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PHARMACOLOGY AND TOXICOLOGY OF THE MONOVALENT CARBOXYLIC IONOPHORES

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INTRODUCTION

The ionophores (Greek *ion,* ion; *phore,* carrier) are defined as compounds which form lipid soluble, dynamically reversible, cation complexes which act as vehicles for transporting ions across biological membranes. The resultant changes in transmembrane ion gradients and electrical potentials often produce profound effects on cellular function and metabolism which underlie the unique pharmacological properties of the ionophores.

History

The biological activity of the ionophores was first recognized through their effects on the metabolism of mitochondria. Valinomycin was reported to be a powerful uncoupler of oxidative phosphorylation (1), whereas nigericin and dianemycin produced an unusual substrate selective inhibition of mitochondrial respiration (2). The uncoupling activity of valinomycin was eventually ascribed to its ability to stimulate the energy dependent uptake of K^+ by mitochondria (3, 4), whereas nigericin promoted the energy independent passive egress of mitochondrial K^+ (2, 5). Ultimately it was recognized that valinomycin and nigericin are the prototypes of two major subclasses of ionophores: (*a*) *neutral ionophores,* such as valinomycin, form *charged complexes* with cations which serve to transport the latter down their *electrochemical* gradient; (*b*) *carboxylic ionophores,* such as nigericin, form *electrically neutral* zwitterionic complexes with cations which serve to promote an electrically neutral *exchange diffusion* of cations, e.g. a Na^+-for-K^+ exchange across membranes (5, 6).

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The neutral ionophores carry charge and are capable of grossly distorting biological membrane and action potentials, which presumably accounts for their high toxicity. The exchange-diffusion transport induced by carboxylic ionophores is less disruptive to biological systems; hence they are better tolerated and generate more interesting alterations of the function of isolated cells, organs, and even intact organisms. Consequently this review will concentrate on the physiological and pharmacological properties of the carboxylic ionophores and more specifically on the *monovalent* carboxylic ionophores.

Mechanism of Ionophore-Mediated Transport

Both ionophore subclasses form complexes by enveloping cations and displacing their solvation shell with strategically placed oxygen atoms of the backbone, which serve as ligands. The limited flexibility of the molecular backbone defines a cavity of a preferred size which favors interaction with ions of a specific ionic radius, hence producing a high degree of ion selectivity (3, 6). The relationship between the affinity and selectivity of cation *complexation* to the kinetics and selectivity of cation *transport* involves multiphasic interconversion between different conformers of a given ionophore and their multiphasic interaction with the transported cation. The ion selectivity of ionophore-cation complexation under equilibrium conditions is reflected, to some degree, in the ion selectivity of dynamic ion transport. A resolution of the principal component reactions of transport for both ionophore subclasses has been detailed in a prior review (7).

For optimal transport kinetics, complexation affinity must be neither too low to favor complexation nor too high to hinder release of the transported ion species. Behavior of the ionophore conformers in the various regions of the polarity continuum of the membrane is also critical (8, 9). Additional complications arise from interaction of the ionophore with the nitrogenous bases of the phospholipids within the membrane (9). These factors have presumably been optimalized for cation transport through natural selection for ionophores produced by microorganisms. Synthetic ionophores reported to date, e.g. the crown polyethers (10), have been designed primarily for strong and selective cation affinities and lack the other molecular attributes required for rapid transport kinetics.

Ionophore Structures

About one hundred natural ionophores have been reported to date. The following description of a select ionophore armamentarium will encompass the range of cation selectivities and transport modes determining their potential as pharmacological agents and usefulness as tools in biology.

The first ionophore in Figure 1, valinomycin (*A*), is a cyclic depsipeptide

with a covalently bonded 36 atom backbone which folds into a necklace 4 Å high and 10 Å wide; six carbonyl oxygens ligand the cation occupying the central cavity diameter (11). It is a neutral ionophore with an equilibrium selectivity for K^+ over Na^+ of about 10,000 to 1 (3, 6).

The nactins [Figure 1 (*B*)] are a series of homologous hydroxyacid esters which form a macrotetralide with a covalently bonded 32 atom backbone which folds into a configuration like the seam of a tennis ball (12). Four carbonyl and heterocyclic ether oxygens deployed at the apices of a cube ligand the cation. K^+: Na^+ selectivity is lower than that of valinomycin; the higher homologs have higher transport turnover numbers and lower K^+: Na^+ selectivities (13).

The crown polyether, dicyclohexyl-18-crown-6 [Figure 1 (*C*)], is the prototype of a series of totally synthetic ionophores containing an 18 atom covalently bonded ring of ether oxygens separated by carbon atoms (10). These ionophores are usually devoid of asymmetric centers and molecular chirality, and complexed cations are not enveloped as snugly as the natural

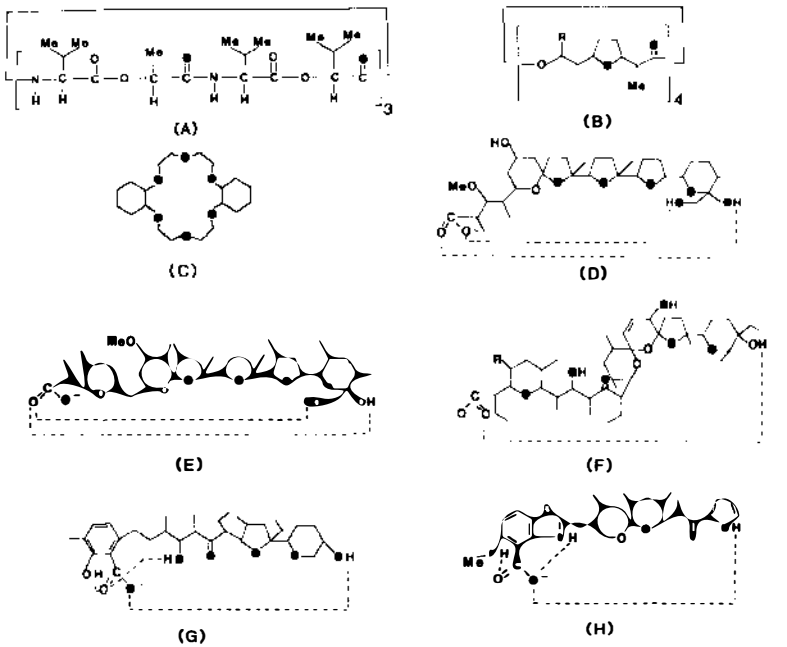

Figure 1 Structures of representative ionophores. (*A*) Valinomycin, (*B*) Macrotetralide Nactins. In the series nonactin, monactin, dinactin, trinactin, tetranactin the R groups change progressively from four methyl groups to four ethyl groups. (*C*) Dicyclohexyl-18-crown-6, (*D*) Monensin, (*E*) Nigericin, (*F*) R=H, Salinmycin; R=Methyl, Narasin; (*G*) Lasalocid A, (*H*) A23187; the filled-in oxygen atoms are indicated by x-ray crystallography to be the principal liganding atoms.

ionophores (1). An exception is the dinaphthyl crown ethers, which have a high degree of gross molecular chirality and have been used to resolve racemic amines (14). In general they have poorer cation affinities, transport turnover numbers, and ion selectivities than do their natural counterparts (6). They are of less interest for their biological effects than for their use as catalysts for organic synthesis, where their high chemical stability is an advantage.

