oxide and sulfide copper ores (*12*). We think it unlikely that an Inca metalworker would confuse bismuth with tin or copper minerals and that, if there had been accidental admixture of minerals, most of the bismuth would have been eliminated during smelting. If the bismuth were derived from the copper ore, we expect that other impurities would be detected in the alloy, as in European bronzes in which bismuth is present (*5*). They are not found. Native bismuth was probably used to make the alloy. Could bismuth have been added to copper by mistake instead of metallic tin? Native bismuth has a color and surface texture quite different from tin and, in a society in which metals were relatively scarce, it is unlikely that they would have been handled carelessly. Furthermore, if it was intended to make tin bronze, too much alloy addition was used—most bronze from Machu Picchu contains about 5 percent tin (*2*). It is most likely that the alloy used for the llama-head casting was deliberately made by the addition of native bismuth to molten tin bronze, which could be done easily since the denser bismuth would sink to the bottom of the melt before much was lost by vaporization.

Use of bismuth bronze would facilitate making the composite knife in several ways. Addition of small amounts of bismuth to bronze helps make sounder castings (*13*). Better adhesion to the stem would be obtained because of reduced shrinkage during solidification, although the amount of bismuth used is less than that required to obtain expansion upon solidification in most alloys where this effect has been studied (*14*). The addition of bismuth may have produced better adhesion between the head and the stem because of more effective wetting at the interface. But it is possible that the Inca smith may have simply wanted the whiter color of the bismuth bronze. These results show a new dimension of metallurgical sophistication in Inca metalwork.

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Phosphorylation Events During Müllerian Duct Regression

Abstract. *Regression of the fetal rat Müllerian duct in vitro was stimulated by sodium fluoride in the absence of Müllerian inhibiting substance. The action of Müllerian inhibiting substance was inhibited by sodium vanadate, adenosine 5′-triphosphate, and several related nucleotides in the presence of manganese ions. Epidermal growth factor specifically inhibited the substance, but only with manganese ions present. Insulin, platelet-derived growth factor, and nerve growth factor had no effect. These results suggest that dephosphorylation of membrane proteins mediates the action of Müllerian inhibiting substance.*

Much descriptive information has accumulated on Müllerian duct regression (*1*); until recently, however, little was known about the molecular events by which Müllerian inhibiting substance (MIS) causes regression. Our observations (*2*) that EDTA imitated the action of MIS and that Zn^{2+} inhibited MIS in organ culture provided clues to understanding this complex developmental mechanism. We proposed that the action of MIS may be mediated by an extracellular enzyme that is modulated by Zn^{2+} and that this effect is reversed by chelation of Zn^{2+} with EDTA.

The potential link between MIS and epidermal growth factor (EGF) was surmised following the report of an unusual phosphotyrosyl protein phosphatase that is cell membrane–bound and inhibited by Zn^{2+} (*3*). This enzyme hydrolyzed phosphate tyrosine bonds in membrane proteins phosphorylated by an EGF-stimulated protein kinase independent of adenosine 3′,5′-monophosphate (cyclic AMP) (*4*). Even more intriguing was the association of specific tyrosyl protein kinase and phosphatase activities in membrane vesicles from rat cells transformed by Rous sarcoma virus (*5*). Oncogenes of several RNA tumor viruses have also been found to be tyrosine-specific protein kinases (*6*) showing similarities to the EGF-stimulated kinase (*7*). Since MIS inhibits growth of human ovarian and endometrial carcinoma cell lines in vitro and in nude mice (*8*), it is possible that MIS might act through dephosphorylation of membrane proteins, thus opposing the action of EGF or even the process of transformation. Studies of the effects of various agents, including epidermal and other growth factors, on Müllerian duct regression in vitro now suggest that MIS causes regression by antagonizing the effect of an EGF-sensitive, tyrosine-specific protein kinase in the Müllerian duct.

