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ACTIVE IMMUNIZATION OF SWINE AGAINST ZEARALENONE

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ACTIVE IMMUNIZATION OF SWINE AGAINST ZEARALENONE

By

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ABSTRACT

Active Immunization of Swine Against Zearalenone

By

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Height of vaginal epithelium (VE) was investigated as a sensitive measure for monitoring efficacy of active immunization against zearalenone (Z). Twelve prepubertal gilts were ovariectomized (OVX) and fed Z for 3 d. Vaginal samples were collected before initial feeding and 24 h after the last feeding of Z. Vaginal samples were examined histologically and mean height of VE determined. Gilts fed 50, 150, and 350 μg Z/kg body weight (BW) had greater height of VE after feeding Z than before feeding Z ($P<.05$). Rate of increase in height of VE was greater for gilts fed 150 or 350 μg Z/kg BW than for gilts fed 50 μg Z/kg BW ($P<.025$). An indirect competitive enzyme-linked immunosorbent assay (ELISA) was devised for determination of Z in porcine urine. Limit of detection was 10 ng Z/ml urine. Mean within assay coefficient of variation (CV) for each point in five standard curves was 5.9%. Between assay CV for naturally contaminated urine samples in low and high regions of the standard curve were 10.9% (n=18) and 8.8% (n=20), respectively. To determine effects of immunizing pigs against Z, 24 pigs were OVX and allotted to nonimmunized (NI) or immunized treatments (I). A Z-bovine serum albumin (BSA) conjugate was injected at

wk 0 and 4 to achieve immunization. At wk 10, Z specific antibodies could be detected after a 10^7 dilution. At this time, nonimmunized and immunized pigs were allotted by weight to receive no Z (NZ) or 150 μg Z/kg BW (Z) for 3 d. Immunization had no effect independent of exogenous Z ($P>.20$). Height of VE increased more rapidly ($P<.01$) in I/Z and NI/Z pigs than NZ controls. Height of VE from I/Z gilts was greater than VE in NI/Z gilts after 3 ($P<.05$) and 10 d ($P<.005$). Percentage of ingested Z excreted in urine in 8 h following administration of Z was higher in I gilts than NI gilts ($P<.05$). From these data, we conclude that 1) VE in OVX gilts responds in a dose related manner to low doses of Z, 2) a precise ELISA procedure is applicable to the determination of Z in urine, and 3) active immunization of swine against Z potentiates estrogenic effects of Z on height of VE, and also increases excretion of Z equivalents in urine.

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I. LITERATURE REVIEW

Introduction

The following areas of Z research will be discussed: chemical properties, occurrence, metabolism, mechanism of action, clinical effects on swine, and current methods of control for Z mycotoxicosis. Literature reviewed will pertain mainly to relationships between Z and porcine biology, and effects of Z on commercial swine production. Research performed on other species will be reported when swine have not been used as the experimental model.

Relevant Chemical Properties

Z is chemically described as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone. It has a molecular weight of 318.36 and a molecular formula of $C_{18}H_{22}O_5$. Purified Z is a white, crystalline compound with an absorption maxima in methanol at 236 (E=29,700), 274 (E=13,909), and 316 (E=6,020) nm (Mirocha et al., 1977). Z is practically insoluble in water (0.002 g/100ml), however Z is quite soluble in aqueous alkali, methylene chloride, ethyl acetate, ethanol and benzene (Hidy et al., 1977). In a review of Z chemistry, Shiphandler (1975) characterized reactions in synthesis and metabolism of Z and metabolites of Z.

Occurrence

Contamination of swine feedstuffs with Z is difficult to predict. Visual quantification of pinkish Fusarium mold

cannot be used to predict contamination of grain with Z because proliferation of Fusarium fungi does not necessarily mean that Z will be present. Proper environmental conditions are required for a lengthy period of time for fungi to enter into a secondary metabolic stage where mycotoxins are produced (Abramson and Mills, 1985). Researchers have demonstrated, however, that Z production is correlated with plant density, soil fertility, insect damage, cool and damp weather conditions (especially between anthesis and soft dough stage in corn), mechanical damage during harvest, and improper storage conditions (Mirocha et al., 1977; Sutton et al., 1980; Montani et al., 1988). These factors are variable even within an area because of differences in cropping practices and quality of equipment used for harvesting and storage. Therefore, it is not surprising that incidence of Z in feedstuffs is usually localized and difficult to predict.

Presence of Z in a variety of grains and feeds has been reviewed by numerous authors including Bennett and Shotwell (1979), and Kuiper-Goodman et al. (1987). Contamination of feedstuffs with Z has been reported in many countries including Korea (Lee et al., 1985;1986), Australia (Blaney et al., 1984; 1986), Denmark (Hald and Krogh, 1983), Sweden (Olsen et al., 1986), United Kingdom (Buckle, 1983), and France (Jemmali et al., 1978). However, this review will concentrate on incidence and concentration of Z in North American agricultural commodities.

In the United States, only limited surveys of feeds and grains for Z have been performed. Z was found in 45% of

samples from the 1975 Virginia wheat harvest, however Z was not detected in wheat from Virginia wheat from 1976 to 1980 (Shotwell and Hesseltine, 1983). Zearalenone concentrations of .1 to 8 μg/g (mean .66 μg/g) were found in 40 of 342 corn and feed samples from the 1981 Illinois harvest (Cote et al., 1984). In 209 corn and wheat samples submitted from throughout Michigan (1986 crop), 41% and 18% contained Z at concentrations greater than 200 and 500 ng/g, respectively (Hart, 1987). A sample of "refusal corn" from Indiana (1972 to 1973) contained 5 μg/g Z, .2 μg/g α-zearalenol, 20 μg/g deoxynivalenol (DON) and 16 μg/g 15-acetyl DON, while another sample contained .3 μg/g Z, 3 μg/g DON and 16 μg/g 15-acetyl DON (Abbas et al., 1986). Stahr et al. (1981) analyzed "normal" grain and reported Z in 11 and 17% of samples in concrete stave silos or stored dry, respectively. Over half of Z-positive samples contained more than 4 μg/g Z, while remaining feeds contained between .5 and 4 μg/g Z.

Periodically, Z has proven to be an important mycotoxin in parts of Canada. Williams (1985) reported that of 61 samples of corn imported for food usage, only 1 sample contained a detectable concentration of Z (200 ng/g; minimum detection level was 30 ng/g). However, 23 of 81 domestic samples contained detectable concentrations of Z with a mean of 96.7 ng/g and a range of 13 to 475 ng/g. Funnel (1979) conducted a six year survey of suspect feedstuffs in Ontario (1972 to 1977) and reported a mean concentration of 3.85 μg/g in Z-positive samples with a high of 141 μg/g. Twenty percent

had concentrations of Z between 1 and 10 μg/g; 3% were between 10 and 100 μg/g. Incidence of Z in feedstuffs ranged from 9 to 22% and was strongly correlated to rainfall in August (Sutton et al., 1980). Z seems to be much less of a problem in western Canada. Prior (1981) reported that in 475 samples of suspect feedstuffs from western Canada (1975 to 1979), only two corn samples and one cereal sample contained Z (0.5 μg/g). Similarly, one of 271 samples analyzed from across western Canada contained Z (1 ug/g; Andrews et al., 1981).

