaccharide processing of those glycoproteins that reach the appropriate site occurs normally even in monensin-treated cells [35,37,68,121]. However, these observations could be explained as well if processing of oligosaccharide chains of different secreted glycoproteins occurred at different sites, only some of which were sensitive to monensin [53,68].

The ability of monensin to effectively 'freeze' processing of molecules at a particular stage had lead to its use in identifying transitory synthetic intermediates. Examples include the insulin receptor where several polypeptide precursors have been described [65], the intracellular accumulation of non-cleaved precursors of pituitary hormones that occur in the presence of monensin [66], and dissection of the pathway for secretion of gonadotropin by cultured human trophoblastic cells [54].

In some instances, the effect of monensin may be to redirect, rather than block, the movement of Golgi apparatus-derived product. For example, under normal conditions, proteins of developing seeds accumulate in a central vacuole which then partitions into smaller units of storage protein (i.e, the protein bodies). However, when treated with monensin, the Golgi apparatus-derived transport of the protein vicilin in pea cotyledon was redirected from the vacuole to the plasmalemma and the newly synthesized vicilin was released from the cotyledon cells to accumulate between the plasmalemma and cell wall [112]

Monensin inhibition of Golgi apparatus function is sufficiently well established [25,42,61,121] that the phenomenon is used widely as one criterion for verifying the passage of a biochemical entity through the Golgi apparatus. Thus, based on partial monensin inhibition, Hammerschlag and co-workers [49,50] concluded that passage through the Golgi apparatus was an obligatory step in the intracellular routing of materials in fast axonal transport. Bartalena and Robbins [51] showed that monensin impeded the exit of thyroxin-binding globulin from the Golgi apparatus without affecting the terminal glycosylation of the protein. Yanagashito and Hascall [40] reported that monensin reduced and delayed transport of both secretory and membrane-associated forms of proteoglycans, suggesting passage through the Golgi apparatus of rat ovarian granulosa cells in culture Similarly, an involvement of the Golgi apparatus in the transport of sulfatides to myelin [92] and phytohemaglutinin to protein bodies in bean cotyledons [55] were deduced from monensin inhibition Flickinger and co-workers [48] using [^{3}H]leucine, showed that all, or nearly all, of the protein secretory product of mouse epididymis principal cells pass through the Golgi apparatus in times approximately equivalent to those reported in other tissues. This transfer of product from Golgi apparatus to the cell surface was largely blocked by monensin

III-B 2 Swelling response of Golgi apparatus and processing defects

Swelling of Golgi apparatus cisternae observed in the electron microscope following fixation with glutaraldehyde is, perhaps, the most consistent visual in vitro demonstration of a monensin-induced effect on a membranous cell compartment [34,41,61,121,148–150] (Figs 3 and 4). In animal cells, swelling usually occurs at a minimum monensin concentration of about 10^{-7} M [37,151], although monensin effects have been reported at even lower concentrations (Refs. 148 and 152, and subsection *III-B 7*) In plant cells and *Euglena*, the minimum concentration of monensin to elicit a response is about 10^{-5} M [57,122,149,152,153].

The swollen cisternae usually appear devoid of contents by electron microscopy (Figs. 3 and 4) but an electron-dense substance may be precipitated through the osmium tetroxide-zinc iodide reaction (unpublished data) Although all cisternae of the Golgi apparatus may swell in response to monensin (Fig. 3 and 5A), the major effect appears to be associated with the mature, or trans, parts of the Golgi apparatus stacks (Figs 4 and 5B) [148,149,152].

Griffiths and co-workers [118] showed that monensin inhibited the transport of viral membrane proteins from medial to trans Golgi apparatus cisternae, thus indicating a monensin block between medial and trans cisternae Monensin also blocked trimming of the high mannose bound to the viral membrane proteins and their conversion to complex oligosaccharides Similarly, Niemann and co-workers [72] found that monensin blocked glycosylation of E1 glycoprotein of corona virus in infected mouse cells. Srinivas and co-workers [68] reported failure to process simple endo-H-sensitive to

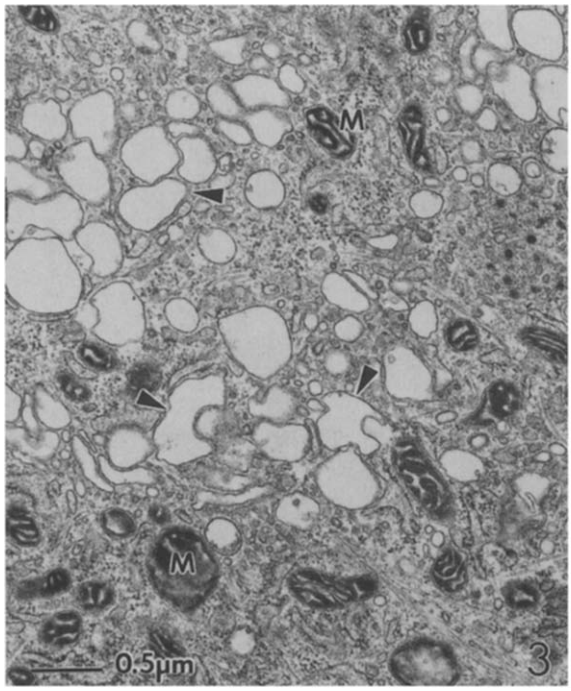

Fig 3 A V79 cultured cell incubated 1 h in medium containing 10^{-7} M monensin Golgi apparatus cisternae (arrowheads) were swollen and appeared empty Mitochondria (M) were condensed. Endoplasmic reticulum and other cellular constituents were of near-normal appearance

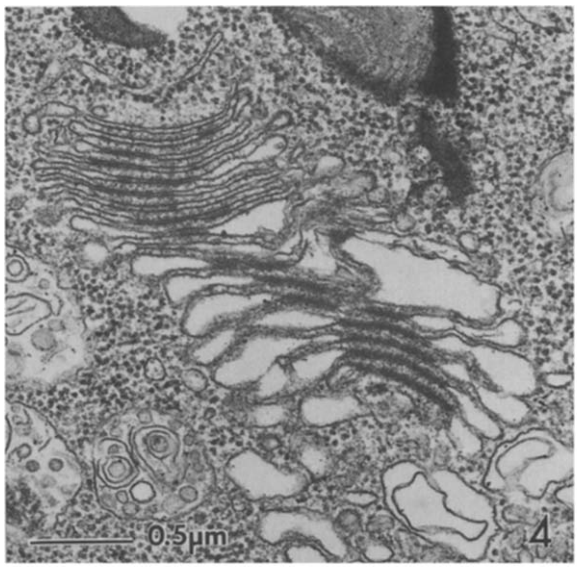

Fig 4 Transverse section through a *Euglena golgi* apparatus from a cell exposed to 10^{-5} M monensin for 1 h. Swelling of cisternae was similar to that observed in animal cells (compare with Fig 3) but the large number of cisternae allowed for visual localization of the monensin effect Swelling was limited to the trans half (toward the bottom of the micrograph) of the Golgi apparatus and this pattern was consistent even at monensin concentrations of 10^{-4} M and exposure times as long as 48 h

complex endo-H-resistant oligosaccharides and reduced efficiency of cleavage of the PrENV glycoprotein precursor to gp70 for Eveline mouse cells infected with Friend murine leukemia virus These findings indicate a block prior to entry into the Golgi apparatus Also, in cultured hepatoma cells, transport of vesicular stomatitis virus (VSV) G protein was arrested prior to acquisition of endo-H resistance, suggesting a block early in the processing pathway [42]. Strous and co-workers [49] showed that monensin affects primarily the galactosyltransferase-containing cisternae of the Golgi apparatus based on studies of the metabolism, localization, and biosynthesis of *N*- and *O*-linked oligosaccharides of galactosyltransferase in HeLa cells. The accumulation of incompletely processed glycoproteins indicates either an up-stream accumulation of secretory materials behind a Golgi apparatus blockage by monensin [44] or a monensin block near the exit site from endoplasmic reticulum [68].