Regression of the fetal rat Müllerian duct was detected by a graded organ-culture assay (*9*). The urogenital ridge of the female rat fetus was removed at 14½ days of gestation and placed in organ culture for 72 hours. Serial sections of the ridge were examined for regression of the Müllerian duct and graded on a scale of 0 to 5, with 0 representing no regression and 5 complete regression. MIS was partially purified from newborn calf testes (*10*) and portions, with or without various test solutions, were mixed 1:1 with the standard culture medium.

Sodium fluoride (> 1 m*M*), reported to weakly stimulate the same phosphotyrosyl-protein phosphatase that was inhibited by Zn^{2+} and stimulated by EDTA (*3*), induced almost complete Müllerian duct regression (Fig. 1A). With < 0.6 m*M* sodium fluoride the duct was intact and with intermediate concentrations it was only partially affected. This result is analogous to that seen with EDTA (*2*), except that the fluoride concentration causing complete regression was about twice that of EDTA. Sodium vanadate, an inhibitor of phosphotyrosyl protein phosphatase (*11*), progressively inhibited MIS-induced regression at concentrations > 5 μ*M* (Fig. 1B). MIS activity was suppressed almost completely by 37.5 μ*M* sodium vanadate.

Since the action of MIS could be mimicked by agents that activate a tyrosyl protein phosphatase, we surmised that agents which stimulate tyrosine phos-

phorylation, such as EGF, might inhibit regression. EGF suppressed MIS activity in a dose-dependent manner, with Mn^{2+} an obligatory cofactor (Fig. 1C). EGF alone had no effect on MIS-induced regression, while 1 m*M* Mn^{2+} itself, unlike Zn^{2+}, had only a limited effect (Fig. 1D) (*2*).

Dimethyl sulfoxide (DMSO) is also capable of activating the EGF receptor in the absence of growth factor (*12*). DMSO had no effect on MIS activity in the absence of Mn^{2+}; however, with 1 m*M* Mn^{2+}, 0.5 and 1.0 percent DMSO caused a statistically significant partial suppression of the activity (Fig. 1E). The dependence of this inhibition on Mn^{2+} is consistent with the requirement for metal ions by the EGF receptor (*4*).

Prompted by the effects of modulators of tyrosyl protein phosphatase and kinase on the Müllerian duct in vitro, we investigated nucleotide substrates for tyrosyl protein kinase. Adenosine 5′-triphosphate (ATP) (1 m*M*) inhibited the action of MIS in a dose-dependent manner (Fig. 1F), but again only with 1 m*M* Mn^{2+}. Hence subsequent studies were performed with 1.0 m*M* Mn^{2+} in the medium. Other nucleotides (1 m*M*) completely inhibiting MIS action in the presence of Mn^{2+} were guanosine 5′-triphosphate (GTP), adenosine 5′-monophosphate, β-nicotinamide adenine dinucleotide, β,γ-imidoadenosine 5′-triphosphate, β,γ-methyleneadenosine 5′-