To assist in evaluating consequences of dietary concentrations of Z, a daily "no adverse effect level" of 60 μg Z/kg body weight (BW) has been established for reproductive effects in swine (Kuiper-Goodman, 1987). Therefore, a 100 kg gilt consuming 4 kg feed/d would have diminished reproductive performance if dietary Z was 1.5 μg/g or higher. This concentration of Z is not found in an average feed sample, however reproductive problems with lower concentrations of Z may occur if other dietary mycotoxins are present. Large scale annual analysis of grain needs to be performed to determine a percentage of grain samples in which this concentration of Z would be found. With the development of inexpensive and rapid techniques such as enzyme-linked immunosorbent assay (ELISA; Warner et al., 1986) and selective adsorption of mycotoxins (SAM), these data should become available from surveys and evaluation of grain for domestic or import/export sales. Financial losses to industry are difficult to estimate, but results from a survey indicate

that one million dollars of hog production may have been lost in Ontario in 1973 to 1974 due to contamination of feedstuffs with Z (Trenholm et al., 1985). Although not a problem on an average year, serious financial problems are caused when swine consume sufficient Z to lower reproductive performance.

Metabolism

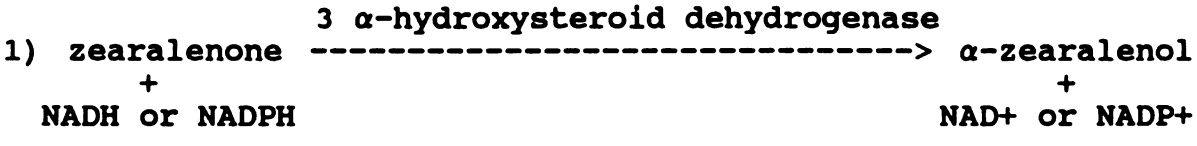
Complete knowledge of distribution, metabolism and mechanism of action is necessary if clinical effects of Z on swine are to be understood, and methods designed to combat Z mycotoxicosis. Briefly, Z is ingested by swine and is absorbed through the gut into blood. Zearalenone can then be transported to 1) insensitive tissues, 2) sensitive target tissues where it exerts estrogenic effects, or 3) liver where it is reduced and/or converted to a more soluble glucuronide form. Zearalenone and Z metabolites are excreted mainly in urine conjugated to glucuronic acid.

When rats ingest diets containing 250 ppm Z, about 50% of Z is absorbed from the intestine into systemic circulation (Smith 1980a). A smaller percentage of Z is absorbed at higher doses (90 mg Z/kg BW; Mirocha et al., 1981). Once absorbed and into systemic circulation, Z becomes associated with hydrophobic regions of serum albumins and sex hormone binding globulins (Martin et al., 1978) as Z is essentially insoluble in aqueous solution. I assume that Z, like steroids and thyroid hormones, enters tissues by the free intermediate mechanism. Hormones must have a plasma $t_{.5}$ less than 24 h to enter tissues by this mechanism (Pardridge, 1981). Research

supports this assumption for although plasma $t_{.5}$ for [^{3}H]Z has been calculated in chickens as 89 h after a 5 mg Z/kg BW dose (Mirocha et al., 1982), plasma $t_{.5}$ for free Z would be much shorter. No free Z was detected in plasma collected from gilts 30 min after they were fed 95 μg Z/kg BW (detection limit .5 ng/ml; Olsen et al., 1985a).

According to the free intermediate mechanism, transport of protein-bound hormones into tissues is determined by 1) capillary transit time, 2) unidirectional dissociation rate, and 3) membrane permeability-surface area product. This model would predict that concentrations of Z in liver would be much greater than in brain because of the five fold longer transit time of blood in liver over brain, and because liver has a much larger surface area of capillaries (Pardridge, 1981). Mirocha et al. (1982) reported concentrations of Z in edible tissues as a function of time in chickens dosed with [^{3}H]Z. Low and constant radioactivity was observed in abdominal adipose tissue, skin, heart, blood and plasma during 48 h following intubation with Z, and high values were recorded in liver soon after dosing with Z. Hepatic radioactivity gradually decreased during the 48 h period.

When Z is spontaneously dissociated from carrier protein and diffuses through capillary cells into liver, Z is metabolized along 2 major pathways as detailed below.



UDP-glucuronosyltransferase isoenzymes
2) zearalenone ----------> zearalenone-glucuronic
(α-zearalenol) acid conjugate
+
glucuronic acid

Metabolism of Z to α-zearalenol was demonstrated with rat liver in vitro (Kiessling and Pettersson, 1978) and in rat, rabbit, pig, cow and man in vivo (Mirocha et al., 1981). Absorption and metabolism of Z are rapid processes since Z and α-zearalenol have been detected in plasma as early as 30 min after feeding a gilt 80 μg Z/kg BW, and detectable concentrations remained in plasma for 5 d posttreatment with Z (Olsen et a., 1985a). Olsen et al. (1981) identified the Z-reducing enzyme as 3 α-hydroxysteroid dehydrogenase. In rat liver, enzyme activity was located in microsomes with NADH as cofactor, and in microsomes and cytosol with NADPH as coenzyme. Considerable differences between species exist in ability to reduce Z (Olsen and Kiessling, 1983; Pompa et al., 1986). Of note is that of five species examined, swine have one of the greatest abilities to form the more highly estrogenic α-zearalenol. Indeed, plasma concentrations of α-zearalenol were 1.5 to 6 fold higher than Z for 8 h following feeding of 80 μg Z/kg BW (Olsen et al., 1985b).

Although not specifically demonstrated with Z, toxins in general are conjugated to glucuronic acid by a two step process. First, phenolic compounds are converted to reactive nucleophilic metabolites by phase one enzymes (probably

cytochromes P450) and second, intermediates are conjugated to glucuronic acid or sulfate by UDP-glucuronosyltransferase isoenzymes or sulfotransferases, respectively (Bock et al., 1987). All Z in plasma and urine is conjugated to glucuronic acid only (Olsen et al., 1985b). Conjugating enzymes consist of enzyme families with overlapping, but distinct specificity. Thus some toxins (eg. benzopyrene-3,6-quinone) have monoglucuronide and diglucuronide forms (Bock et al., 1987). Since identification of Z-glucuronide conjugates has been accomplished via unspecific enzymatic hydrolysis with β-glucuronidase, exact location of glucuronic acid moiety or moieties is unknown. Zearalenone has two hydroxy groups, and two ketone moieties available for reduction (as in C6' reduction of Z to α-zearalenol). Therefore Z could be conjugated at 1 or more of 2 different positions, and Z metabolites at 1 or more of 4 different positions. Glucuronidation increases solubility of compounds in aqueous solutions and facilitates excretion by kidney (Kulkarni and Hodgson, 1980). In swine fed low doses of Z (80 μg Z/kg BW), almost 100% of toxin excreted in urine is conjugated to glucuronic acid (Olsen et al., 1985a). In contrast, only 31% of total urinary Z was conjugated at higher doses (90 mg Z/kg BW; Mirocha et al., 1981). No fecal excretion of conjugated Z or metabolites has been reported (Smith, 1982), and comparison of fecal and urinary [^{3}H] following oral or topical administration of [^{3}H]Z revealed that in rats, very little Z is excreted fecally via bile (Smith, 1980a)

Enterohepatic recirculation may have confounded this result to some degree.