Monensin effects on Golgi apparatus have been observed in a wide range of plant and animal species and appear to be a universal response to the topical application of monensin. As pointed out above, monensin action is exerted on the trans half of the stacked cisternae (Fig 4), often near the point of exit of secretory vesicles [1,34,61,57,118,149,152] or, especially at low monensin concentrations or short exposure times, sometimes in the midregion of the stacked cisternae [148,149]. Intracellular transport may be blocked [35,42,49,68,117,121], often within minutes after exposure to monensin [88,106,125,152,154]. Swollen units usually accumulate near the Golgi apparatus [147,152]

III-B 3 Accumulation of Golgi apparatus elements in the presence of monensin

A monensin effect is quite rapid in both animal and plant cells; i e , changes in Golgi apparatus have been observed after only 2–5 min of treatment [85,149,152, 155]. These early effects have been documented particularly well is suspension cultures of carrot (*Daucus carota* L) [152]. When carrot cells were exposed to monensin at 10^{-5} M (which is approximately the minimum concentration that will elicit a strong monensin response in plants), production of secretory vesicles ceased and, almost immediately, an increased number of cisternae in the dictyosome stacks was observed. An average of one additional cisterna per stack was formed within the first 2–4 min of monensin treatment and, in some experiments, a second cisterna was formed within about 6 min. These effects occurred without significant swelling of cisternae. Thereafter, vacuoles, representing intact swollen cisternae, began to accumulate in the cytoplasm at a rate of about one every 3–4 min (Fig. 6). The mechanism postulated for this momentary increase of dictyosome cisternae was that monensin, acting on the trans pole of the dictyosome, blocked normal formation

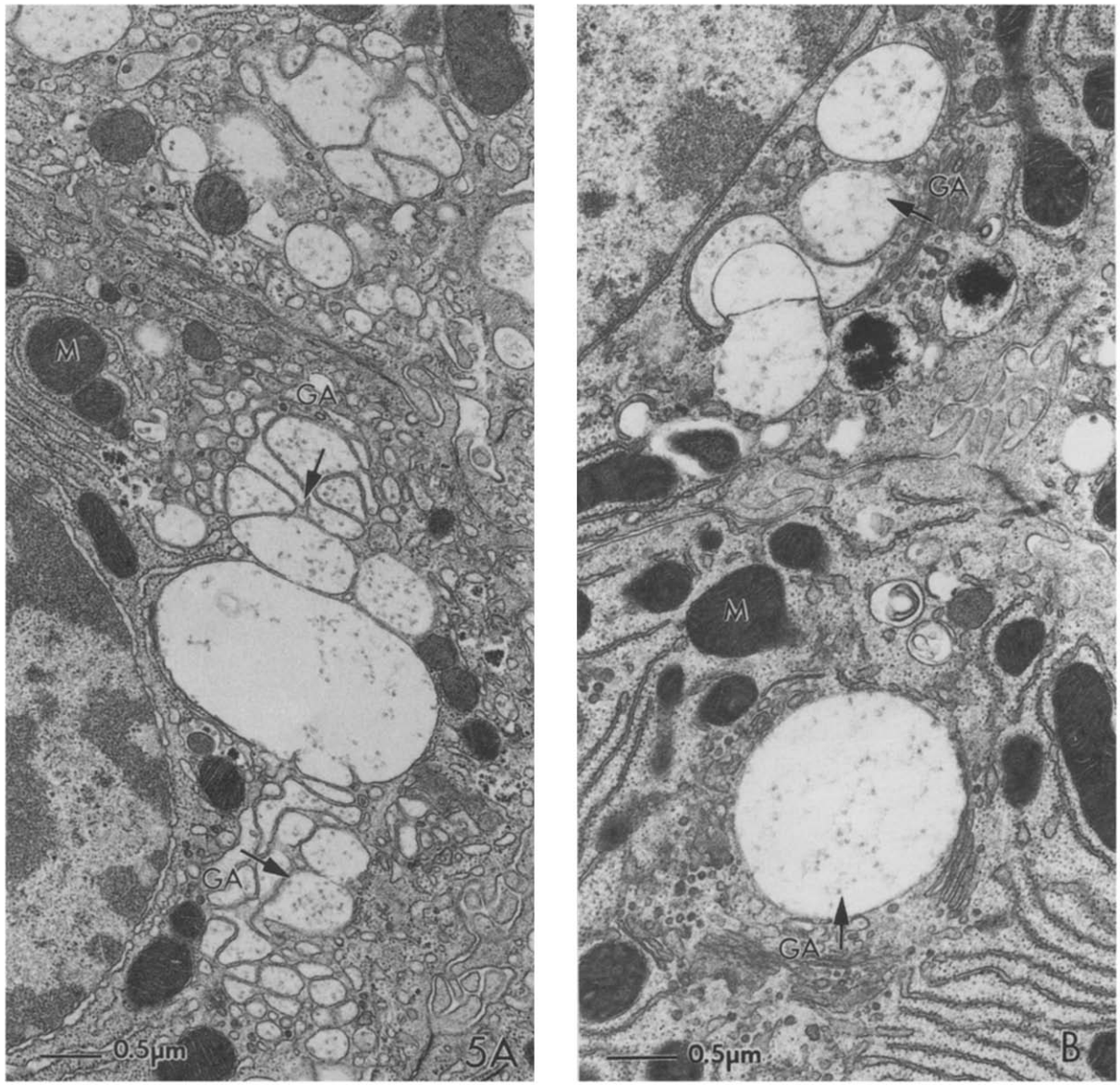

Fig 5 Micrographs from 200 μm thick slices of 9-day-old rat liver incubated 30 min in medium containing 10^{-5} M monensin (see Morré et al [125] for details of procedure) The two samples differed only in that the one illustrated in (A) was adjacent to a 'natural' (i e. uncut) surface of the liver lobe, whereas (B) was from a cell near the 'cut' surface of the tissue slice In both instances, some Golgi apparatus (GA) cisternae were swollen, however, in (A) swelling was progressive from cis to trans pole (direction of arrow) whereas in (B) swelling was confined to the trans cisterna Note that mitochondria (M) were condensed

of secretory vesicles but did not block the formation of new cisternae at the cis face of the apparatus However, as the trans cisternae began to swell, the swollen cisternae were eventually released as intact units that neither fragmented nor integrated (fused) with other cellular constituents (e.g., plasma membrane). With the scale producing green alga *Pyramimonas inconstans*, exposures of 1 to several hours to monensin resulted in disorientation of the Golgi apparatus and disruption of scale morphogenesis [94]. These effects were reversible

More recent studies indicate that a similar pattern of swelling and accumulation of cisternae in the cytoplasm occurs in cultured animal cells [151]. When H-2 hepatoma cells were treated for varying times with 10^{-8} to 10^{-5} M monensin, one swollen cisterna per stack of cisternae was produced after 6–8 min of treatment During this time, approximately one additional cisterna per stack was formed (Fig. 7, inset). As the cisternae vesiculated, vacuoles began to appear in the cytoplasm These large swollen vacuoles were formed at the rate of one every 5–6 min for about 20–25 min for a total of 4 such vacuoles. After this, no new vacuoles were formed (Fig. 7) Throughout, the number of normal cisternae remained constant indicating the formation of new cisternae to compensate for swelling and vacuolization of the trans Golgi apparatus elements At high monen-

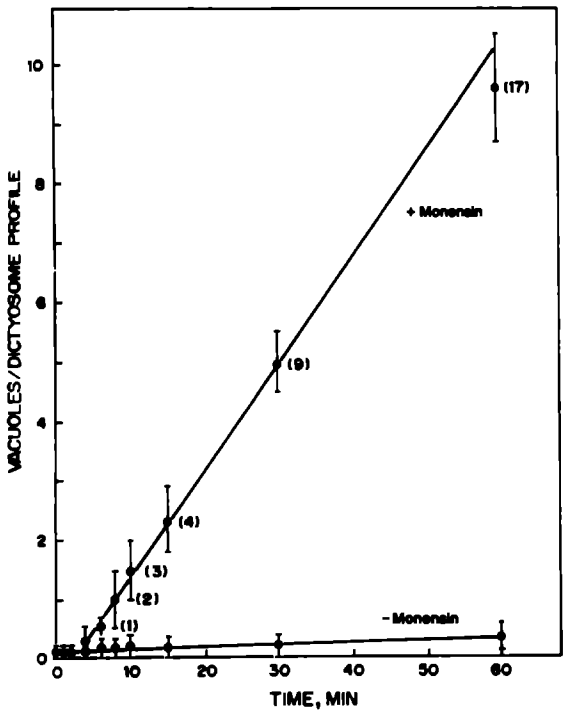

Fig 6 Graph illustrating number of vacuoles (swollen cisternae) per dictyosome profile per section in suspension cultures of carrot following exposure to monensin Trans cisternae swelled and then formed into spherical vesicles (vacuoles) that slowly migrated from the dictyosomes into the cytoplasm New cisternae apparently formed on the cis faces of the dictyosomes to replace those lost to vesiculation The number of vacuoles formed per unit time was proportional to the turnover of cisternae and indicates that, in these cells at least, membrane (and cisterna) formation continued even in the presence of monensin (From: Boss, Morré and Mollenhauer [156].)

sin concentrations, the vacuoles were larger and appeared more rapidly than at low concentrations of monensin but the kinetics of vacuole formation were qualitatively similar. However, by 2 h following treatment with 10^{-6} M momensin, all cisternae of the Golgi apparatus appeared as vacuoles. The swollen trans compartments that accumulate in the Golgi apparatus region with monensin inhibition may contain regions that are clathrin coated; e.g., condensing secretory material (proinsulin) in pancreatic cells [43].