Monensin [Figure 1 (*D*)] is a typical carboxylic ionophore with a quasi-linear array of heterocyclic rings. The chirality of the molecule is such that it is inclined to fold into a 24 atom ring stabilized by head to tail hydrogen bonding. The liganding oxygens are staggered and form more of a cage than a planar girdle about the complexed cation (15). It readily forms complexes only in the ionized form which obtains at physiological pH and above. The electrostatic neutralization of the cation by the carboxylate determines that monensin transports in the exchange diffusion mode even though the charged carboxylate is distal to the cation and is not involved in a true ionic bond. Monensin exhibits an affinity preference for Na^+ over K^+ of about 10 (6).

Nigericin [Figure 1 (*E*)] is closely related to monensin in structure except at the carboxylate end, where an additional ring occurs. The chirality of its 25 atom backbone is such that the carboxylate impinges on the central cavity, forming a true ionic bond with the complexed cation (16, 17). The conformational difference from monensin is such that the K^+:Na^+ affinity is inverted, nigericin preferring K^+ over Na^+ by a factor of 100 (6). Although the kinetic selectivities observed for ionophore-mediated K^+-for-Na^+ exchange in erythrocytes is not as extreme as its equilibrium affinities, the Na^+ over K^+ preference for monensin, and the K^+ over Na^+ preference for nigericin, are clearly evident (18).

Lasalocid (X-537, RO 2-2985) [Figure 1 (*F*)] is smaller than the previous two ionophores and its backbone contains but 20 atoms (19, 20). The carboxyl is attached to an aromatic ring which provides both a fluorophore (21), useful as an environmental probe, and a chromophore which, together with the ketonic carbonyl, can serve as circular dichroic probes for molecular chirality (9). Because of its inherent tendency to dimerize (19), it can form electrically neutral complexes with biologically important divalent ions such as Ca^{2+} and Mg^{2+} (22). Lasalocid has a wide range of complexation affinities and transport capabilities, encompassing not only inorganic polyvalent and alkali ions but also primary amines such as catecholamines (22). Despite the extensive use of lasalocid made since its introduction as a Ca^{2+} ionophore, we would advise against its continued use as a definitive tool for perturbing biological systems. The extent to which selective divalent or monovalent carboxylic ionophores produce more or less similar effects on various biological systems has mechanistic significance. Unambiguous

interpretation of the specific effects of an ionophore with the broad specificity of lasalocid is difficult.

Salinomycin [Figure 1 (*G*) (23)] and its methyl homolog, narasin (24), have been selected from the remaining monovalent selective ionophores because of their potential development as agricultural feed supplements and drugs. They are distinguished by the occurrence of three consecutive rings fused through two spirane carbons. Their $K^+ : Na^+$ selectivities generally lie between those of monensin and nigericin (25).

A23187 [Figure 1 (*H*)] is unique in its content of heterocyclic rings containing nitrogen (26). Like lasalocid it has aromatic moieties which give rise to chromophores and a fluorophore (21). Its fluorescent properties have been used extensively to characterize its environmental transitions within biological and artificial membranes (27). It is often referred to as a divalent ionophore transporting Ca^{2+} much more efficiently than either Na^+ or K^+ (28). It can complex Li^+ reasonably well and Na^+ weakly (28), and we find it also complexes trivalent La^{3+} and tetravalent Hf^{4+}. Perhaps it would be best described as having a preference for high charge density cations which would encompass all polyvalent cations and the smaller alkali ions. This can be explained as a consequence of the occurrence of nitrogen atom ligands which require cations of higher charge densities than do oxygen atoms to form strong ion-induced dipole bonds. A23187 has been used extensively in biological studies because of its selective ability to permit the metabolic trigger ion, Ca^{2+}, to enter cells and raise cytosolic Ca^{2+} activity. Extensive reviews of its effects on tissues and cells have recently been compiled by Reed (29, 30).

Ionomycin is a more recently introduced divalent, i.e. Ca^{2+} selective ionophore. This property presumably arises from its possession of a second anionic moiety, an enol group, which, together with the head carboxylic group, enables ionomycin to form 1 : 1 electrically neutral complexes with divalent ions (31). Its biological applications will presumably be similar to those of A23187. For those readers who wish to pursue the chemical, physical, and biological properties of divalent carboxylic ionophores in further depth, several reviews are recommended (7, 29, 30, 32–35a).

Equilibria of Ionophore-Mediated Transport

One property of carboxylic ionophores which is seldom emphasized is their ability, at physiological pH, to transport protons in the form of their undissociated free carboxylic acids. Given enough time the carboxylic ionophores will establish the equilibrium for all ions they can transport appreciably:

$$\frac{[M^+]_{in}}{[M^+]_{out}} = \frac{[N^+]_{in}}{[N^+]_{out}} = \frac{[X^{n+}]_{in}^{1/n}}{[X^{n+}]_{out}^{1/n}} = \frac{[H^+]_{in}}{[H^+]_{out}}.$$

For M^+ and N^+ in biology the obviously important candidates are K^+ and Na^+ and with some ionophores catecholammonium ions and other nitrogenous bases. In general, with the prevailing transcellular gradients, the alkali ion competent ionophores will promote an intracellular ingress of Na^+ in exchange for an equivalent egress of K^+. For X^{n+} the obvious candidates are Ca^{2+} and Mg^{2+}. Since the Mg^{2+} gradients in tissues are close to one, i.e. $\sim$ 5 mM $[Mg^{2+}]_{in}$ and $\sim$ 5 mM $[Mg^{2+}]_{out}$ metabolically significant changes in intracellular $[Mg^{2+}]$ are unlikely, hence the biologically significant divalent movement is the entry of Ca^{2+} into the cell thermodynamically propelled by an activity gradient of $\sim 10^5$. It should be borne in mind that the intracellular rise in Ca^{2+} is not easily predicted from the quantity of Ca^{2+} entering the cell; much of the Ca^{2+} initially present is sequestered and the entering Ca^{2+} will be likewise sequestered to some extent by existing intracellular devices. Alternatively, a Ca^{2+} ionophore could also raise cytosolic intracellular Ca^{2+} activity by releasing intracellular Ca^{2+} from a membrane-enclosed sequestered source such as the mitochondria.