Mechanism of action

The exact mechanism by which Z adversely affects reproduction has not been determined. However, it is known that free Z diffuses through plasma membranes and binds to estrogen receptors (ER) in reproductive and other sensitive tissues. Subsequent translocation of the Z-ER complex into cell nuclei initiates expression of specific genes which cause an "estrogenic response". Estrogen concentrations are highly regulated in vivo, and thus, inappropriate influx of estrogen from exogenous sources , such as dietary Z, has been implicated as the source of various reproduction problems in swine and other domestic livestock.

The ER is associated with the nuclear membrane and contains hormone and DNA binding domains (Koike et al., 1987). Zearalenone and Z metabolites bind specifically to, and compete with, estradiol-17β for hepatic (Powell-Jones, 1981), uterine (Kiang et al., 1978; Katzenellenbogan et al., 1979; Greenman et al., 1979), mammary (Boyd and Wittliff, 1978) and hypothalamic ER (Kitagawa et al., 1982). Z and Z metabolites have different affinities for the ER with the binding affinity of Z about 10% of that of estradiol-17 β (Kiang et al., 1978). The relative binding affinities for rat uterine ER are estradiol-17β > α-zearalanol > α-zearalenol > β-zearalanol > Z > β-zearalenol (Tashiro et al., 1980). Once Z is bound to ER, the receptor complex translocates into the nucleus. Duration of nuclear retention for receptor complexes

is longer for Z (Kiang et al., 1978) and α-zearalenol (Katzenellenbogan et al., 1979) than for estradiol-17β. α-Zearalenol is about 3 to 4 fold more estrogenic in vivo than Z (Hurd, 1977). The DNA binding domain of ER binds specific genomic sites and allows expression of a number of early events typical of estrogen response (Clark, 1977). Administration of exogenous Z to mice increased RNA, DNA and protein, as well as increased cellular permeability to ^{14}C-α-aminoisobutyric acid, ^{14}C-(3-O-methyl)-glucose and ^{3}H-uridine (Ueno and Yagasaki, 1975). There are several theories concerning the mechanism whereby mitogenic effects of estrogen are mediated in estrogen-sensitive cells; however, the exact mechanism for estrogens and thus Z, has not been fully determined at cellular or molecular levels (Soto and Sonnenschein 1987).

Zearalenone has specific activities in many tissues in vivo, and thus administration of Z can enact a wide range of biological alterations. Zearalenone is an uterotrophic compound (Stob et al., 1962; Ueno and Tashiro, 1981) that causes vaginal cornification in rodents and monkeys (Kumagai and Shimuzu, 1982; Parekh and Coulston, 1983; cit Kuiper-Goodman et al., 1987). Hobson et al. (1977) reported that in ovariectomized rhesus monkeys, Z induced a decrease in secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) for 3 to 15 h after injection. This was followed by a surge of LH 36 to 72 h after injection, but not always an increase in FSH. Long and Diekman (1986)

fed 108 mg Z to gilts on various days postmating and reported reduced secretion of prolaction, LH, and estrogen. In a later paper (Long et al., 1988), they reported that feeding Z to gilts decreased concentration of intrauterine Ca^{2+} and increased concentrations of intrauterine Zn and Mg on d 11 and 13 postmating. It has been reported that Z depressed plasma concentrations of testosterone in boars (Berger et al., 1981) and interfered with enzymes that metabolize testosterone in the human prostate gland (Thouvenot and Morfin, 1980). Also, 6-mo gilts fed 10 ppm Z for 2 wk had depressed mean serum concentrations of LH compared with controls. This effect was not observed 1 wk after withdrawal of dietary Z. These biological alterations, either separately or in combination are probably involved in disruption of reproductive performance in animals administered Z.

Clinical effects

Clinical vulvovaginitis from feeding moldy corn was first reported by Buxton (1927) and later by McNutt et al. (1928). Many case reports were subsequently published detailing isolated cases of gross Z mycotoxicosis due to ingestion of contaminated feedstuffs by swine (Pullar and Lerew, 1937); Koen and Smith, 1945; McErlean, 1952; Bristol and Djurickovic, 1971; Miller et al., 1973). Among clinical signs reported in female pigs and their *in utero* offspring were prolonged estrus, mammary development, vaginal prolapse, neonatal mortality, stillbirths and small litters. Reduced libido was observed in male swine.

Following isolation (Stob et al., 1962) and

characterization (Mirocha et al., 1967) of a uterotrophic compound named Z (also called F2) from Fusarium infected grain, several studies were performed to determine effects of high dietary concentrations of Z (>10 ppm) on swine. Christenson et al. (1972) fed 500 to 600 ppm Z to pigs for 64 d and reported that females had increased uterine weight, and males had decreased weight of testis. Chang et al. (1979) fed 25 to 100 ppm of purified Z to female pigs during preestrus, gestation or both preestrus and gestation. Multiple reproductive disorders were produced including infertility, constant estrus, pseudopregnancy, diminished fertility, reduced litter size, smaller offspring, fetal malformation, juvenile hyperestrogenism and probably fetal resorption.

It was not until the 1980's that studies were performed to determine effects of lower concentrations of Z (more commonly seen in feedstuffs) on swine reproduction. Edwards et al. (1987b) demonstrated that inclusion of 10 ppm Z in diets of gilts for 48 d (145 to 193 d of age) delayed attainment of puberty. However, this effect was not observed when 10 ppm Z was included in diets of prepubertal gilts for 2 wk (180 d of age; Green et al., 1987). When 5 or 10 ppm Z was included in diets of postpubertal, nonpregnant gilts between d 5 and 20 of the estrous cycle, estrous cycles were extended (Edwards et al., 1987a). Young and King (1986a) fed gilts 0, 3, 6, or 9 ppm Z starting the day following pubertal estrus. Gilts were inseminated artificially at each subsequent estrus. A linear increase in number of animals

pseudopregnant with increasing dose of Z was observed, as well as a linear decrease in number of gilts mated at second heat and farrowed. Edwards et al. (1987b) found that weaning to estrus interval was longer in sows fed 10 ppm dietary Z for 14 d preweaning. No other variables of fertility were affected. In summary, Z causes reproductive problems in gilts and sows even when included in the diet at less than 10 ppm.