The response of Golgi apparatus of liver slices to monensin was qualitatively similar to that with hepatoma cells in culture [154]. With liver slices, a fraction enriched in vacuoles was isolated and demonstrated to contain the trans Golgi apparatus markers, galactosyltransferase, and thiamine pyrophosphatase, in ratios similar to those of Golgi apparatus proper [154]. In barley aleurone layers, the α-amylase and acid phosphatase activities that accumulated within aleurone cells following treatment with monensin, were localized in cellular components with buoyant densities intermediate between endoplasmic reticulum and mitochondria and cosedimented with latent inosine diphosphatase activity, a putative Golgi apparatus marker in plants [45]. Heupke and Robinson [46] reported a shift to higher density of Golgi apparatus membranes from monensin-treated barley cells, a response no obvious from work with mammalian cells. The Golgi apparatus cisternae that accumulated behind a monensin block in Semliki Forest virus-infected BHK cells bound viral nucleocapsids, and the resulting increase in density permitted their separation by gradient centrifugation from other Golgi apparatus elements [146].

The effects of monensin on Golgi apparatus, at least up to several hours of exposure, appear to be fully reversible [83,94,152,154] In carrot cells, normal secretory activity was resumed within 20 min after transfer of cells to a monensin-free medium; although, in these cells, the vacuoles formed during the monensin block, remained in the vicinity of the Golgi apparatus for several hours or more, even after apparently normal secretory activity had resumed However, with the longer treatment times of several hours [98] or days [95], growth in both plant and animal cells, and cell spreading in animal cells, became irreversibly inhibited

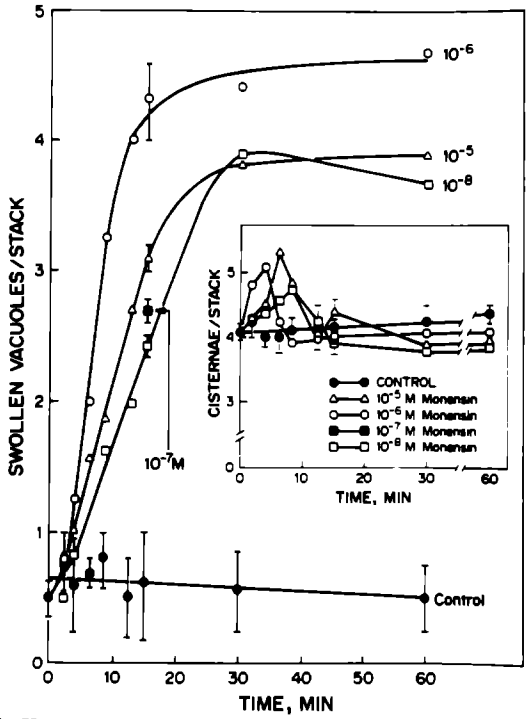

Fig. 7 Kinetics of formation of cisternae and vacuoles (swollen cisternae) by Golgi apparatus of H-2 hepatoma cells in culture following exposure to monensin The number of cisternae per stack increased by about one cisterna (average) after 4–6 min of monensin treatment and then declined to the control level at about 10 min (see insert) This decline in number of cisternae occurred concomitantly with the formation of vacuoles (see main curve). The cisternae of the cis poles appeared relatively normal for about 20 min of treatment during which time an average of four vacuoles per dictyosome was formed However, by 120 min of treatment, all cisternae had become vacuolated and normal-appearing cisternae were no longer present (From Morré, Minnifeld and Mollenhauer [151].).

III-B 4 Mechanism of monensin-induced swelling of Golgi apparatus cisternae

Monensin apparently causes swelling of Golgi apparatus cisternae through a Na^+-in/H^+-out exchange across the membranes leading to a net uptake of $Na^+ + Cl^-$ and entry of water [157,158] Evidence in support of this concept was provided by studies with isolated chromaffin granules which lysed readily after brief exposure to monensin in Na^+- or K^+-containing isotonic media For swelling to occur, the membrane must normally be impermeant to cations as is known for the chromaffin granule. The chromaffin granule membrane contains a H^+-ATPase which is electrogeneic and, in the presence of a permeant anion, acidifies the granule interior to pH 5 5. Thus, the operation of this pump in the presence of monensin drives net salt uptake [158]. To test whether net salt uptake driven by the presence of a proton gradient also would explain the monensin-induced swelling of Golgi apparatus cisternae, wild-carrot cells in suspension culture were treated with drugs and inhibitors known to interfere with proton gradients [156] Monensin-induced swelling of Golgi apparatus in situ could be inhibited by the protonophore, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydazone (FCCP), But was only little affected by the inhibitor of lysosomal acidification, quercetin, or by the lysosomotropic amines, chloroquine, and ammonia. Cyanide also dramatically decreased swelling, and arsenate (with prolonged treatments) reduced the number of swollen cisternae Organic acids, by providing a readily permeable counterion, promoted monensin-induced swelling. These data imply that the monensin-induced swelling of Golgi apparatus cisternae involves a proton gradient at or near the mature poles of the Golgi apparatus Because monensin induces a $1 \cdot 1$ Na^+/H^+ exchange, and since the Van't Hoft factors for H^+ and Na^+ are practically the same [159], the osmolarity of the cell content should not increase to cause swelling without a net proton influx One explanation would be that the pH of Golgi apparatus vesicles is highly regulated Proton translocating ATP hydrolyzing enzymes (H^+-ATPases) are associated with several components of cells that develop acidic interiors such as endosomes, coated vesicles, lysosomes, and trans Golgi apparatus cisternae [160]. Evidence for the presence of an H^+-ATPase has been the demonstration of ATP-dependent vesicle acidifiation in Golgi apparatus isolated from rodent liver [161–163], corn coleoptiles [164], and sycamore cells [165] Acidification was demonstrated both by [^{14}C]methylamine uptake and by spectrophotometric assays of acid-quenched dye fluorescence (Acridine orange, Neutral red, or Quinacrine)

Several lines of evidence confine the Golgi apparatus H^+-ATPase to the trans cisternae. As emphasized in the preceding section, the early in situ effects of monensin are frequently localized to the trans faces of the Golgi apparatus The resultant swelling, which is proposed to be due to the accumulation of osmotically active ions in exchange for protons [156,166], occurs predominantly in trans cisternae Additionally, a basic congener of dinitrophenol (3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine, DAMP), which concentrates in acidic compartments as shown in fibroblasts by immunocytochemistry is present only is cisternae and vesicles associated with the trans faces of the Golgi apparatus [84] Moreover, DAMP rapidly leaves these compartments when cells are incubated with monensin, thus further indicating that accumulation of DAMP is due to the acid pH Some involvement of a low pH compartment is evidenced by the observation that some monensin effects on processing, i.e , proteolytic conversion of proalbumin [64], are mimicked by amines However, in promyelocytic leukemia cells, processing of myeloperoxidase, while blocked by both monensin and chloroquine, was not affected by NH_4^+ cations, thus indicating that processing is not necessarily influenced by pH-dependent mechanisms [67] These results were interpreted as indicative of processing in Golgi apparatus based on inhibition of transport by monensin and chloroquine rather than processing in lysosomes and other late, acidic compartments involving a pH-dependent mechanism [67]

Confirmation of a trans location of the H^+-ATPase has come from free-flow electrophoresis separations of Golgi apparatus yielding cis, medial, and trans compartments in fractions of differing electrophoretic mobility [154,167]. In these separations, proton pumping activity was found exclusively in the most electronegative fractions coming from the trans-most Golgi apparatus region.