The above equilibrium expression contains no terms for the ion selectivity of a given ionophore. However, a given carboxylic ionophore not only has characteristically different propensities for transporting different alkali cations, e.g. Na^+ and K^+, but also for transporting H^+ (18, 35). This becomes important in describing the kinetics of carboxylic ionophore-mediated transport. For example, monensin promotes a faster movement of extracellular Na^+ into erythrocytes than an egress of intracellular K^+. In order to preserve electroneutrality a transient egress of H^+ occurs, temporarily raising the pH of the erythrocyte interior until the K^+ egress can catch up with the Na^+ uptake. The converse obtains with nigericin which produces a transient lowering of the pH of the erythrocyte. Even though on gross analysis the shifts in K^+ and Na^+ will balance, the pH effects, although transient, may have metabolic consequences that should not be overlooked. Our selected ionophore armamentarium contains one monovalent ionophore, monensin, which produces a transient intracellular pH rise; one, nigericin, which produces a pH drop; and one, salinomycin, which produces negligible intracellular pH transients because it transports K^+ and Na^+ at almost equal rates (18).

CARDIOVASCULAR EFFECTS OF IONOPHORES

Our early studies of the complexation affinity spectrum of ionophores failed to uncover any which could transport the physiologically important cation Ca^{2+}. In 1970, in the course of investigations of the structure of lasalocid, Paul and his coworkers described a well-defined crystalline barium complex of lasalocid (19). This prompted us to examine the ability of this ionophore

to complex and transport Ca^{2+} in model systems, which indeed it did (22). This, in turn, suggested that in view of the important role of Ca^{2+} in the myocardial contractile cycle, the cardiovascular system might show interesting responses to lasalocid. We did confirm that lasalocid produced definite positive inotropic and chronotropic effects on the isolated perfused rabbit heart (22), which led to experiments on the instrumented anesthetized dog (36).

The response of the intact dog to lasalocid is considerably greater than that of the isolated rabbit heart. Graded responses of the contractility index, LV dP/dt, were measured from 0–2 mg/kg i.v. lasalocid; 2 mg/kg more than tripled the control values and a half maximal response was obtained at 0.75 mg/kg. At this dose, heart rate remained constant, aortic systolic and diastolic pressure rose, as did cardiac output, while left ventricular diastolic pressure and peripheral resistance fell. The effects persisted as long as 4 hours (37).

Despite the increase in cardiac work induced by lasalocid, blood withdrawn from the coronary sinus showed a spectacular fourfold increase in oxygen content. This implied that coronary flow increased disproportionately greater than total cardiac output, which was confirmed by direct measurement with an electro-magnetic flow probe. Increases in myocardial oxygen consumption were remarkably small (37).

Despite our initial premise that the cardiovascular properties of lasalocid arise from its ability to transport the pharmacologically active cations Ca^{2+} and the catecholamines, we found that other selectively monovalent carboxylic ionophores, devoid of this capacity, are more potent in evoking similar cardiovascular effects. Included in this list are nigericin, dianemycin, monensin, X-206 (Roche), and A-204 (Lilly) in order of increasing potency (37). The list has subsequently been extended to include the monovalent specific carboxylic ionophores salinomycin (38, 39), grisorixin (40), and alborixin (40). The indirect involvement of catecholamines in the cardiovascular properties of all carboxylic ionophores is implicated, since their effects were partially inhibited by propranolol or practalol (36, 37).

Schwartz et al (41) and Osborne et al (42) corroborated, in the main, the basic hemodynamic response pattern obtained by our laboratory with anesthetized dogs. The Schwartz group further claims to have refuted an uncited report from our laboratory that "reserpinized preparations respond like controls"; however, no reference is provided (41). We are unaware of any such published report from our laboratory, nor is it consistent with our early published observation of partial inhibition of the hemodynamic effects of lasalocid by β-adrenergic blockers (36, 37). The dose of reserpine utilized by Schwartz et al, 220 mg/dog (7–10 days), is sufficient to produce severe deleterious effects, e.g. weakness, severe diarrhea, so that it is not possible

to be certain whether the lack of subsequent response to lasalocid (no data given) was due to depletion of catecholamines or to the generally impaired state of the dog (43). Furthermore, their report of no adverse effects at 5.5 mg/kg lasalocid is not consistent with the premature ventricular contractions we usually observed above 2 mg/kg. Their report that lasalocid restored to normal values (125 mm Hg) the mean arterial pressure of two dogs which had been placed in a state of shock by "exteriorizing" their intestines (43) suggests that ionophores might be useful in treating shock.

These observations were extended in subsequent papers demonstrating that lasalocid produced equivalent hemodynamic effects in conscious chronically instrumented dogs (43, 44). Significant drops in regional resistance in the renal and iliac arteries were found (43), calling into question the earlier claim of lack of significant peripheral resistance responses (41).

Ionophores and Shock

DeGuzman & Pressman reported that two dogs in cardiogenic shock induced by ligating a lower branch of the left anterior descending coronary artery were restored to their preshock hemodynamic state by 1 mg/kg lasalocid (37). Lasalocid sustained the pressure of 10 dogs in a defined state of hemorrhagic shock (mean aortic pressure 40–50 mm Hg) while only one of eight animals so treated survived without lasalocid administration (44). Ten dogs in shock produced by commercial *E. coli* endotoxin (Difco) and treated with 0.15 mg/kg salinomycin survived for 4 hours and had their hemodynamic parameters restored towards normal, whereas three of ten endotoxin-treated dogs failed to survive without the ionophore (39).

McCaig & Parratt reported that monensin was not effective in reversing endotoxic shock in five cats (45); however, they observed that 4 of 11 control cats failed to respond to a relatively high dose of monensin (0.25 mg/kg). In our laboratory six cats, including two with induced right heart failure, all responded to monensin, although the responses were less than those usually seen in dogs. We also obtained typical hemodynamic responses to monensin in conscious dogs and rabbits, anesthesized sheep, and one pig. This pattern suggests that marked species differences in responses to monensin are unlikely and we feel that some procedural difficulty might have occurred in administering monensin to the refractory cats.