The effects of Z appear to be relatively short term, therefore no persistent reproductive problems have been observed following removal of low concentrations of Z from diets.

The effects of Z on boars has been investigated. Berger et al. (1981) observed reduced libido scores in boars fed 40 ppm Z from 14 to 18 wks of age. However, Ruhr (1979) observed that feeding up to 200 ppm Z for 8 wk did not decrease the reproductive potential of mature boars. Young and King (1986b) fed 0, 3, 6, or 9 ppm Z from 32 d to 145 or 312 d of age. None of the tested dietary concentrations of Z reduced libido, however percent motility of sperm was significantly decreased and a trend was observed for lower total and gel free volumes of semen. In conclusion, Z has detrimental effects on the reproductive performance of boars, however boars do not appear to be as sensitive as gilts and sows to Z.

Methods for Control and Decontamination

Currently, methods for controlling contamination of feedstuffs with Z are inadequate. Usually, farmers do not determine concentrations of mycotoxins in their feed until it

has been stored, and/or a problem observed in livestock. If concentration of Z were determined in grain or feed, then isolated areas of high concentration of Z may be missed due to inherent sampling errors. When reproductive problems or a high concentrations of Z are observed, then contaminated feed is withdrawn from breeding herd diets, and sold or diluted with noncontaminated grain (less than 50 ppb) for ingestion by grower/feeder pigs. "Z-free" grain is purchased for consumption by the breeding herd and replacement gilts. This procedure can be inadequate in years of widespread Z contamination since uncontaminated grain may be unavailable or only available at high cost. Also, it may be impractical to remove and sell corn stored under anaerobic, high moisture conditions. Therefore, better methods for detection and control/decontamination of Z contaminated feeds must be found.

Prevention of Z production is probably the best long term solution to contamination of animal feedstuffs with Z. Some insecticides inhibit production of Z in grain or liquid cultures (Wolf and Mirocha, 1973; Berisford and Ayres, 1976) and applications of fonofos, maneb, and carbaryl to corn inoculated in the field with Fusarium roseum significantly reduced concentrations of Z in grain (Draughon and Churchville, 1985). In addition, plant geneticists are developing corn and cereal varieties that are resistant to invasion and proliferation of Fusarium species. This appears to be a practical and economic long term method to control Z

contamination of feedstuffs.

A number of processes have been identified which decontaminate Z in feeds. Chemical treatment of corn with formaldehyde, ammonium hydroxide (Bennet et al., 1980), chlorine (Sarudi et al., 1979), Gasol (acids and unspecified compounds; Kallela and Saastamoinen, 1982) and 2% lime water (Abbas et al., 1988) have been reported to substantially reduce the concentration of Z in feedstuffs. However, fairly extensive detoxification periods and in some cases elevated temperatures are necessary for decontamination of Z.

Various dietary treatments have been explored as means to alter absorption and metabolism of Z. Elevated dietary fiber or protein, or inclusion of 5% synthetic anion exchange zeolite in feeds has been demonstrated to ameliorate detrimental effects of Z on feed efficiency, feed consumption and body weights of rats fed 250 ppm Z for 14 d (Smith, 1980b). Feeding diets with high-protein, or high-protein and alfalfa increased concentration of free Z excreted in urine. Dietary zeolite (5%) decreased urinary excretion of conjugated Z and α-zearalenol. Increased quantities of Z were fecally excreted in rats fed high alfalfa and protein (Smith, 1982). Inclusion of 15% alfalfa in diets of weanling swine decreased Z-induced uterine enlargement (James and Smith, 1982). However, when 15% dietary alfalfa and 10 ppm Z were fed during lactation and for 21 d postweaning, no prophylactic effect was observed on the extended weaning to estrus interval caused by Z. In addition, a significant decrease in feed intake was reported when 15% dietary alfalfa

was fed (Young et al., 1988). Green et al. (1988) found that dietary progesterone increased serum progesterone concentrations, but failed to counteract Z mycotoxicosis when 1 mg Z/kg BW was fed to gilts on d 4 to 15 postmating.

A sorbent chemically similar to zeolite, aluminosilicate, binds aflatoxin *in vitro*, and to reduce bioavailability of aflatoxin *in vivo* (Phillips, 1987). Orr (1987) demonstrated that .5% aluminosilicate in diets contaminated with Z and deoxynivalenol improved average daily gain and feed efficiency of swine. However, further research needs to be performed to determine if improvement in growth variables was dependent on inclusion of dietary aluminosilicate.

Amount of diet as well as dietary composition is important in modulation of toxic effects of Z. Kiritsy et al. (1987) reported that toxic effects of 1 mg Z/kg BW were less on rats given a reduced feed intake treatment. Although dietary treatments can reduce toxicity of Z, they do not appear to be practical, economic methods for routine incorporation into commercial swine production.

Another prophylactic method with potential to neutralize toxic effects of Z is immunization against Z. Antibodies specific to Z have been generated previously for use in a RIA (Thouvenot and Morfin, 1983). Pestka et al. (1985) characterized cross reactivity of Z metabolites with antibodies generated against Z-bovine serum albumin (BSA), determined an optimum immunization protocol, and devised an

indirect competitive ELISA for determination of antibody titer.

Z and estrogens work through the same mechanism (Katzenellenbogan et al., 1979), therefore, similar biological alterations in metabolism may be observed with immunization against Z as with immunization against estrogens. Immunization against estradiol has increased fetal growth of rats (Spencer et al., 1986) and increased secretion of LH in monkeys (Cowchock et al., 1972), both responses consistent with removal of endogenous estrogens by ovariectomy. Ferin et al. (1969) demonstrated that diethylstilbestrol (DES), a nonsteroidal estrogen not inhibited by antisera for estradiol-17β, restored the LH surge in rats after ovulation had been blocked by antibodies to estradiol-17β. Furthermore, Ferin et al. (1968) demonstrated that in rats, antisera to estradiol-17β inhibited the increases in uterine weight and vaginal cornification evoked by endogenous estrogen production following treatment with human chorionic gonadotropin. They also reported that antisera to estrogen administered before subcutaneous delivery of tritiated estradiol-17β inhibited uptake of radioactivity into uterus, vagina and pituitary gland; however, antibodies did not inhibit diethylstilbestrol-induced increase in uterine weight. In contrast, Wise and Ferrell, (1984) concluded that immunization against estradiol potentiated estrogenic activity after finding that heifers immunized against estradiol had increased ovarian weight, greater number of

large follicles, increased average daily gain and improved feed efficiency over controls. Cowchock et al. (1972) observed that in monkeys immunized against estrogen, vaginal epithelium was persistently cornified. This was similar to constant vaginal estrus syndrome in rats that are constantly producing estrogens in response to gonadotropin administration. Finally, although no statistical analysis was performed, mice immunized against Z appeared to have higher uterine weights than nonimmunized controls fed 0, 10 or 20 ppm Z (Pestka, unpublished data). Therefore, based on these literature, it is difficult to predict whether immunizing swine against Z will neutralize or potentiate estrogenic effects of Z.