Griffing and Ray [166] have offered the suggestion that acidification of cisternal lumina may be part of an osmotic mechanism to compress and flatten the cisternae The latter, for example, might aid in the transfer of content into secretory vesicles. The inward pumping of protons would tend to favor the exit of Na^+ and K^+ out of the cisternae Furthermore, as the pH falls, those monovalent cations remaining would tend to combine with acidic groups of the Golgi apparatus membranes, further reducing the osmolarity of the cisternal content relative to that of the external cytoplasm. Thus, water would be driven osmotically out of the cisternae to both compress the cisternae, as seen along a pronounced cis to trans gradient for plant Golgi apparatus [168], and, perhaps, to account for the condensation of secretory materials in condensing vacuoles and other trans Golgi apparatus compartments (e.g , ref 169).

Other cell compartments, including endocytic vesicles [170,171], lysosomes [172,173], multivesicular bodies [174], and coated vesicles [175–177] have H^+-ATPases. All use H^+-ATPases to acidify their interiors and the enzymes responsible have been solubilized from lyso-

somes and reconstituted into liposomes [178]. Vacuole (tonoplast) membranes [179], and possibly also plasma membranes of fungi [180], contain H^+-ATPase. Similarly, a gradient of acidification within the endocytic pathway has been indicated from immuno-electron microscopy with protein A-colloidal gold and monospecific antibodies to the weak base primaquine [181].

However, not all compartments with H^+-ATPases (e.g., cells, vacuoles, lysosomes, coated vesicles) swell in response to monensin. Cell and/or vacuole swelling may be limited due to the very large internal volumes involved. The contraction of contractile vacuoles of *Paramecium* was inhibited reversibly by monensin and in a manner dependent upon the presence of Na^+ [91] but marked swelling was not observed. Little is known about the swelling response, if any, of lysosomes, coated vesicles and other endocytic compartments in response to monensin inhibition. Their functions, however, are inhibited by monensin as will be emphasized in the section that follows.

Carboxylic ionophores strongly inhibit proton uptake by photosynthetic preparations [19]. In chloroplasts, swelling of thylakoids (inner membrane compartments) but not of the space between inner and outer plastid membranes has been observed to result from monensin treatment [182]. Thylakoid swelling, in contrast to swelling of mitochondrial cristae and of Golgi apparatus cisternae, was reduced upon incubation in darkness, again suggesting a relationship between swelling in the presence of monensin and the light-driven proton gradient used for photophosphorylation [182] Mitochondria have an outwardly directed energy-linked proton pump and do not swell with monensin (rather, they tend to condense, see Figs. 3, 5A and 5B) while the light-driven proton pump of chloroplasts and chromatophores, and that of the Golgi apparatus pump, are directed inward causing the vesicles to swell.

III-B.5. Secretory pathways bypassing the monensin block

Evidence for a secretory pathway bypassing the Golgi apparatus in the monensin-blocked cells is provided by Kubo and Pigeon [183] who investigated the effects of monensin on the synthesis and expression of membrane IgM of a human lymphoblastoid line. They found altered processing of both μ, κ chains and incomplete terminal glycosylations. Yet, transport of the altered molecules was observed. That the aberrant processing did not influence markedly the membrane expression of the IgM is consistent with a secretory pathway bypassing the Golgi apparatus in monensin-blocked cells. Similarly, a dual secretory pathway, only one part of which was susceptible to monensin, was deduced from studies of α-amylase secretion in rice seed scutellum [100]. In Zea maize roots, monensin inhibited secretion of α-amylase but not polysaccharide slime [58].

The blocked secretion that results in the intracellular accumulation of secretory products frequently is not absolute. Some portion of the material synthesized is released from the monensin-inhibited cells and this material frequently exhibits an abnormal type of post-translational modification. For example, those proteoglycans from chicken embryo chondrocytes secreted in the presence of monensin are vastly undersulfated [37,39,74,75]. Thus, membrane and secreted molecules leaving the cell following a monensin block appear to have been denied the full range of processing enzymes they would normally encounter during transit through the cell However, whether the incompletely processed molecules bypass one or more particular intracellular compartments (e g , the Golgi apparatus), or whether they pass through functionally incomplete compartments, remains to be determined. During maturation of Uukuniemi virus in baby hamster kidney cells, monensin appeared to inhibit a terminal step of virus assembly, but not the expression of virus membrane glycoproteins G_1 and G_2 at the cell surface. These findings suggest that both G_1 and G_2 could enter a functional transport pathway in the presence of monensin that bypasses the trans Golgi apparatus compartment to become expressed at the cell surface [99]. Evidence for a Golgi apparatus bypass has been presented in liver where secretory lipoproteins may move directly from endoplasmic reticulum to the cell surface without direct Golgi apparatus involvement [184,185].

At concentrations of 10^{-7} M or higher, monensin inhibited secretion of albumin, transferrin, and VSV proteins G and X destined for delivery to the cell surface to the same extent in rat hepatoma cells [42]. This was taken as evidence that the same vesicles were used by all four proteins in their movement from Golgi apparatus to the plasma membrane However, the time required to move from ER to the Golgi apparatus, based on sensitivity to endoglycosidase H, differed for secretory and membrane proteins. An even more striking observation was that following the monensin block, secretory proteins accumulated in an endo-H-sensitive form, whereas, membrane proteins were already endo-H-resistant. This strongly implies that membrane and secretory proteins are not in the same compartment initially and would support the concept of peripheral input of secretory proteins into the secretory vesicles of the Golgi apparatus, at least in liver [42,186,187]

Alonso and Compans [103] provided evidence for two distinct pathways of glycoprotein transport in Madin-Darby canine kidney (MDCK) cells only one of which was blocked by Monensin However, in baby hamster kidney (BHK21) cells, both influenza virus and VSV maturation were sensitive to monensin. The VSV particles were synthesized in both MDCK and BHK21 cells, but transport to the cell surface was blocked only in the MDCK cells Thus, there appear to be two

distinct pathways of transport of glycoproteins to the plasma membrane in MDCK cells, only one of which is blocked by monensin There is no information on the nature of the alternative transport vesicle that carries influenza virus to the cell surface of MDCK cells if, in fact, a vesicle is involved.

Melroy and Jones [45] reported accumulation of normally secreted α-amylase within barley aleurone layers after monensin treatment. However, only isozyme 2 was secreted normally whereas isozymes 1, 3 and 4 were not secreted Also, in the perfused rat liver, monensin treatment has less of an effect on biliary secretion than on secretion of plasma proteins [47].

Similarly, in the transport of HLA-DR α- and β-chains [71], processing of *N*-linked carbohydrate chains to full endo-H resistance occurs However, with the associated I-chain, processing of both *O*- and *N*-linked carbohydrate chains is inhibited, and carbohydrate chains remain predominantly endo-H susceptible. Here, the processing of membrane-associated proteins that occurs despite a monensin block may reside in intercalary cisternae that constitute the Golgi apparatus region recently termed medial [118,146]

III-B 6. Endocytosis

There is now considerable evidence for monensin-susceptible compartments in the endocytotic pathway. Transfer of product to secondary lysosomes [83] as well as virus penetration into cultured cells [78] are impaired by monensin. Stein and co-workers [188] have shown that monensin blocks transferrin recycling by causing internalized ligand to accumulate in the perinuclear region, primarily in multivesicular bodies, of the K562 cells used in the study. Based on studies of HRP uptake in rat fibroblasts, Wilcox and co-workers [79] suggested that inhibition of endocytic events may be the consequence of an inhibition of membrane recycling within the cell rather than a direct effect of monensin at the cell surface.