Ionophores and Coronary Flow

The effects of the carboxylic ionophores on coronary flow in intact animals appear to be separate and distinct from their effects on the myocardium. Coronary flow rises prior to the manifestation of contractility responses, peaking about 10 min after administration of lasalocid and then subsiding (37, 41). The resolution of the coronary and myocardial effects of the

carboxylic ionophores is more distinct in the case of monovalent ionophores, e.g. monensin (Figure 2) and salinomycin. With these ionophores the coronary response subsides considerably after peaking about 10 min after ionophore injection, whereas LV dP/dt peaks at 15 min and plateaus. At lower doses of monensin, 2–25 μg/kg, its effect is almost entirely coronary selective and in the absence of an increase in aortic pressure, cardiac output and LV dP/dt, the increase in coronary flow obviously must be due entirely to a drop in coronary resistance (37, 46–48). In contradistinction to the myocardial effects of carboxylic ionophores, the coronary responses are not inhibited by β-adrenergic blockers, e.g. propranolol (37, 47). Labeled microsphere distribution indicated that monensin-induced increase in coronary flow is divided approximately equally between the epicardium and endocardium (49). The increased coronary flow did not improve the delivery of blood to a localized ischemic zone produced by partial occlusion of the left anterior descending coronary artery (49). Salinomycin, on the other hand, was reported to improve the ratio of endocardial to epicardial blood flow (50).

Role of Catecholamines in Effects of Ionophores

Although the inhibition of the contractile stimulation of ionophores by β-adrenergic blockade clearly implicates an ionophore-mediated release of catecholamines, the level of plasma catecholamines released by salinomycin reaches only one half the level required for an equivalent stimulation of

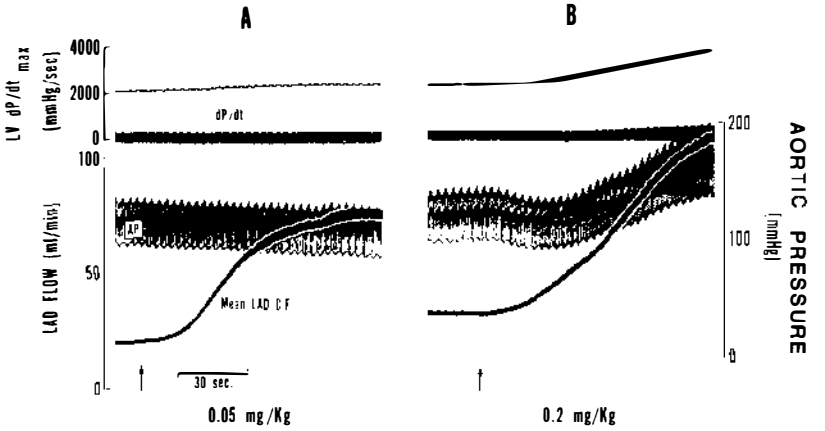

Figure 2 Effect

Contractility (LV dP/dt max) and aortic pressure (AP) were unaffected, whereas mean left anterior descending coronary flow (LAD C.F.) began rising within 10 sec and reached fourfold control value within 2 min and AP rose after a transient drop. Despite the initial fall in AP, coronary flow rose almost immediately due to a drop in coronary resistance.

contractility by direct infusion of epinephrine. Due to the modest stimulation of ventricular oxygen consumption by salinomycin, the mechanical efficiency of left ventricular hydraulic work was doubled compared to control values, an effect not produced by catecholamines alone (51). Similar increases in hydraulic work efficiency have been obtained with lasalocid (37) and monensin (49).

The responses of the intact animal to carboxylic ionophores have their counterparts in their effect on organ and subcellular preparations. The inference that lasalocid and A23187 can transport Ca^{2+} across biological membranes was verified by their ability to release Ca^{2+} from a preparation of loaded vesicles derived from the sarcoplasmic reticulum (21, 52, 53).

Our initial observations of the inotropic effects of lasalocid on the perfused guinea pig heart were extended to perfused rat heart by Schaffer et al (54). They also showed that lasalocid would restore the contractility of a heart inhibited by low Ca^{2+} (0.1 mM) in the perfusion fluid or whose access to extracellular Ca^{2+} was blocked by Ruthenium Red. In retrospect it is surprising that in these experiments not only heart work, but also coronary flow, was inhibited by reserpine and propranolol, since coronary dilatation does not appear to be mediated through catecholamines. Smaller increases of work and coronary flow were obtained with A23187. The results were interpreted entirely in terms of the ability of A23187 and lasalocid to mediate a direct transport of Ca^{2+} across membranes, coupled with the ability of lasalocid to transport catecholamines (54).

Levy et al reported that lasalocid also stimulated the contractility of a striated muscle preparation, with or without electrical stimulation. They also reported that whereas low levels of lasalocid (4.6 μM) stimulated the contractility of the electrically driven isolated rabbit atrium, higher levels (13.8 μM) induced a rise in resting tension, i.e. contracture (55). Comparable results were obtained with electrically driven human atrial appendage (56).

Role of Na^+ in Effects of Ionophores

The earlier reports on the biological activity of lasalocid, in the main, presumed its effects to be related to its ability to transport Ca^{2+} and catecholamines (22, 41, 54). Recognition of the ability of the selectively monovalent ionophores to produce comparable cardiovascular effects at even lower concentrations brought this assumption into question and implicated a rise in intracellular Na^+ as the pivotal process (7, 37). Devore & Nastuk (7) came to a similar conclusion based on the ability of lasalocid to reduce the resting potential of frog cutaneous pectoris muscle and the hyperpolarization of ionophore-treated muscle by replacing Na^+ in the medium with choline$^+$. Tetrodotoxin did not block the effect, indicating that the ionophore was the vehicle for Na^+ ingress rather than the voltage-

dependent Na^+ channels. Although in retrospect we concur with their conclusions, their reasoning does not seem rigorous. The resting potential is usually assumed to be dominated by the K^+ diffusion potential. Ingress of Na^+ via exchange-diffusion, in and of itself, would not affect the membrane resting potential markedly were it not for the fact that it would be coupled to an equivalent egress of K^+. They also noted a reduction of the amplitude of induced action potentials which do depend primarily on the Na^+ diffusion potential, although the latter relationship is not discussed in detail (57).