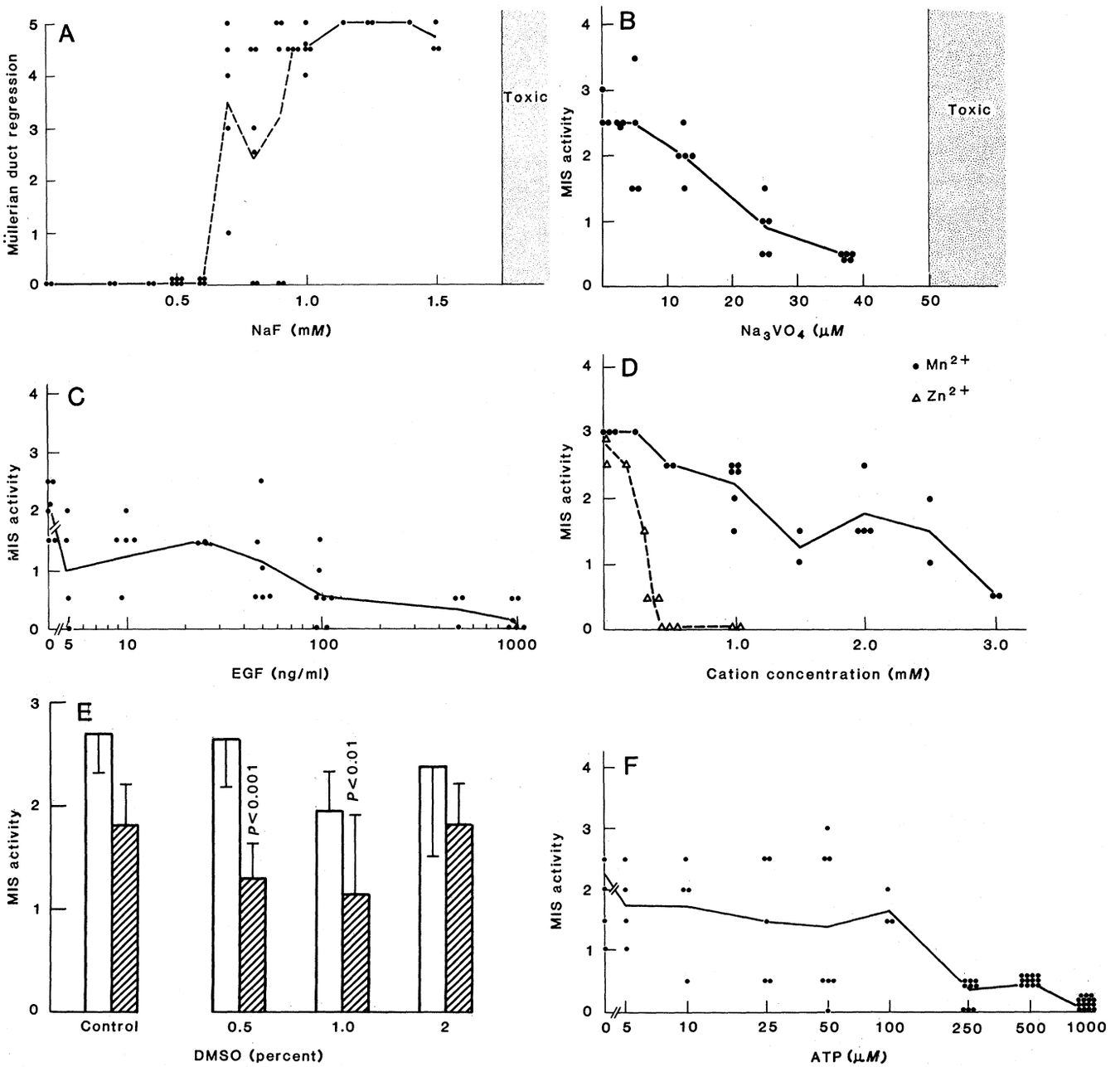

Fig. 1. (A) Regression of the Müllerian duct in vitro with increasing concentrations of sodium fluoride in the absence of MIS. (B) Effect of sodium vanadate on regression induced by a fixed amount of semipurified MIS. (C) Effect of EGF (Receptor Grade, Collaborative Research) on MIS-induced regression in the presence of 1 m*M* $MnCl_2$. EGF-dependent inhibition was not seen in the absence of $MnCl_2$. (D) Relative effects of $MnCl_2$ and $ZnCl_2$ on MIS-induced regression. (E) Effect of DMSO on MIS-induced regression without (open bars) or with (shaded bars) 1 m*M* $MnCl_2$. Each bar represents the mean ± standard deviation for at least eight separate organ culture observations. MIS activity was significantly suppressed by 0.5 and 1.0 percent DMSO with 1 m*M* $MnCl_2$ present. DMSO had no effect on the Müllerian duct in the absence of MIS ($N = 5$). (F) Inhibition of MIS action by ATP in the presence of 1 m*M* $MnCl_2$. ATP-dependent inhibition was not observed without $MnCl_2$. In (A) to (D) and (F) each data point represents one organ culture result; the lines show means for the individual assays at each dose.