Maxfield [85] reported that 6 μM monensin resulted in an increase in internal pH of endocytic vesicles of cultured mouse fibroblasts from 5 0 to above 6 0 to account for its effects on receptor-mediated endocytosis Similarly, Marsh and co-workers [78] concluded that the inhibition of Semliki Forest virus penetration into cultured cells was the result of this increase in pH of endocytic vacuoles and lysosomes above pH 6 0, the threshold for fusion activity of viral membranes Monensin has also been shown to inhibit lysosomal degradation of protein by affecting lysosomal pH [90] and to abolish asialoglycoprotein degradation in cultures of rat hepatocytes through a pH shift in prelysosomal endocytic vesicles [80]. Using digital image analysis, Tyco and co-workers [86] showed that monensin raised the pH of endocytic vesicles in cultured human hepatoma cells and caused a ligand-independent loss of receptors

In other studies, monensin did not prevent internalization of ^{35}S-labeled proteoglycans by rat ovarian granulosa cells although their intracellular degradation was completely inhibited [40]. Yet, degradation pathways involving proteolysis of both dermatin and heparin sulfate and limited endoglycosidic cleavage of heparin sulfate continued. These findings, while consistent with an involvement of both acidic and nonacidic compartments, show that monensin inhibition is primarily on those processes that normally occur in acidic compartments such as endosomes or lysosomes by raising their pH. Similarly, with isolated hepatocytes, Whittaker et al [89] found no effect of monensin on insulin internalization but, rather, an impairment of its degradation once internalized.

Rustan and co-workers [56] suggested that monensin inhibits both endo- and exo-cytosis by a similar mechanism, namely, disruption of proton gradients Their conclusions were based on studies of rat hepatocytes in which monensin inhibited both secretion of very-low-density lipoproteins, and binding and degradation of asialofetuin Both secretion and receptor binding were markedly decreased after only 15 min of monensin treatment although no effect on protein synthesis was observed. However, secretion was more sensitive to monensin than endocytosis, suggesting that monensin independently inhibits endocytic and secretory functions although the mechanisms may be similar.

Marnell et al [87] explained the monensin block of the cytotoxic effect of diptheria toxin on a similar basis Following endocytosis of the toxin, the toxin was assumed to penetrate the membrane of the endosome and enter the cytoplasm in response to an acid environment By neutralizing the ability of endosomes to acidify their interiors, monensin, like the lysosomotropic amines, was able to block the low pH-dependent dissociation of receptor-ligand complexes and subsequent release of ligands either to the cytoplasm (viruses and toxins) or to lysosomes (endocytosed proteins such as LDL). This in turn would prevent recycling of receptors and membrane and eventually bring endocytosis to a halt due, not necessarily to an inhibition of the uptake processes per se, but perhaps, to blockage of an internal step very similar to that believed to be blocked at the Golgi apparatus.

Also consistent with similar modes of monensin inhibition in processing both endocytic and exocytic vesicles are findings that a single mutation in Chinese hamster ovary cells impaired both Golgi apparatus and endosomal functions in parallel Included were the monensin sensitive steps of virus and toxin penetration from endosomes into the cytoplasm and of Golgi apparatus-associated maturation of Sinbis virus [81]. The alterations correlated with losses of ATP-dependent

vacuole acidification as if the ATPase of endosome and Golgi apparatus shared a common genetically regulated subunit. Ono et al. [189] studied a monensin-resistant mutant of mouse Balb/3T3 cells which also proved to be a poor host for either vesicular stomatitis virus or Semliki Forest Virus multiplication. The mutant cells resistant to monensin, bound virus normally and contained acidic compartments. However, movement of virus from the cell surface to the endosome and lysosome compartments was extremely slow.

Thus, the ability of monensin to block processing of endocytic vesicles by making prelysosomal compartments less acidic, suggests a mechanism for perturbation of endocytosis based on its ionophoric properties The mechanism could be similar to the monensin-mediated exchange of monovalent alkali ions for protons that induces, by osmotic means, the observed swelling of Golgi apparatus cisternae. Clearly, monensin does not interfere with the uptake and binding of particles at the cell surface. Monensin is ineffective against activities that occur at the cell surface. Hedin and Thyberg [76] showed that uptake of IgG prebound to the cell surface was unaffected by monensin Similar findings have been made in studies of receptor-mediated endocytosis of various other ligands [78,80,83]. However, monensin may secondarily affect internalization through depletion of monensin-sensitive receptor sites at the cell surface. This would occur if the cell surface receptors are recycled back into the cell and then blocked in the post-Golgi region by monensin so that they could not return to the cell surface [63,80,88]. Thus, monensin inhibition of endocytic events seems to be at the site of transfer from endocytic vesicles to lysosomes [40,63,76,93] or, in monensin-sensitive endosomes, inhibition of the dissociation of ligand-receptor complexes [81]

III-B 7. Low-dose and anomalous swelling responses by monensin

Most animal cells show a dose-related response to monensin that falls off rapidly at monensin concentrations less than 10^{-7} M Consequently, most studies of monensin effects use monensin concentrations of 10^{-7} M or higher However, a few reports indicate a cellular response at monensin concentrations less than 10^{-7} M For example, receptor capping by lymphocytes is stimulated by low concentrations of monensin in the range 10^{-7} to 10^{-9} M and inhibited by monensin concentrations above 10^{-7} M [96] Cultured adrenal chromaffin [62] and heart [190] cells also are stimulated by low concentrations of monensin. Though these effects occur at concentrations below the threshold effects for most Golgi apparatus responses, they still presumably result from increased levels of cytoplasmic sodium due primarily to the ionophore insertion at the plasma membrane

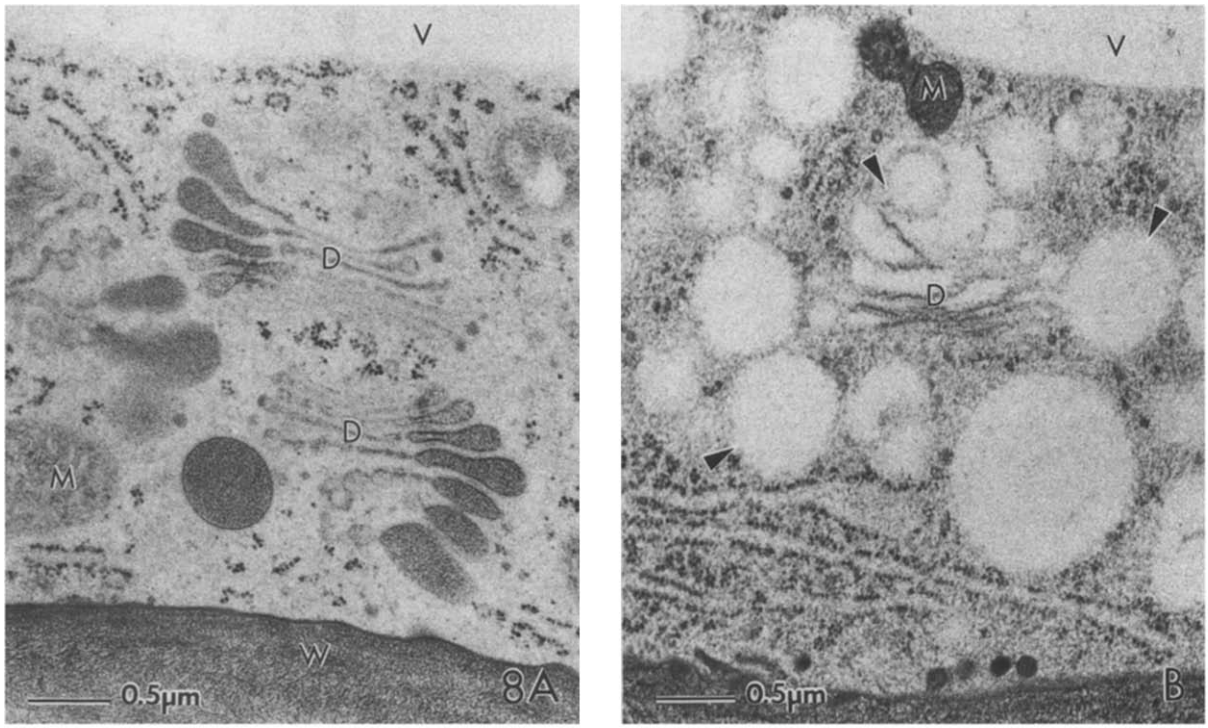

Fig 8 (A) Outer cap cells from a control (nontreated) maize root tip preserved by freeze-substitution [192]. The form of dictyosome (D) was normal and similar to that following glutaraldehyde/osmium tetroxide fixation (B) Same except that root tip was treated for 0 5 h with 10^{-5} M monensin before being preserved by freeze-substitution. The trans cisternae and/or secretory vesicles (arrowheads) were swollen, and mitochondria (M) were condensed (compare with mitochondria of A) W, cell wall, V, central vacuole