Meier et al compared the effects of monensin, lasalocid, and A23187 on the action potential of canine Purkinje fibers. From the alteration of the action potentials interpreted by phase plane analysis it was concluded that monensin and lasalocid both increase intracellular Na^+ but A23187 does not (58). Sutko et al confirmed the shortening of the Purkinje fiber action potential by monensin and extended these findings to nigericin. They also observed a 9 mV drop in the resting potential with nigericin but not with monensin (59). Gelles, on the other hand, reported that lasalocid hyperpolarizes the resting potential of canine Purkinje fibers (60).

Bassett et al found that a 20 min incubation of cat ventricular muscle with 10^{-6} M monensin increased subsequent K^+-induced contracture, even in the presence of α- and/or β-adrenergic blockade. This effect required Na^+ in the incubation medium and was interpreted in terms of increased intracellular Na^+ increasing the intracellular Ca^{2+} available to the contractile proteins upon depolarization (61). The ability of the ionophore-mediated rise in intracellular Na^+ to increase the availability of intracellular Ca^{2+} is also implicated by the evidence that progressively increasing concentrations of monensin first stimulate the contractility of the perfused spontaneously beating rabbit heart and eventually cause contracture (62).

Biological Interaction between Na^+ and Ca^{2+}

Ca^{2+}, or its complex with the ubiquitous calmodulin, is the immediate activator for a number of intracellular processes. Among these are exocytosis of storage granules, contractility, secretion, lymphocyte capping, and the events triggered by fertilization of eggs. In general, activatable cells are quiescent at 10^{-7} M Ca^{2+} activity and are stimulated to express their specialized functions in graded fashion as Ca^{2+} activity rises through 10^{-6} M to 10^{-5}–10^{-4} M. One source of the triggering Ca^{2+} is the relatively high level of extracellular Ca^{2+} ($> 10^{-3}$ M) which needs only an increase in membrane permeability to rush into the cell. An alternate source of Ca^{2+} for activation exists within the cell itself since most cells contain $> 10^{-3}$ M gross chemical Ca^{2+}, maintained at a free or activity level of 10^{-7} M or less by virtue of being sequestered into various pools (63).

The biological synergism between Ca^{2+} and Na^+ has long been empirically recognized but it is only lately that insight into the relationship has been gained. Some of these mechanisms have recently been reviewed (63). A rational basis now exists for an intracellular rise in Na^+ making Ca^{2+} more available to intracellular receptors, e.g. calmodulin and troponin C, either by displacing intracellularly sequestered Ca^{2+} (64) or by bringing extracellular Ca^{2+} into the cell via an exchange diffusion carrier, the so-called "Baker pump" (65). A good precedent for an increase in intracellular Na^+ mobilizing intracellular Ca^{2+} is the mechanism for the inotropic effect of the cardiac glycosides, which is generally attributed to a rise in intracellular Na^+ due to inhibition of the Na^+ pump (66).

Ca^{2+} specific carboxylic ionophores, A23187 or ionomycin, can mimic physiological Ca^{2+}-mediated cell activation by increasing membrane permeability to Ca^{2+}. If extracellular Ca^{2+} is required for activation, presumably the cell membrane would be the critical target for the ionophore. If, on the other hand, extracellular Ca^{2+} is not required, as in activation of the sea urchin egg (67, 68), the presumptive ionophore target would more likely be membranes surrounding intracellular Ca^{2+} pools, e.g. the mitochondria or sarcoplasmic reticulum.

Cell activation by monovalent ionophores can be attributed to their ability to promote a one-for-one entry of Na^+ in exchange for intracellular K^+. Since intracellular Na^+ activities are low, about 10 mM, a one-for-one, thermodynamically spontaneous exchange of intracellular K^+ for extracellular Na^+ would cause a greater relative increase in intracellular Na^+ than decrease in intracellular K^+. This implies that the rise in intracellular Na^+ is apt to be more physiologically significant than the drop in K^+. As explained above, the rise in intracellular Na^+ would induce a rise in intracellular Ca^{2+} activity, hence cell activation. In the case of the adrenal cell the release of catecholamines by monensin does not require extracellular Ca^{2+}; hence in this case the source of Ca^{2+} for activation of secretion is intracellular (69).

Side Effects of High Concentrations of Ionophores

The majority of reports of cell activation by A23187 require 10^{-6}–10^{-5} M ionophore (29, 30), whereas monensin acts in the range 10^{-8}–10^{-6} M, e.g. adrenal cells (69) and beating heart cells (70). Monensin, an ionophore which raises intracellular Ca^{2+} indirectly is often considerably more potent in activating cells than the Ca^{2+} selective ionophore A23187. Indeed, monensin is capable of producing strikingly different effects in the 10^{-6}–10^{-5} M range than it does in its lower ranges. Table 1 illustrates the stimulation of lymphocyte capping by different monensin levels. Capping, which we presume is a function of Ca^{2+} excitation of the lymphocyte cytoskeleton,

is maximal at 10^{-8} M monensin and is less apparent at 10^{-7} M. At 10^{-6} M monensin it is inhibited below control values and even more so at 10^{-5} M. We interpret this as signifying that 10^{-8} M monensin is capable of fully activating the cells, and that higher ionophore levels cause surface membrane damage which results in inhibition of the capping process. The only effect previously reported in lymphocytes with A23187, occurring in the range of 10^{-6}–10^{-5} M, is an inhibition of capping (71).

We believe the ease with which increased cytosolic Na^+ releases enough Ca^{2+} to activate cells has definite physiological significance. Although we stop short of suggesting that physiological cell activators actually work by triggering a sudden inrush of Na^+ per se, other control factors, e.g. hormones, may cause long-term alterations in intracellular Na^+ thereby providing bias for modulating the extent to which a given stimulus raises intracellular Ca^{2+} activity.

All naturally occurring ionophores must have comparable intermediate polarity in order to be compatible with both the polar region of biological membranes at which complexation and decomplexation take place, and the low polarity membrane interior traversed by the complex. This assures that all ionophores are amphipathic and have a certain propensity to affect biological membranes by virtue of their detergency, particularly at higher concentrations. Thus the high concentrations of A23187 usually employed in the literature to activate cells may mask the true picture of the events triggered by a specific rise in cytosolic Ca^{2+} activity by altering the structure and integrity of the cell membrane. Even when Ca^{2+} activation is achieved, Ca^{2+} may enter the cell through a damaged plasma membrane rather than through the ionophore-mediated transport.