II. RESPONSE OF VAGINAL EPITHELIUM TO ZEARALENONE

Introduction

Body weight, feed consumption, feed efficiency, activity of 3 α-dehydroxysteroid dehydrogenase, liver enlargement and kidney enlargement have been used as response variables to investigate prophylactic properties of fiber, protein and zeolite on Z mycotoxicosis. These response criteria were functional as research tools for investigating estrogenic effects of 250 ppm dietary Z for 4 wk in rats. However, these response variables were insensitive to lower concentrations of Z, and it was difficult to repeat these dietary effects when swine were fed 10 or 50 ppm Z for 4 wk (Smith, 1980a; James and Smith, 1982). Therefore, these response variables are inadequate to quantify efficacy of prophylactic treatments for Z mycotoxicosis in swine since these variables are sensitive only to extreme concentrations of Z, and not concentrations which cause problems in commercial swine production.

Uterine weight is a more sensitive variable with which to estimate estrogenic effects than those discussed above, and has been used to demonstrate ameliorating effects of alfalfa on Z mycotoxicosis. However, gilts had to be fed a relatively high dietary concentration of 10 ppm Z for 4 wk to cause detectable differences in uterine weight (James and

Smith, 1982). Other disadvantages of uterine weight as an indicator of *in vivo* effects of Z are that large numbers of pigs are required per treatment, repeat measures are not possible and pigs must be sacrificed. Since residues from feeding Z prevent salvage of carcasses for food, experimental costs are greatly increased. Therefore, uterine weight is functional as a gross indicator of Z mycotoxicosis, but is not a practical response variable sensitive to low concentration of Z delivered for short periods of time.

Vaginal cornification bioassays have been used to quantify effects of Z in rats (Kumagai and Shimizu, 1982) and primates (Parekh and Coulston, 1983; cit Kuiper-Goodman, 1987), but this technique cannot be applied directly to swine because porcine vaginal epithelium does not undergo cornification (Cole, 1930). However, porcine vaginal epithelium does respond to estrogen. Kurtz et al. (1969) fed 1 to 50 mg Z or administered 0.5 to 2 mg estrogen intramuscularly to prepubertal gilts, they reported effects of both Z and estradiol on VE as 1) squamous metaplasia, and 2) an increase in mitosis. Based on this research, we hypothesized that height of porcine vaginal epithelium may exhibit dose response to Z.

We performed several preliminary studies and observed that in prepubertal gilts (approximately 50 kg), depth of VE was highly variable within vaginal segments and histological sections. I could not detect differences in height of VE after .25 to 3 ppm dietary Z. Most VE had already undergone

squamous metaplasia, and stratified cuboidal epithelium had been lost from the lumenal surface in some places. Pigs fed 1 or 3 ppm Z had marked vulvar swelling after 3 d on treatment. It is possible that younger pigs would not have such variable pretreatment VE, however, in light of the fact that ultimately, we wanted to test estrogenic effects of Z in much heavier pigs (approximately 100 kg), I decided that this would not be an acceptable animal model, even if a dose response could be quantified.

To test the hypothesis that ovarian estrogen production contributed to variation in pretreatment height of VE, and to insensitivity of VE to exogenous Z, we ovariectomized gilts and monitored height of VE. Ovariectomy decreased baseline height of VE, decreased variation found in height of VE (partially by reducing crypt proliferation), and changed most lumenal VE to a cuboidal-type cell. Thus, the ovariectomized prepubertal gilt was the animal model used to test the hypothesis that increase in height of VE is related to dose of Z.

Materials and Methods

To determine dose response of VE to Z in swine, 12 pigs were ovariectomized and housed in gestatation crates for a recovery period of at least 2 wk. Basal diets contained less than 50 ng Z/kg feed as tested with an ELISA (Warner et al., 1986). Pigs weighed 58.5 +/- 5.2 kg at the initiation of the experiment. Long acting antibiotics were administered before initiation of the experiment to minimize complications due to

vaginal infections. Four pigs were allotted by weight to dietary treatments of 50, 150 or 350 μg Z/kg body weight (BW). Z was dissolved in 100% ethanol, and allotted by volume onto 200 g of basal diet. After evaporation of ethanol, 200 g meals were fed once daily for 3 d following a 12 h withdrawal of feed.

Three vaginal samples were collected per d on d 0 and 3. Samples were collected with alligator forceps from about 20 cm anterior to the vulva. Samples were fixed/stored in 10% formalin until they were embedded in wax, sectioned 7 μm thick, and stained with hematoxylin and eosin. Depth of VE was estimated at a magnification of 400X by comparison to an ocular grid. Measurements were taken if epithelial surfaces adjoining lamina propria and lumen were parallel (within 10%) for a length of 140 μm. Means were calculated within pig and treatment, slopes were estimated using linear regression, and differences between slopes determined using a t-statistic (Gill, 1978).

Results and Discussion

Response of VE in OVX gilts to Z is shown in Table 1. All treatments increased height of VE from their respective baselines ($P<.05$). Rate of increase in height of VE was greater for gilts fed 150 and 350 μg Z/kg BW as compared with gilts fed 50 μg Z/kg BW ($P<.025$).

Our preliminary experiments revealed that ovariectomy substantially reduced height and variation of VE. In addition, ovariectomy increased sensitivity of VE to Z.

This could be because ovariectomy removed the major source of circulating endogenous estrogens. Since binding affinity of Z for ER is about 10% of that for estradiol-17 β, VE would be more responsive to Z if competition from estrogens was withdrawn. Kurtz et al. (1969) reported that VE in three of eight control gilts (mean BW was 34 kg; range was 14 to 59 kg) had undergone squamous metaplasia similar to gilts fed estrogen. They concluded that these prepubertal gilts could produce endogenous estrogens in sufficient quantities to cause changes in VE.

Based on the work of Kurtz et al. (1969), we initially intented to quantify change in VE cell type from stratified cuboidal to squamous. This type of analysis would have been subjective relative to the method we developed as there appears to be a spectrum of cell shapes between cuboidal and squamous. Thus, subjective judgements would have had to be made as to whether a given cell were more "cuboidal-like" or "squamous-like". Although relative proportions of cell types would probably be correlated to mean height of VE, counting and recording number of cell types would have been considerably more time consuming than estimating mean height of VE.

We observed variation in sloughage (or loss in processing) of the stratified cuboidal-type epithelium covering squamous cells following Z-induced proliferation. This layer was small relative to total height of VE and distortion of actual height would be small.