Swelling of Golgi apparatus cisternae occurs in a wide range of plant and animal cells (see subsection III-B 2) and may be a universal response to monensin poisoning. However, in plants, and to a lesser extent animals, swelling is influenced by the fixative used to preserve the cells Specifically, morphological evidence of swelling is less (in animal Golgi apparatus) or nonexistent (in plant Golgi apparatus) when the tissues are fixed in potassium permanganate as compared to fixation in glutaraldehyde/osmium tetroxide [122]. These effects could be due either to fixation artifacts or to differences between plant and animal Golgi apparatus (e.g , Ref. 168) This problem was evaluated by comparing the images of Golgi apparatus preserved by various chemical fixatives as well as preservation by freezing and low temperature substitution in acetone and osmium tetroxide. Presumably, the image following freeze substitution would reflect the true ultrastructure more closely than the image following chemical fixation. The results of freeze substitution in both animal cells [191] and maize root (Figs 8A and B), show swollen Golgi apparatus cisternae following monensin exposure in a pattern similar to that observed after glutaraldehyde/osmium tetroxide fixation [192]. However, using video-enhanced light microscopy and cultured bovine mammary epithelial cells, a marked swelling response to monensin was observed only after the addition of glutaraldehyde fixative to the monensin-incubated cells (Morré, D J., Mollenhauer, H H., Spring, H., Trendlenberg, M , Morré, D M. and Kartenbeck, J , unpublished data). Thus, whether monensin-induced swelling occurs in vivo or is in response to aldehyde fixations remains an important questiion.

No swelling was observed in Golgi apparatus of protoplasts of carrot cells freshly prepared by digestion of cell walls when exposed to monensin even though such a response was obtained in the same cultures prior to wall dissolution [193]. Similarly, in thin slices of liver incubated in monensin, the Golgi apparatus adjacent to cut edges of tissue slices showed a different swelling response than Golgi apparatus adjacent to the uncut natural surfaces of the lobe (unpublished data) Adjacent to a cut edge, fewer cisternae swelled and those that swelled were only in the most trans positions The basis for such differences is unknown but might, for example, indicate changes in cisternal proton pumping ability, or monensin uptake, in response to changes in the physiological state of the Golgi apparatus brought about by the tissue excision

IV. Toxicity studies

The mechanism by which monensin interacts with coccidia and rumen microflora is well documented [26,194–201] However, the interaction between monensin and the tissue of the host animal is less well understood even though the clinical manifestations of monensin poisoning are well known. Most striking are differences between the in vitro monensin effect observed in cultured plant and animal cells, and plants, and the in vivo effects observed in animals

When used at recommended levels, either as a coccidiostat for poultry [1,202] or for cattle [8,30,202], monensin seldom causes poisoning Nonetheless, misuse of the product, usually from improperly mixed or improperly distributed feed, may cause toxicosis and death [203–213]. Horses, ponies and other equine species are particularly sensitive to monensin poisoning [12,209, 214]. The median lethal dosage (LD_{50}) for the horse is 2–4 mg monensin per kg body weight compared to 50–80 mg per kg body weight for cattle and 200 mg per kg body weight for poultry [12,214,215] In mammals, the physical signs of monensin toxicosis commonly include anorexia, diarrhea, depression, sweating, ataxia, palpitations of the heart, and sudden death following exercise [204,205,209,211,214–217] Stiffness of hindquarters and swollen gluteus muscles [210,211], elevated pulse rate [211,217,218], and ECG abnormalities [207,208,214,217] also have been reported In fowl, the outstanding signs of monensin toxicosis are drowsiness, excessive thirst, anorexia, depression and paralysis [205,212,213] Marked congestion in a variety of organs also has been noted [205] Severely poisoned birds may die in sternal recombency [205]. Routine clinical tests on serum from horses poisoned by monensin may show abnormally high values for blood urea nitrogen, total bilirubin, creatine kinase, lactate dehydrogenase and aspartate aminotransferase [204,207–209,211] However, clinical manifestations are often variable making interpretation and diagnosis difficult. Moreover, serum levels of sodium, potassium, chlorine, calcium, phosphorus, and urea may remain at near-normal levels following monensin treatment [211].

In poisoned mammals and fowl, generalized congestion, hemorrhage, and macroscopic injury to striated muscle [210,211,214,219], spleen [205,212], lung [212], liver [209], and kidney [209,211,212,220,221] have been noted. The most consistent microscopic observation in ponies, cattle, pigs and fowl, has been cardiac myocyte degeneration and vacuolization [150,208,217,219–225]

In animals poisoned with relatively high doses of monensin, an initial condensation of heart mitochondria is often seen (Fig 9) However, with longer exposure times, some mitochondria swell and vacuolate with an almost total loss of matrix substance (Fig 9). Intracristae spaces generally remain unchanged with swelling being restricted to the mitochondrial matrix This is followed by loss or dilution of matrix components and a reduction in size of cristae so that the mitochondria appear as empty vacuoles with residual cristae Typically, only some mitochondria in a particular fiber become swollen and appear as vacuoles, and

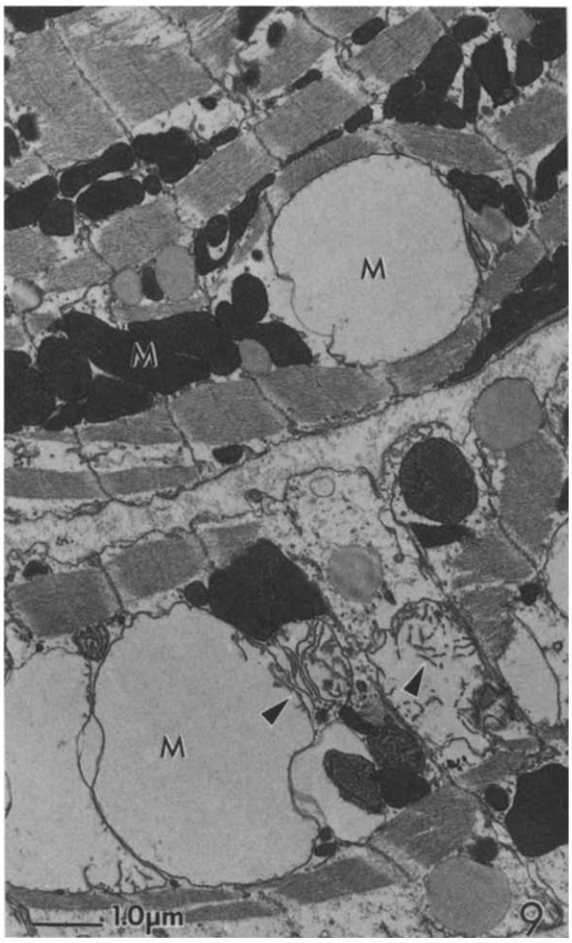

Fig 9 Section of left ventricle from a rat treated with a single intraperitoneal injection of 40 mg monensin per kg body weight Most mitochondria (M) were either condensed or swollen In swollen mitochondria, cristae (arrowheads) were greatly reduced in extent but, otherwise, were of approximately normal thickness This dosage level (e.g , 40 mg monensin per kg body weight) is quite high for rats and often resulted in significant generalized damage to the muscle fiber as illustrated in the lower part of the micrograph However, mitochondrial swelling (but not condensation) may occur as well at lower monensin dosages, even when no fiber damage can be identified ultrastructurally Swelling and vacuolization of mitochondria are progressive with time whereas mitochondrial condensation was dose-dependent and often occurred rapidly