Table 1 Effect of monensin on lymphocyte receptor capping[a]

Monensin	Percent capped cells[b]
Control	37.2
5×10^{-9} M	31.0
5×10^{-8} M	73.0
5×10^{-7} M	53.0
5×10^{-6} M	17.4
5×10^{-5} M	4.0

[a] Cultured mouse T-lymphoma cells were exposed to monensin in RPMI 1640 medium at room temperature for 30 min followed by monoclonal rat antibodies against Thy-1 (15 min, 37°) and rabbit antirat immunoglobulin (15 min, 37°). Cells were then fixed with 2% paraformaldehyde and stained with fluorescence-conjugated goat antirabbit immunoglobulin for microscopic examination. (Unpublished observations of L. Bourguignon and B. C. Pressman.)

[b] Standard deviation ± 5%.

Mechanism of Action of Ionophores on Coronary Flow

If ionophore-augmented cytosolic Ca^{2+} activity induces or strengthens contractility, how then can the coronary vasodilatory properties of the carboxylic ionophores be explained? The hypothesis we favor is that the ionophores stimulate the release of humoral substances from various cell types of the coronary vessel which activate relaxation of the smooth muscle cells. Pressman & Lattanzio have reported the stimulated release of adenosine into the effluent of the perfused rabbit heart, which could stimulate the adenosinergic relaxation receptors of the coronary vessels (72). More recently we have also observed a partial inhibition of the ionophore-mediated release of labeled arachidonate metabolites from perfused hearts loaded with labeled arachidonate, as well as the partial inhibition of salinomycin-induced coronary vasodilatation by the prostaglandin synthetase inhibitor indomethacin. This supports the likelihood that carboxylic ionophores act at least in part by stimulating the release of prostaglandins (73). Prostacyclin is a known smooth muscle relaxant (74) and its production could be initiated by a rise in cytosolic Ca^{2+} activity, which would stimulate activation of phospholipase A_2, which in turn would release arachidonic acid from phospholipids. This mechanism is suggested by the report of Knapp et al who have observed stimulation of prostaglandin release by A23187 from kidney minces (75). Thus adenosine and prostacyclin are both candidate agents for mediating the carboxylic ionophore-induced relaxation of coronary smooth muscle.

Occurrence of Ionophores in Higher Organisms

The prostaglandins themselves have also been reported to act as ionophores, as have a prostaglandin polymer, PGBx (76), various fatty acids, and even phosphatidic acid (77, 78). We are inclined to be skeptical of these reports, since one would predict that lipophilic acids could ion pair with cations and render them lipid soluble. Thus the ability of a given metabolite to form lipid soluble complexes to some extent with cations is not determinative for an ionophore, nor is marginal capacity for transporting ions across lipid barriers. Such properties of putative ionophores must be quantitatively comparable with those of authentic ionophores in order to be considered significant. Lipids and their oxygenated derivatives lack the backbone rigidity of true ionophores and hence cannot form well-defined complexation cages, nor do they flip-flop between limited conformational options in the manner required to facilitate translocation of cations across membranes (79). Ion complexation with true ionophores is also facilitated by the presence of strategically situated oxygen atoms in the chain which permit multidentate chelation.

In the liposomal Ca^{2+} transport system used by Serhan et al PGBx and phosphatidic acid appear to have about one half the transport activity of ionomycin and A23187; however, their transport was compared over a 5 min interval vs 10 s for the ionophores (77). Normalized activity for the test agents would appear to be about one fiftieth that of the ionophores. The activity of polyenic acids, e.g. linoleic and linolenic, depended on prior auto-oxidation, which we assume occurred during isolation; reduction of these products with $SnCl_2$ destroyed their inophorous activity (77). Blondin reported the extraction of lipoidal inophores from mitochondria, but these fatty acid oxidation products had low activity compared to authentic ionophores of microbiological origin and the complicated extraction procedure provided ample opportunity for a chemical transformation of natural polyenic lipids favorable for ion pairing to cations (80).

In general we doubt whether true ionophores occur in higher organisms. If they did they would be able to equilibrate between various biological membranes, imparting to each similar permeabilities regardless of their function within the cell. Shamoo and his collaborators have also claimed to have isolated ionophorus proteins from biological systems high in transport activity, e.g. mitochondria and Na^+-K^+ ATPase; however, it is difficult to understand how they could possibly be mobile carriers in their native state since they were obtained from digests of macromolecular material (81, 82). The principal criteria they cited for ionophores, cation binding, and the ability to cause electrical conductivity changes in black lipid membranes, in our opinion, are not definitive for mobile carriers, which the ionophores are by definition.

Effects of Ionophores on Other Organs and Cells

The recent reviews by Reed encompass many papers describing cell activation by the Ca^{2+} ionophores A23187 and lasalocid (29, 30). In the present review we have chosen to focus on the monovalent carboxylic ionophores which activate cells indirectly by raising cytosolic Na^+, since this effect is more selective and of greater pharmacological potential. We believe that these ionophores will ultimately be found capable of producing many, but not all, of the effects ascribed to A23187. As previously pointed out, the ability of lasalocid to transport both Ca^{2+} and Na^+ effectively complicates determination of which of these ions is primarily responsible for the many effects ascribed to this ionophore, hence we have chosen not to dwell on the extensive lasalocid literature in this review. We refer the reader to Reed for a comprehensive compendium of lasalocid effects (29, 30).

Monensin has already been shown to duplicate the earlier observed effects of A23187 and lasalocid in releasing amylase from mouse parotid acinar cells (83). The effect was associated with enhanced $^{22}Na^+$ uptake and net

Ca^{2+} efflux indicative of a rise in intracellular Ca^{2+} activity derived from intracellular Ca^{2+} stores. Knapp et al found that monensin and nigericin mimic A23187 in releasing prostaglandins from renal medullary preparations. The release is dependent on Ca^{2+} being present in the medium. On the other hand, this group found monensin and nigericin incapable of substituting for A23187 in stimulating prostaglandin production by platelets (75). Monensin also stimulates catecholamine secretion from cultured chromaffin (69) and pheochromocytoma (84) cells. Feinstein et al found that monensin and nigericin release serotonin from human platelets. However, they ascribe the effect directly to the reduction of the transcellular Na^+ gradient rather than to a Ca^{2+}-dependent mechanism (85).