Results from conservative statistical analysis indicated that rate of increase in VE from pigs on 50 μg Z/kg BW would not have been different from control pigs fed no Z. The increase in height of VE from pigs fed 50 μg Z/kg BW was compared statistically against pooled pretreatment data from 11 pigs. Statistical analysis was performed as if height of VE did not change in this 3 day period. Only 2 degrees of freedom were allowed for statistical comparison. It is quite likely that a dose of 50 μg Z/kg BW would cause an increase in height of VE compared to controls fed no Z if more pigs were assigned per treatment.

The lowest dose to cause significant change in height of VE was 150 μg Z/kg BW. When height of VE is plotted as a function of dose, it appears that 150 μg Z/kg BW is in the upper portion of the dose response curve. Therefore, 150 μg Z/kg BW would be the optimal dose to detect a difference in response of VE to Z under a prophylactic treatment. This dose would not be as sensitive an indicator for detecting potentiation of estrogenic effects of Z as it would be for detecting neutralization. Finally, 150 μg Z/kg BW is approximately 3.75 ppm dietary Z if a 100 kg gilt consumed 4 kg/d. This is 2.5 fold greater than the dose of Z determined by Kuiper-Goodman et al. (1987) as the "no adverse effect level" for reproductive effects in pubertal pigs. In addition, this would probably be a concentration of Z that would cause economic problems due to decreased reproductive performance. Therefore, this is a practical concentration of

Z to use when investigating methods for neutralization of estrogenic effects of Z.

Samples were collected from the more highly invaginated section of vagina, approximately 20 cm anterior to the vulva (range 15 to 25 cm). Variation in height of VE was small relative to baseline heights of VE. Therefore, any anatomical differences with distance into vagina appear small relative to mean height of VE.

Since height of VE increases in relation to dose of Z, feeding 150 μg Z/kg BW and monitoring height of VE can be used to evaluate preventative or therapeutic treatments for Z mycotoxicosis.

TABLE 1: EFFECT OF ZEARALENONE (Z) ON HEIGHT OF VAGINAL EPITHELIUM IN OVARIECTOMIZED GILTS

Z[b]	Height of vaginal epithelium, μm[a]	
	Pretreatment	Posttreatment
50	18.8 (2.0)[c]	34.8 (4.4)[de]
150	23.2 (3.0)[c]	87.0 (10.4)[df]
350	18.8 (3.2)[c]	98.0 (4.4)[df]

[a] Mean with standard error of mean in parenthesis.
[b] μg Z/kg BW, meal-fed daily for 3 d.
[c,d] Means within a row with different superscripts differ (P<.05).
[e,f] Means within a column with different superscripts have different rate of vaginal epithelial increase (P<.025).

III. ELISA FOR DETECTING ZEARALENONE IN URINE

Introduction

High pressure liquid chromatography (HPLC; James et al., 1982) and liquid chromatography (LC; Olsen et al., 1985b) procedures have been developed for detection of free and conjugated forms of Z and Z metabolites. Monitoring excretion of Z in urine can help determine mechanism through which prophylaxis of Z mycotoxicosis is mediated (Smith, 1982). However, present techniques require extensive purification procedures and complex equipment, and thus large scale analysis of urine is difficult due to time and economic restraints. Therefore, our second objective was to develop and validate an enzyme-linked immunosorbent assay (ELISA) for rapid and inexpensive determination of Z equivalents in urine.

Materials and Methods

All inorganic chemicals and organic solvents were reagent grade or better. Tween 20, hydrogen peroxide, 2,2' azinobis(3-ethylbenzthiazoline sulfonic acid), egg albumin (ovalbumin, grade II) and B-glucuronidase (enzyme activity of 588,000 units/g; bovine liver type B-1) were purchased from Sigma Chemical Co. (St Louis, MO). Crystalline Z was kindly donated by International Minerals and Chemicals Corporation (Terre Haute, IN).

A β-glucuronidase solution was prepared by adding 3.4 mg enzyme (3000 Fishman units) to 10 ul of .2 M sodium acetate buffer (pH 5.5). Naturally contaminated urine (500 µl), 400 µl .2M sodium acetate buffer (pH 5.5) and 10 µl of β-glucuronidase solution were mixed, and incubated for at least 16 h. Zearalenone was extracted by adding 500 µl ethyl acetate, vortexing, and centrifuging at 16000 x g for 1 min. Upper layer (ethyl acetate + Z) was removed and placed in a 1.5 ml tube. The extraction procedure was repeated. Ethyl acetate and Z mixture was dried to residue under nitrogen and residue was resolubilized in 500 µl of 40% methanol. Samples were vortexed to ensure resolubilization.

Monoclonal antibody (MAb) to Z was diluted 1:1500 in 1% ovalbumin (in distilled water) and added 1:10000 to .1M sodium phosphate buffer (PBS; pH 7.5). Samples of 125 µl were air-dried in microtiter wells (Immunolon Removawells, Dynatech Laboratories, Alexandria VA) as described by Warner et al. (1986). MAb coated plates were washed three times with 300 µl of .025% (v/v) Tween 20 in PBS (PBS-Tween) and contents aspirated with a Dynatech Miniwash (Dynatech Laboratories, Inc., Alexandria, VA). A 50 µl aliquot of sample and 50 µl of Z standard (0 to 500 ng Z/ml in 100% methanol) were added to MAb coated wells. Concentration of Z in a 6 µg/ml solution was checked spectrophotometrically and dilutions made for standards. One milliliter samples were dried to residue under nitrogen and stored at -21^{o}C until needed.

Z-horseradish peroxide (Z-HRP) conjugate was prepared as per Warner et al. (1986), diluted 1:400 in 1% ovalbumin-water, and 50 µl added to each well. Plates were incubated 1 h at 37°C and bound Z-HRP determined by the method of Pestka et al. (1982) on a Dynatech microelisa Minireader II. A logit-log transformation was applied to standard curves and data using ELISANALYSIS (J.H.Peterman, University of Alabama at Birmingham). A correlation coefficient of at least .99 and slope between .8 and 1.1 was required for a standard curve to be considered valid (Butler et al., 1987).

Results and Discussion

To determine concentration of free or total Z in porcine urine, a direct competitive ELISA was devised from procedures of Warner et al. (1986), Dixon et al. (1987) and James et al. (1982). A typical ELISA standard curve is presented in Figure 1.