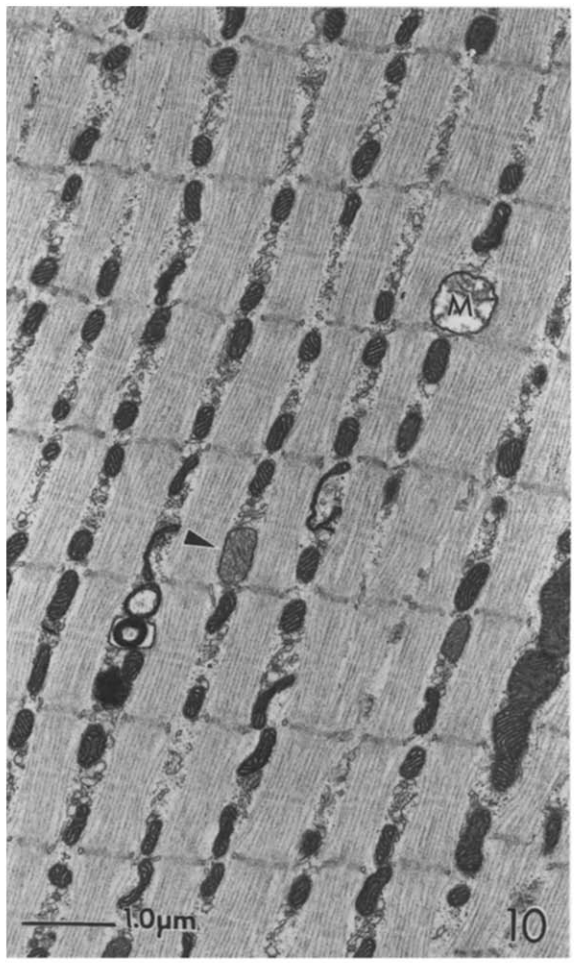

Fig 10 Section of diaphragm from rat treated with one intraperitoneal injection of 40 mg monensin per kg body weight. Only a few mitochondria in the diaphragm were swollen (e.g , M), a few were normal (arrowhead), but most were mildly condensed Swollen mitochondria always appeared randomly distributed through a fiber although significant differences in mitochondrial swelling were often noted between fibers (e g , see Fig. 12)

these are randomly distributed throughout the fiber (Fig. 10). We have identified early stages of mitochondrial vacuolation and swelling in both the rat and pony (unpublished data), but these mitochondria were seldom plentiful, suggesting that transition to the vacuolated state, once started, was relatively rapid Non-swollen mitochondria may appear condensed, especially after exposure to high levels of monensin (Fig. 9 and 10). Granulation of mitochondria (Fig. 11) was observed only occasionally and those granules that were present appeared similar to the tricalcium phosphate granules often associated with normal mitochondria [226,227]. However, whether monensin-induced granules contain

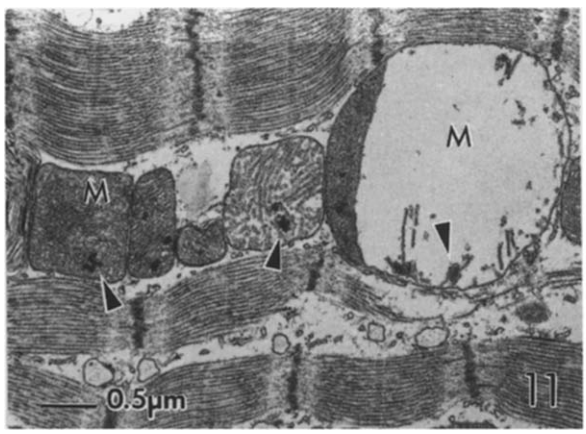

Fig 11 Section of the left ventricle of a pony treated with monensin Mitochondrial (M) granules are illustrated at the arrowheads

Ca^{2+} has not been determined. Granules like these have been observed in ischemic and reperfused hearts and are considered indicative of calcium overload [228–231]. Ca^{2+} overload may be a potential cause of cell death or cellular dysfunction in ischemia [228,232–237] although the effects are reversible if the cell is not too severely damaged [228,229] Exaggerated mitochondrial swelling has also been observed immediately following reperfusion of hearts rendered ischemic by occluding blood flow for a few minutes [228,229]

Whether excess mitochondrial Ca^{2+} occurs as a result of monensin is not clear Under appropriate conditions, either monensin or Na^+ in excess can block Ca^{2+} accumulation and promote its release from both mitochondria and sarcoplasmic reticulum over a broad range of monensin and Na^+ concentrations [20,24,238] It is probable that the release of Ca^{2+} by monensin can occur as the result of an increase in cytoplasmic Na^+ from a monensin shuttle although monensin (at relatively high concentrations) has been shown to release Ca^{2+} directly in a Na^+-independent manner from cardiac sarcoplasmic reticulum [24] In all probability, however, monensin is not present in myocytes at high concentrations since swelling of Golgi apparatus cisternae (a characteristic response to monensin) is never observed in these cells. The picture is further complicated by the fact that Ca^{2+} release patterns may vary according to type of cell (e g., white vs red muscle cells [239]) as well as the availability of extracellular Ca^{2+} [110]. Although not related to this report, it may be of some interest to note that both mitochondrial condensation and granulation are much more intense in X-537A-treated animals (and cultured cells as well) than in comparable animals (or cells) treated with monensin [21].

The percentage of affected mitochondria varied markedly between muscle types and species of animal. Analyses of striated muscle in ponies showed that there was a much greater (50–100-times) likelihood of finding altered mitochondria in heart tissues than in diaphragm or appendicular muscle [224] A similar relationship existed in rats except that most swollen mitochondria were in the diaphragm [224]. Some antibiotics (e g , tiamulin and avoparcin) may act synergistically with monensin to induce shifts in the distribution of swollen mitochondria [240] and other cellular damage. Differences in distribution patterns of swollen mitochondria also were observed between red and white muscle fibers of the rat diaphragm [224]. Red and white fibers were differentiated structurally by size, mitochondrial content, and Z-band configuration [34,241–243] Swollen mitochondria were present in all fiber types when the number of affected mitochondria was small. However, when large numbers of swollen mitochondria were present, the distribution pattern was heavily skewed toward the white muscle fibers (Fig. 12). A differential

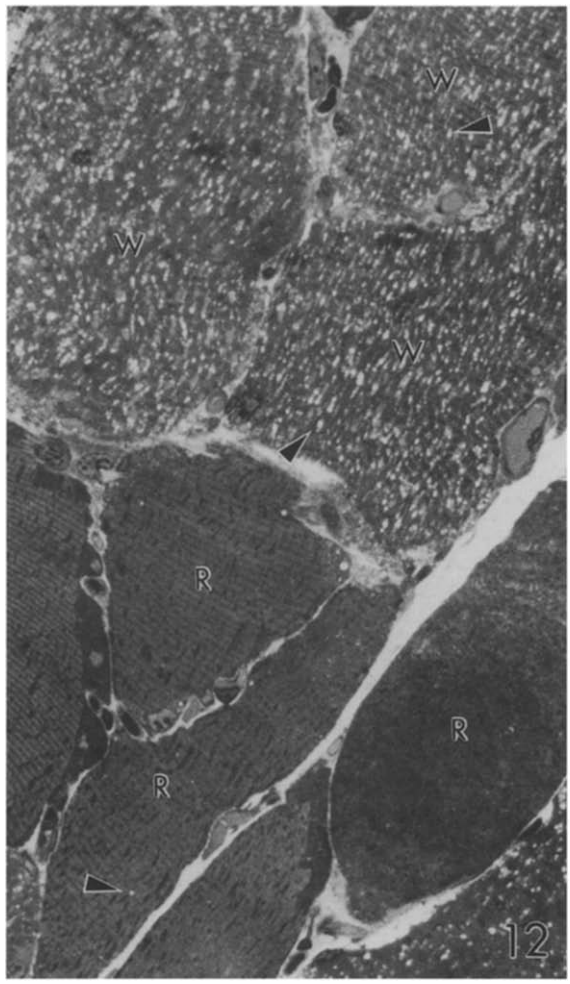

Fig 12 Light micrograph of rat diaphragm illustrating swollen and vacuolized mitochondria (arrowheads) Most aberrant mitochondria were in white (W) muscle fibers and only a few were in red (R) muscle fibers

effect of monensin was also noted by Van Vleet and co-workers [217] who observed in swine severe damage in the diaphragm, vastus lateralis, semitendinosus, triceps and intercostal muscles; moderate damage in longissimus lumborum muscle, and little (or minimal) damage in tongue. Damage was greatest in muscles containing a high proportion of type 1 fibers These distribution patterns, coupled with the characteristic form of the degenerating mitochondria, are not common observations and, therefore, may be strong indicators of monensin poisoning in animals.

Swollen mitochondria have not been observed in any of the nonmuscle cells of the heart, diaphragm, or appendicular tissues or in liver, adrenal, or kidney cells Thus, monensin administered to mammals in vivo tends to induce mitochondrial changes only in selected tissues and/or types of muscle fibers The mechanisms for these mitochondrial changes and reasons for the specificity are not known. These problems are compounded by the fact that mitochondrial condensation,

but not subsequent swelling and degeneration, occurs in cells exposed topically to monensin.