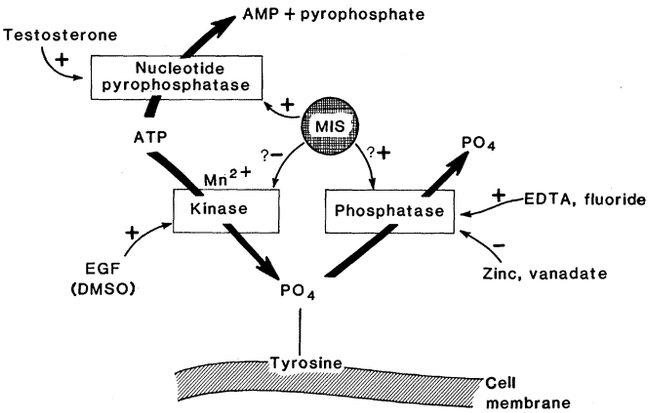

Fig. 2. Working model for the mechanism of MIS action, showing potential interactions that may control membrane phosphoprotein levels. Known modulators that either activate (+) or inhibit (−) relevant enzyme activities are indicated. In stimulating regression, MIS seems to simultaneously activate nucleotide pyrophosphatase in the presence of testosterone (*20*). Question marks indicate potential interaction sites. The overall result of this model would be a decrease in the phosphoamino acid content (possibly phosphotyrosine) in plasma membrane proteins of Müllerian duct cells.

triphosphate, α,β-methyleneadenosine 5′-triphosphate, and adenosine 5′-*O*-(3-thiotriphosphate). Partial inhibitors at 1 m*M* were uridine 5′-triphosphate, cytidine 5′-triphosphate, guanosine 5′-monophosphate, adenosine 5′-diphosphate, β-nicotinamide adenine dinucleotide phosphate, cyclic AMP, adenosine, pyrophosphate, *p*-nitrophenylphosphate, uridine 5′-diphospho-*N*-acetyl glucosamine, uridine 5′-diphosphogalactose, guanosine 5′-diphospho-D-mannose, and guanosine 5′-diphosphoglucose. At 0.5 m*M*, only ATP and GTP significantly inhibited the action of MIS ($P < 0.01$, Wilcoxon rank-sum test). Guanosine 3′,5′-monophosphate was ineffective in suppressing MIS, as were the phosphorylated derivatives of tyrosine, threonine, and serine (< 5 m*M*).

The polar nature of the nucleotides inhibiting regression suggested that they would not readily permeate cells of the urogenital ridge (*13, 14*), but rather would exert their effect extracellularly. ATP has an extracellular effect on cell permeability (*15*), membrane-bound protein kinase (*13, 16*), phosphoprotein phosphatase (*17*), ATPase (*18*), and nucleotide pyrophosphatase and phosphodiesterase (*19*). Of these, only nucleotide pyrophosphate and phosphodiesterase have a sufficiently broad specificity to accept all the nucleotide species with an inhibitory effect on MIS and might be necessary to prevent excessive accumulation of extracellular nucleotides that could perturb cell permeability (*15–19*). Analogously, cell-surface pyrophosphatase may be necessary for the rapid turnover of extracellular nucleotide pools in the way that cyclic AMP phosphodiesterase modulates this regulatory nucleotide inside cells.

A histochemical technique showed enhanced nucleotide pyrophosphatase activity on the regressing Müllerian duct of the 16-day-old male embryo in vivo. In vitro there was localization of activity on the duct only after incubation with semipurified MIS and testosterone. Müllerian duct staining was also inhibited by the pyrophosphatase substrate ATP (*20*).