Modifications to the standard ELISA procedure have made a substantial improvement in determination of Z in large numbers of samples. Previously used ELISA procedures were modified (see Appendix A) such that effect of time on slope of standard curve was reduced from that illustrated in Figure 2 to that of Figure 3. Previously, determinations of Z in samples pipetted 10 to 15 min after the standard curve were inaccurate as slope of standard curve increased with time after addition to microtiter wells. By adding Z-HRP after all unknown samples had been added to wells, effect of time on slope of standard curve was reduced considerably. Estimations

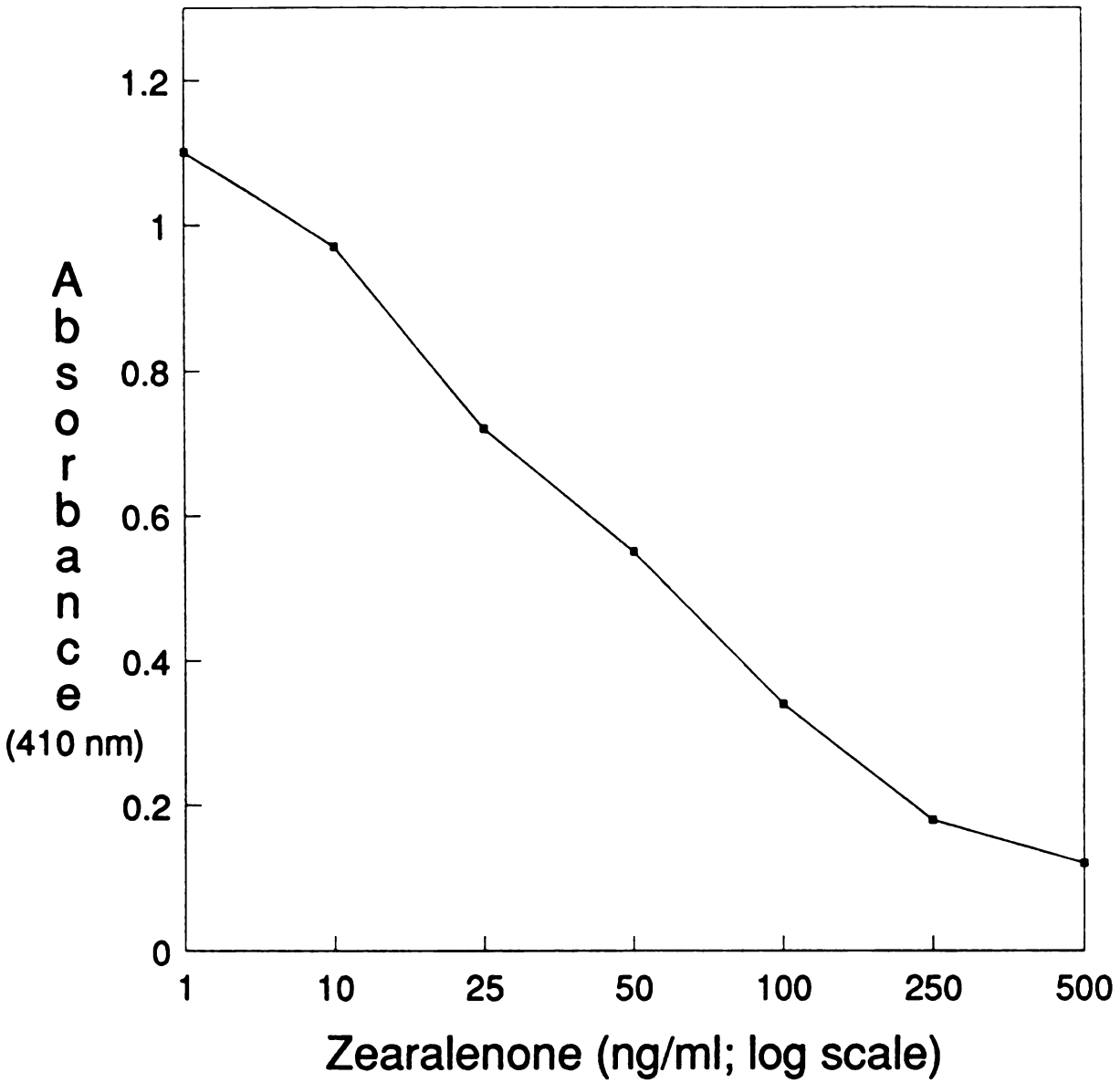


Figure 1. A typical standard curve for zearalenone using a direct competitive enzyme-linked immunosorbent assay. Zearalenone-specific monoclonal antibodies were bound to the solid phase of microtiter wells. Crystalline zearalenone was added in 40% methanol, followed by an equal volume of zearalenone-horseradish peroxidase conjugate in aqueous solution. Wells were incubated at 37°C for 30 min, and bound peroxidase determined in a color reaction.