While mitochondrial aberrations are the primary morphologic indicators of monensin poisoning in striated muscle, other aspects of muscle ultrastructure may show deterioration [217] or may remain relatively normal even with gross mitochondrial damage [224]. Generalized fiber and cell degeneration may occur following monensin poisoning [211,217,223]. The extent of monensin-induced injuries appears to be time and dosage dependent. If death occurs shortly after monensin exposure, there may be little or no recognizable evidence of pathological change, at least in liver, kidney, and striated muscle. Generalized necrosis appears to occur most often when monensin is administered over long periods of time. Even with single doses of monensin, structural aberrations in striated muscle develop progressively over several days and then regress if the animal survives the initial insult (unpublished data). We have not observed permanent injury in either striated muscle or liver of monensin-treated rats although such effects have been noted in other animals [211].

Many of the effects on striated muscle attributed to monensin occur also with other ionophores irrespective of their ion specificities [21,244] This implies, again, that Na^+ is not the direct cause of muscle perturbation but, rather, that the ionic imbalance resulting from the intracellular influx of Na^+ triggers other cellular responses that lead to the observed perturbations. Monensin is known to both inhibit and promote Ca^{2+} accumulation in myocytes depending on the absence or availability, respectively, of external Ca^{2+} stores [109,110]; alter Na^+ gradient-dependent Ca^{2+} transfer through the basolateral plasma membranes of rat small intestine [245]; increase myocardial calcium activity [246], inhibit Ca^{2+} accumulation by cardiac microsomes or cause release of accumulated Ca^{2+} stores [24]; and release Ca^{2+} from microsomes [147,247]. Digitalis and other cardiac glycosides (which increase myocardial contractility) appear to act by altering intracellular Na^+ concentration through inhibition of membrane-bound Na^+-, K^+-activated adenosine triphosphatase which secondarily results in an increase in intracellular Ca^{2+} [see 147]. Calcium ionophores such as lasalocid and A23187 have been suggested as potential probes for studying the effects of calcium imbalance on myocardial function [9]. In retrospect, monensin affects the myocardium in much the same way as do the calcium ionophores and, in some instances, to an even greater extent [9]. These observations suggest that the inotropic effects of monensin may be partially indirect; e.g., through release of histamine and endogenous amines [4], or stimulation of synthesis and/or release of prostaglandin [4,25,248]. Thus, Na^+ balance plays an indirect but critical role in modulating myocardial function However, a calcium-independent catecholamine depleting action of monensin in cultured rat pheochromocytoma cells [249] and in bovine adrenal medullary cells and chromaffin granules [250] suggests that monensin may also play a direct role in altering cellular function. Similarly, Sutko and co-workers [251] showed that both monensin and nigericin produce their effects in guinea pig atria by direct action as well as by releasing catecholamines from tissue stores.

Differences in subcellular responses to monensin, between the whole animal and isolated cells or organs, have been noted by us as well as by others [252]. Thus, swelling of Golgi apparatus cisternae observed in cultured cells, tissue slices, and plant roots and stems, is not an aberration characteristic of the cells of animals poisoned by monensin. Lack of Golgi apparatus welling in animals infers that cells from monensin-poisoned animals are bathed in body fluids containing less than 10^{-7} M monensin which is approximately the minimum effective dose of monensin that will cause swelling of Golgi apparatus cisternae in cultured animal cells. Alternatively, lack of vacuolated and/or swollen mitochondria in cultured mammalian cells and plants generally implies that the vacuolated and/or wollen mitochondria observed in striated muscle from monensin-poisoned animals are secondary effects of monensin poisoning, perhaps caused by a metabolite of monensin [150,224]. Alternatively, monensin could affect the synthesis and/or transport of humoral agents (e.g., catacholamines) which, in turn, would alter muscle homeostasis and lead to the mitochondrial aberrations observed in striated muscle.

For plants, at concentrations of 10^{-5} M, monensin was shown to inhibit germination and growth of ryegrass seedlings [97]. The effects were primarily associated with poor root development and significant reduction of root mass as compared to controls Leaf emergence and leaf mass was only slightly affected. At 10^{-4} M monensin, roots often did not emerge from the seed during germination and root mass of seedlings was often near zero. Under these same conditions, shoot mass was reduced about 50% as compared to controls.

V. Summary

Monensin, a monovalent ion-selective ionophore, facilitates the transmembrane exchange of principally sodium ions for protons. The outer surface of the ionophore-ion complex is composed largely of nonpolar hydrocarbon, which imparts a high solubility to the complexes in nonpolar solvents. In biological systems, these complexes are freely soluble in the lipid components of membranes and, presumably, diffuse or shuttle through the membranes from one aqueous membrane interface to the other. The net effect for monensin is a trans-membrane exchange of sodium ions for protons. However, the interaction of an ionophore with biologi-

cal membranes, and its ionophoric expression, is highly dependent on the biochemcial configuration of the membrane itself

One apparent consequence of this exchange is the neutralization of acidic intracellular compartments such as the trans Golgi apparatus cisternae and associated elements, lysosomes, and certain endosomes. This is accompanied by a disruption of trans Golgi apparatus cisternae and of lysosome and acidic endosome function. At the same time, Golgi apparatus cisternae appear to swell, presumably due to osmotic uptake of water resulting from the inward movement of ions

Monensin effects on Golgi apparatus are observed in cells from a wide range of plant and animal species The action of monensin is most often exerted on the trans half of the stacked cisternae, often near the point of exit of secretory vesicles at the trans face of the stacked cisternae, or, especially at low monensin concentrations or short exposure times, near the middle of the stacked cisternae. The effects of monensin are quite rapid in both animal and plant cells; i.e., changes in Golgi apparatus may be observed after only 2–5 min of exposure It is implicit in these observations that the uptake of osmotically active cations is accompanied by a concomitant efflux of H^+ and that a net influx of protons would be required to sustain the ionic exchange long enough to account for the swelling of cisternae observed in electron micrographs.

In the Golgi apparatus, late processing events such as terminal glycosylation and proteolytic cleavages are most susceptible to inhibition by monensin. Yet, many incompletely processed molecules may still be secreted via yet poorly understood mechanisms that appear to bypass the Golgi apparatus

In endocytosis, monensin does not prevent internalization However, intracellular degradation of internalized ligands may be prevented. It is becoming clear that endocytosis involves both acidic and non-acidic compartments and that monensin inhibits those processes that normally occur in acidic compartments.

Thus, monensin, which is capable of collapsing Na^+ and H^+ gradients, has gained wide-spread acceptance as a tool for studying Golgi apparatus function and for localizing and identifying the molecular pathways of subcellular vesicular traffic involving acid compartments. Among its advantages are the low concentrations at which inhibitions are produced (0.01–1.0 μM), a minimum of troublesome side effects (e.g., little or no change of protein synthesis or ATP levels) and a reversible action. Because the affinity of monensin for Na^+ is ten times that for K^+, its nearest competitor, monensin mediates primarily a Na^+-H^+ exchange Monensin has little tendency to bind calcium.

Not only is monensin of importance as an experimental tool, it is of great commercial value as a coccidiostat for poultry and to promote more efficient utilization of feed in cattle The mechanisms by which monensin interact with coccidia and rumen microflora to achieve these benefits are reasonably well documented. However, the interactions between monensin and the tissues of the host animal are not well understood although the severe toxicological manifestations of monensin poisoning are well known Equine species are particularly susceptible to monensin poisoning, and a common effect of monensin poisoning is vacuolization and/or swelling of mitochondria in striated muscle Other pathological injuries to striated muscle, spleen, lung, liver and kidney also have been noted A consistent observation is cardiac myocyte degeneration as well as vacuolization Differences in cellular response resulting from exposure to monensin (i.e., Golgi apparatus swelling in cultured cells, isolated tissues, and plants vs. mitochondrial swelling in animals fed monensin) suggest that myocardial damage is due either to a monensin metabolite or is a secondary response to some other derivation However, as pointed out by Bergen and Bates [26], the underlying mode of action of ionophores is on transmembrane ion fluxes which dissipate cation and proton gradients Consequently, some or all of the observed monensin effects in vivo in animals could be secondary phenomena caused by disruption of normal membrane physiology resulting from altered ion fluxes.

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