On the other hand, 10^{-7} M monensin and nigericin inhibit the secretion of procollagen and fibronectin from cultured human fibroblasts and the effect is not obtained with A23187. The inhibition is associated with morphological abnormalities in the Golgi system (86). A similar inhibition of the secretion of immunoglobins (87) and acetylcholinesterase (88) has been reported. Although we have concluded that the monovalent ionophores can stimulate Ca^{2+}-dependent exocytotic release of secretory granule products, these authors deduce from morphological observations that the monovalent carboxylic ionophores also interfere with the packaging of such granules by the Golgi system. Tartakoff & Vassalli (89) have also concluded that monensin inhibits secretion from pancreatic acinar cells by impeding protein transit from the Golgi system to the storage granules; monensin also inhibits the Golgi system of fibroblasts (89). Monensin (90), salinomycin (91), and narasin (92) all produce morphological damage to coccids, which may underlie the economically important coccidiocidal effects of the entire class of carboxylic ionophores.

USE OF IONOPHORES AS LIVESTOCK FEED ADDITIVES

Monensin has been widely employed for a considerable time as a feed additive to control coccidiosis, which is endemic in poultry (93–95). More recently it has achieved similar widespread usage in cattle feed, where it increases the efficiency of conversion of feed to meat (96–99). The latter activity may arise from the ability of the ionophore to shift rumin fermentation toward the more energetically efficient propionate pathway away from the less efficient acetate-butyrate pathway, which is associated with the release of calorically significant amounts of methane (100–102).

The effect of monensin against poultry coccidiosis is shared by other

carboxylic ionophores including lasalocid, salinomycin, narasin, dianemycin, and A-204 (95, 103), and we would presume it is a general property of monovalent carboxylic ionophores along with their cardiovascular effects. The practice of feeding farm animals ionophores takes on particular significance in that it ultimately introduces powerful pharmacological agents into man's food supply.

The level of monensin customarily fed to cattle and poultry are 30 and 120 ppm, respectively. Reasonable estimates for daily feed consumption would be 2% and 6% body weight, respectively, per animal (104); hence the net daily through put of monensin/kg would be considerably higher in the chicken (about 7000 μg/kg) than in cattle (about 600 μg/kg). To provide a frame of reference for evaluating these figures, it should be borne in mind that as little as 2 μg/kg injected into a dog is sufficient to produce a marked dilatation of the coronary arteries (48). If monensin is absorbed appreciably from the digestive tract as we have confirmed in the dog, rabbit, and sheep, and appreciable residues of it or its active metabolites remain in the tissues, it is possible that man is presently ingesting pharmacologically significant levels of ionophore in his meat supply, particularly poultry.

Metabolic Fate of Ionophores

Herberg & Van Duyn reported that chickens fed monensin tritiated by the Wilzbach procedure retained about 2 ppm monensin equivalents of radioactivity in muscle (lean) and about 7.7 ppm in the liver. These values decay to approximately 1 and 1.5 ppm, respectively, after 48 hours and to 0.7 and 0.7 ppm after 96 hours. The majority of the radioactivity recovered after 48 hours was distillable as water, however most of it paradoxically was excreted in the feces rather than in the urine (105). The appearance of the tritium as water in the feces was unexplained.

Later studies with ^{14}C-labeled monensin in the steer and rat indicated that virtually all the radioactivity was excreted in the feces, although it was converted into several metabolites (106). Donoho & Amundson call attention to the difficulty of establishing "what is the safety of metabolites found in edible tissues," especially when it is impossible to obtain a sufficient quantity for testing (107). However, the majority of the metabolites found gave rise to a mass spectrographic fragment of m/e 23 (105), i.e. they bound $^{23}Na^+$ tightly, which to us implies that they were putative ionophores. The structures of compounds M1-M5 of Figure 2 of Reference 106 all appear to be candidate ionophores; if we assume these are likely to resemble the chick monensin metabolites, they leave in question the origin of the large amounts of tritiated water reported in Reference 105.

The ^{14}C-labeled monensin fed to steers by Herberg et al (108) was found to accumulate in the liver to the extent of 0.59 ppm. No comparable levels

were found in other tissues, however the wide range of radioactivity (5.5–26 dpm) found in control tissues from animals not fed labeled monensin is unusual.

Monensin Assays

A special "bioautographic" assay has been developed for the routine determination of monensin residues in meat (109, 110). After preliminary purification, methanol extracts of tissue are chromatographed on thin-layer chromatographic (TLC) silica gel plates. Agar, inoculated with a selected strain of *B. subtilis,* is poured over the plates and the monensin estimated from the zone of bacterial growth inhibition. Although standards were added to the original tissue methanol extracts by Donoho & Kline (109), in the variation of the method described by Okada et al, the standards appear to have been applied directly to the TLC plates with a control tissue extract (110). Donoho & Kline claim an assay sensitivity of 0.025–0.05 μg/g and that the method is primarily reliable for determining the presence or absence of ionophores at low levels rather than for their quantitative determination (109). Okada et al claim a detection level as low as 0.0125 ppm, although they include tissue values as low as 0.006 μg/g in their tabulated data (110).

We have developed a purely chemical assay for monensin in acetic acid-methanol extracts of tissues based on its ability to form lipid soluble complexes with $^{22}Na^+$ (111, 112). Variations of this radiochemical assay can be used for the determination of lasalocid and salinomycin, and presumably it would work with most if not all other carboxylic ionophores. Upon calibrating the rise in $^{22}Na^+$ complexation activity in tissue extracts of monensin-fed chickens, our procedure indicates a much larger level of monensin tissue residues, in the neighborhood of 0.5–1 ppm, than does the bioautographic procedure (111). We have subsequently found that chromatography of the concentrated tissue extracts on fluorosil leaves only a fraction of the original activity detectable by the radiochemical procedure and much of the positive reacting material remains at the origin. Since our procedure is versatile enough to permit the assay of monensin standards at various stages of the tissue extraction and concentration procedure without the removal of solvent required by the microbiological step of the bioautographic assay, we have been able to deduce that monensin is subject to chemical transformation when concentrated in the presence of tissue lipids, possibly by free radicals arising from auto-oxidation of polyenic acids. Such products, if they retain their ability to transport Na^+ across biological membranes, would be expected both to show cardiovascular activity and to be detectable by the radiochemical assay, although they may no longer be detectable by the bioautographic assay. In view of the extreme biological

potency of the ionophores it would be important to reconcile the discrepancy between the two available assay systems in order to ascertain the true risk to man of ingesting significant amounts of residual monensin in food.