In addition to EGF, both insulin (*21*) and platelet-derived growth factor (PDGF) (*22*) selectively stimulate tyrosine autophosphorylation in their own respective receptors. However, neither insulin (0.1 to 10 U/ml) nor PDGF (0.001 to 0.5 U/ml) exhibited any inhibition of MIS activity similar to the effect found with EGF and Mn^{2+} (Fig. 1C). Although nerve growth factor (NGF) is not known to act through receptor autophosphorylation, we tested its effect on MIS. As with insulin and PDGF, NGF (1 to 200 ng/ml) did not alter MIS-induced regression with or without Mn^{2+}. The failure of these growth stimulators to affect regression, especially in light of the known effects of insulin and PDGF on membrane protein phosphorylation (*21, 22*), suggests that the suppression of MIS with EGF and Mn^{2+} is a specific event.

A model that may explain our findings is shown in Fig. 2. Müllerian duct cells may contain an "ectokinase" (*14*) that uses ATP (or GTP) translocated from the cytosol (*18*) or added from without as a substrate for phosphorylation of specific membrane proteins. A basal level of phosphorylation, perhaps under the control of growth factors, may be required for Müllerian duct growth and early development. Pyrophosphatase activity could be present to prevent accumulation of extracellular triphosphates (*15*). MIS might activate the ectophosphatase or inhibit the ectokinase in addition to activating the pyrophosphatase, leading to a diminished level of phosphoprotein in the membrane. The inhibitory effect of exogenous nucleotides on MIS could be interpreted as competitive inhibition of nucleotide pyrophosphatase, freeing (endogenous) ATP to act as the substrate for the ectokinase to produce continued phosphorylation. Some of these hypothetical actions of MIS may be initiated by enzymatic properties of the molecule itself (*10*), similiar to those described for NGF (*23*).

Our model for the action of MIS should be useful in further studies of the molecular events in Müllerian duct regression. The link between MIS, EGF, and phosphorylation may be critical if MIS proves to be of value in chemotherapy of female genital tract tumors. Since EGF-induced phosphorylations bear many similarities to those induced by certain transforming viruses, the study of phosphoprotein modulation during regression may shed light on any interaction between MIS and biological tumor control.

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Inhibitors of Poly(Adenosine Diphosphate–Ribose) Synthesis: Effect on Other Metabolic Processes

Abstract. *3-Aminobenzamide and benzamide, purported to be specific inhibitors of the synthesis of poly(adenosine diphosphate–ribose), were used to elucidate possible functions of this biopolymer. These compounds, at frequently used experimental concentrations, not only inhibited the action of poly(adenosine diphosphate–ribose) synthetase but also affected cell viability, glucose metabolism, and DNA synthesis. Thus, the usefulness of 3-aminobenzamide and benzamide may be severely restricted by the difficulty of finding a dose small enough to inhibit the synthetase without producing additional metabolic effects.*

Mono-ADP-ribosylation (ADP, adenosine diphosphate) and poly-ADP-ribosylation are processes belonging to a class of covalent modifiers of proteins (other such processes are acetylation, phosphorylation, and methylation) that alter enzyme function and structural conformation (*1*). The biological significance of poly-ADP-ribosylation is unclear but appears to be related to regulation of several aspects of DNA metabolism as diverse as DNA synthesis (*2*), sister chromatid exchange (*3*), and differentiation (*4, 5*). There is evidence that repair of DNA damage may depend on the synthesis of poly(ADP-ribose) in the nucleus of the injured cell (*1, 6*), causing conformational changes in the DNA (*7*) and increasing the activity of DNA ligase II (*8*).

Much of the evidence for the cellular roles of poly(ADP-ribose) is based on inhibition of the enzyme poly(ADP-ribose) synthetase by compounds such as 3-aminobenzamide (3AB) and benzamide (*9, 10*). Although these compounds inhibit the synthesis of poly(ADP-ribose), the specificity of this inhibition has not been established. It is generally assumed that there are no other significant metabolic effects in undamaged cells (*11*). Our study shows that 3AB and benzamide in concentrations commonly used to inhibit poly(ADP-ribose) synthetase produce adverse effects on other cellular metabolic processes.