The radiochemical assay is reliable and sensitive for determining ionophore levels in plasma where extraction is easier and complications from the coextraction of lipid are minimal. Monensin injected into anesthetized dog is rapidly cleared from the plasma with a $t_{1/2}$ of 2.5 min, which is presumably too rapid for elimination. LV dP/dt lags in response, peaking 5 min after the monensin injection and remaining elevated long after the ionophore disappears from the blood. This confirms that monensin passing through the blood, is rapidly distributed into the tissues, where it may persist for a considerable time (48)

When monensin is fed to unanesthesized dogs it attains a steady state in the plasma, a balance between absorption and redistribution into the tissues. It reaches peak plasma levels in 90 min; appreciable plasma levels remain beyond 3 hours. By comparing the plasma level-time integrals for injected and orally ingested monensin we estimate that virtually all orally ingested monensin passes through the blood on its way to the tissues (48). Preliminary experiments in rabbits confirm that oral absorption is not a species specific phenomenon, although in the herbivorous rabbit absorption is considerably slower (112). Since the movement of ionophores across biological membranes is essentially a physical phenomenon of a molecule crossing an oil-water barrier, and animal intestinal membranes are presumably similar in polarity, we assume monensin encounters little difficulty in crossing poultry and ruminant gut. We have verified that monensin injected directly into the stomach of instrumented anesthetized dogs and sheep rapidly induces an increase in dP/dt and coronary blood flow. Thus the finding of significant levels of monensin in poultry tissue by the radiochemical assay is a priori more reasonable than is the finding that it is absent from the muscle, liver, kidney, and fat of poultry fed 149 ppm monensin for 56 days and not subjected to an ionophore-free clearing period (109). Our most conservative estimates are that poultry fed 120 ppm monensin ad libitum for a week retain 0.7 ppm monensin in the liver and 0.6 ppm in the muscle; after 1 day of purging with a monensin-free diet tissue levels remain about 0.1 ppm (111). These tissue levels can be compared with the maximal levels of monensin residues permitted in meat by FDA standards, 0.05 ppm. (116).

IONOPHORE TOXICITY

The carboxylic ionophores represent double-edged swords for man. On the one hand they have an established economic value for the raising of cattle and poultry. Their hemodynamic and inotropic properties and ability to

increase the work efficiency of the heart suggest they may find therapeutic applications for various types of heart disease. On the other hand the full consequences of triggering all the metabolic processes affected by raising intracellular Na^+ may prove to limit the pharmacological uses of ionophores. This consideration is particularly applicable to the introduction of monensin and other ionophores into the meat supply of the population at large.

Monensin is commonly accepted as being especially toxic to horses (113, 114) and the literature promoting the use of monensin for cattle (Rumensin, Eli Lilly) cautions against permitting horses access to supplemented feed (115). Matsuoka reports that monensin at the level commonly fed to cattle, 31 ppm, had little effect, whereas 125 ppm caused anorexia and killed one of three animals; 279 ppm was fatal to both animals so treated (113). The LD_{50} level of monensin for horses is about 2–3 mg/kg (114), whereas the LD_{50} for cattle is ten times this level (115). A considerable number of reports of accidental poisoning of horses by monensin have been documented.[1]

At a level of over 200 ppm (higher than recommended for chickens) monensin killed large numbers of two turkey flocks (118). The birds showed pale areas in the myocardium and small hemorrhages on the heart fat post mortem.We obtained many verified reports of monensin-related fatal animal toxicity, released by the FDA under the Freedom of Information Act[1]. It is apparent that under the prevailing conditions associated with the mixing of commercial animal feed, a significant number of dosage errors occur. Reports of its accidental feeding to dairy cattle have been made, [1] although this practice is not sanctioned by the FDA. The safety margin for optimal levels of monensin in animal feed are narrow enough for such errors to cause serious consequences. We have observed other species differences. An injected bolus of 150 μg/kg monensin, a dose easily tolerated by dogs, killed two out of four anesthesized sheep.

Several confirmed reports have been made of toxicity of monensin to workers associated with its manufacture or its compounding into feed concentrates. The symptoms include headache, nausea, nosebleed, and skin rash. We ourselves have experienced headache and dizziness while supplementing experimental rations with monensin (111).

Incubation of mouse eye lenses with 10^{-7} M nigericin for 4 hours has been reported to induce opacity, which suggests that cataracts are another hazard which may be associated with the industrial use of ionophores (119).

[1]FDA Adverse Reaction Reports Form 2025, obtainable from the FDA, 5600 Fishers Lane, Rockville, Md. 20857, under Freedom of Information Act.

CONCLUSIONS

The ability of low doses of carboxylic ionophores to stimulate cardiac contractility, cardiac hydraulic work done for a given oxygen consumption, and myocardial perfusion through increased coronary flow, especially to the endocardium, suggests that these agents may be desirable drugs for treating the low cardiac output syndrome associated with a simple myocardial failure or shock. Osborne et al have reported that a single dose of the bromo derivative of lasalocid causes a long term reduction in the blood pressure of hypertensive rats and rabbits, suggesting it might find application in the control of hypertension in man (121). Grisorexin has been found to improve $^{205}Tl^{+}$ imaging of the myocardium (120), a property which could be expected to be shared by most of the carboxylic ionophores. Ionophores would be counter-indicated in the case of digitalis toxicity, where their ability to increase intracellular Na^{+} would exacerbate the consequences of similar action of cardiac glycosides (122).

Epidemiologically the cause for greatest concern is the ability of low levels of carboxylic ionophores to cause a specific dilatation of the coronary arteries. This effect can be seen in the dog at levels of injected monensin as low as 2 μg/kg; human coronaries may be more or less sensitive, but it has already been established that human atrial appendage does in fact respond to lasalocid in more or less the same fashion as do experimental animals (56). In the presence of coronary heart disease the autoregulatory mechanism of the heart dilates those vessels which are partially obstructed in order to induce a compensatory decrease in resistance. Superimposition of an indiscriminate coronary vasodilator would dilate the normal vessels in parallel with the maximally dilated obstructed vessels, thereby diverting blood away from the already partially ischemic region of the myocardium. This phenomenon has been termed coronary steal and has been shown to be produced by the drug dipyridamole (123), now understood to be contraindicated in cases of coronary disease or angina. Since this disease state is present in a large segment of the population, if even a small fraction consume enough beef or poultry to produce minimal coronary dilation, it could lead to a significant increase in the incidence of acute anginal attacks and myocardial damage. In this regard the four carboxylic ionophores which have been evaluated for livestock feed supplements—monensin, lasalocid, salinomycin, and narasin—ought to be further evaluated for their coronary dilatory potency, taking into account their recommended feed levels (for poultry: monensin, 100–120 ppm; salinomycin, 60 ppm; lasalocid, 75 ppm) (95) and the ability of the ionophores to survive food processing.

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