A lymphoid cell line (WIL-2) was maintained in suspension culture in RPMI 1640 medium supplemented with 10 percent fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). In all experiments, the cells were incubated with one of four benzamide analogs for 24 hours at 37°C. In addition to benzamide and 3AB, the metabolic effects of *m*-aminobenzoic acid (*m*-ABOA) and benzoic acid (BOA), two analogs of benzamide that do not inhibit poly(ADP-ribose) synthetase, were examined (*10*).

Cytotoxicity of various concentrations of benzamide, 3AB, *m*-ABOA, and BOA (0 to 20 m*M*) was monitored by cellular release into the medium of the cytoplasmic enzyme lactate dehydrogenase (*12*); none of the four benzamide analogs affected this standard enzyme assay. The rate of glucose oxidation in cells treated with the benzamide analogs (0 to 7 m*M*) was measured by the conversion of D-[^{14}C(U)]glucose (0.5 μCi/ml, 348.2 mCi/mmole) to $^{14}CO_2$ (*13*). DNA synthesis from [*methyl*-^{3}H]thymidine (10 μCi/ml, 74.9 Ci/mmole), [*methyl*-^{3}H]methionine (10 μCi/ml, 80 Ci/mmole) or [^{14}C]glucose (0.5 μCi/ml) in the presence of one of the four benzamide analogs (0 to 5 m*M*) was also determined. DNA was isolated from the cells by extraction with a mixture of

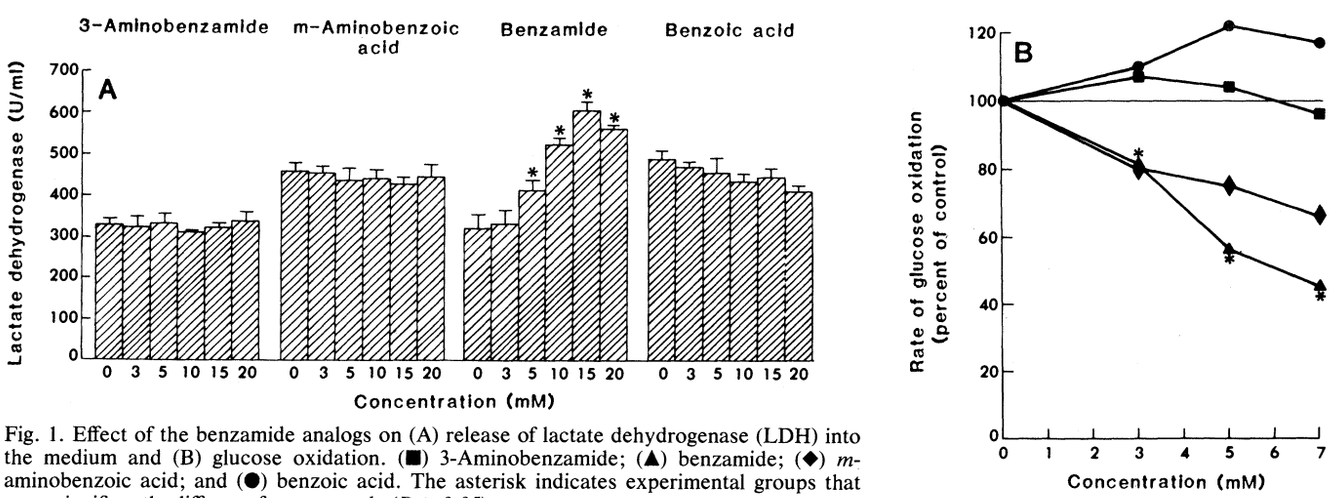

Fig. 1. Effect of the benzamide analogs on (A) release of lactate dehydrogenase (LDH) into the medium and (B) glucose oxidation. (■) 3-Aminobenzamide; (▲) benzamide; (◆) *m*-aminobenzoic acid; and (●) benzoic acid. The asterisk indicates experimental groups that were significantly different from controls ($P < 0.05$).