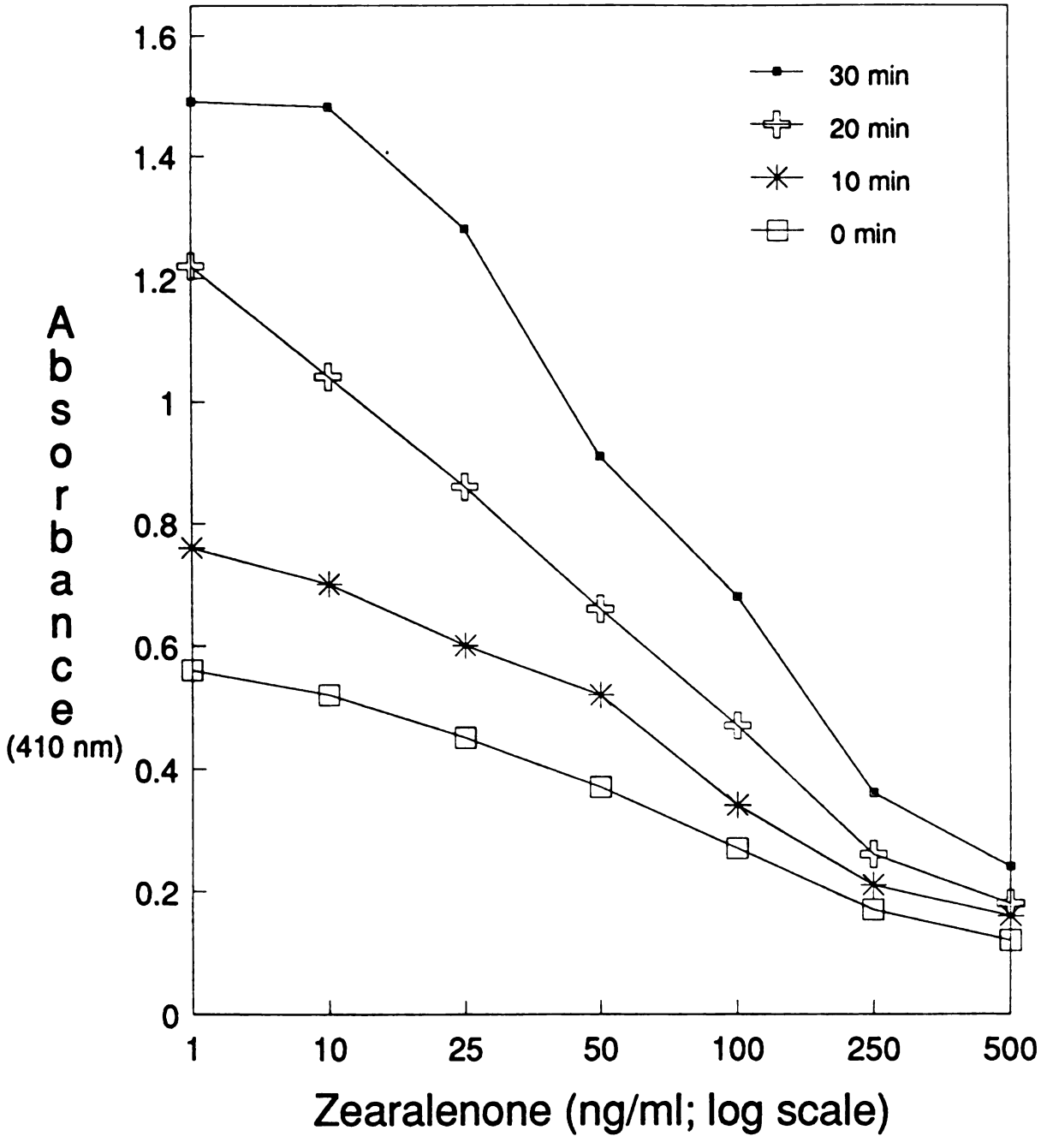


Figure 2. Effect of time on slope of enzyme-linked immunosorbent assay (ELISA) standard curves for zearalenone (Z) using the standard ELISA procedure. Zearalenone (final concentrations of 1 to 500 ng/ml 35% methanol) and horseradish peroxidase conjugate were mixed before addition to microtiter wells coated with Z-specific monoclonal antibody. Standard curves were pipetted 10 min apart for 30 min before incubation and color reaction.

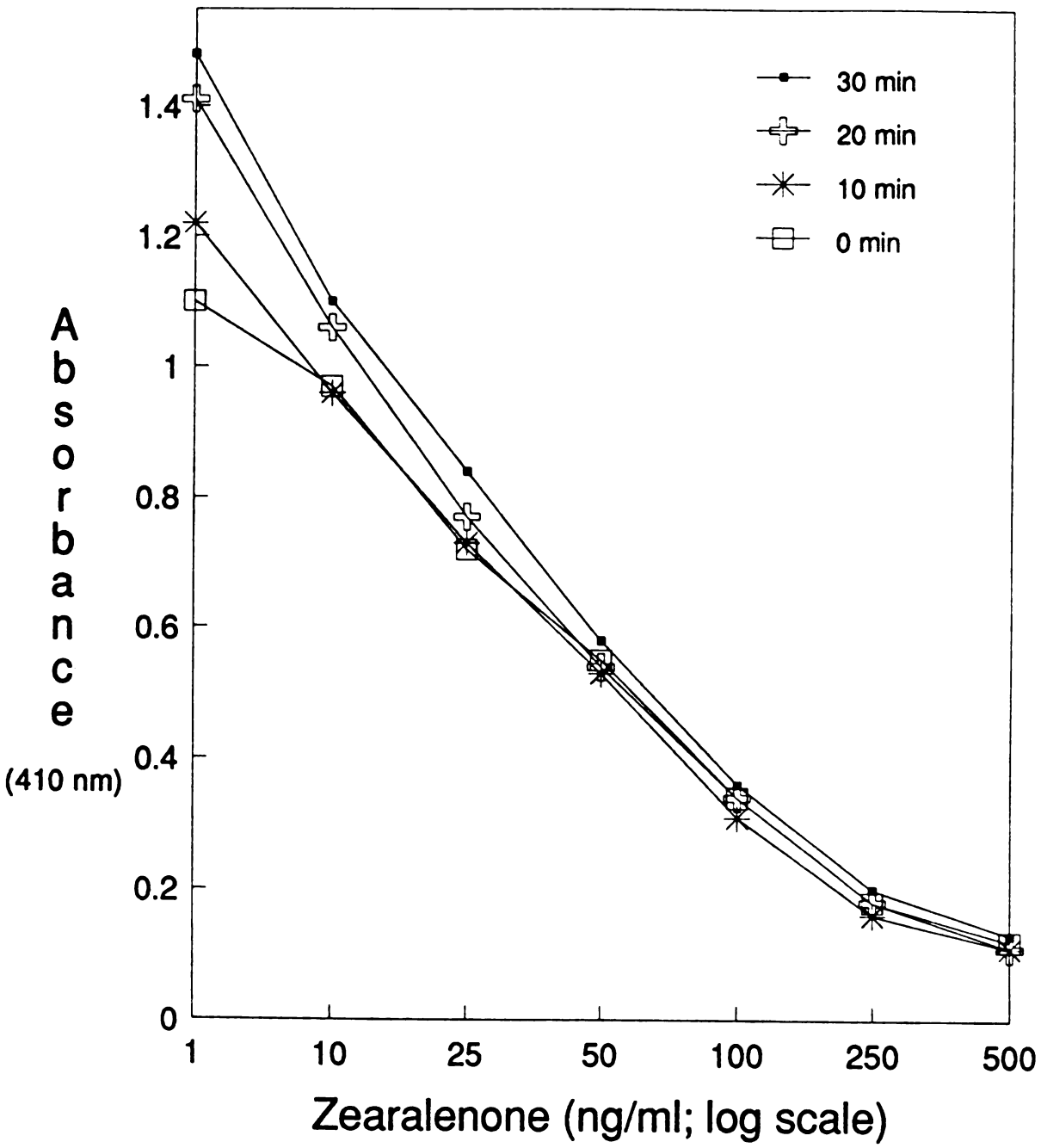


Figure 3. Effect of time on slope of enzyme-linked immunosorbent assay (ELISA) standard curves for zearalenone (Z) using the modified procedure. Standard curves (final concentrations of 1 to 500 ng Z/ml 20% methanol) were pipetted every 10 min for 30 min into microtiter wells coated with Z-specific monoclonal antibody. An equal volume of Z-horseradish peroxidase was added with an octapipette before proceeding with incubation and color determination.

of Z content in samples pipetted at the end of an ELISA plate should be considerably more repeatable.

As part of the ELISA modification process, effect of methanol on specific binding of Z-HRP by MAb was investigated. Results are illustrated in Figure 4. Specific binding of Z-HRP to MAb decreases as concentration of methanol increases above 50%. It appears that high concentrations of methanol irreversibly denatures MAb such that Z-HRP binding is substantially decreased.

Limit of detection for the modified ELISA procedure was 10 ng/ml urine. Concentrations of Z could be determined to 1 ng/ml, but slope of the standard curve was not consistently within the range of .8 and 1.1 after logit-log transformation, and deviated from assay to assay. Theoretical and experimental studies demonstrate that a logarithmic plot of antibody titration curves should produce a linear region with a slope of 1 (Butler et al., 1987). When 1 ng/ml was excluded from analysis, slope of standard curve was between .8 and 1.1, and slopes were more consistent between assays. Also, the effects of time on slope of standard curves were more extreme in the region from 1 to 10 ng Z/ml than with higher concentrations of Z (Figure 3). Since accuracy of estimates determined near the end of an ELISA plate in the range of 1 to 10 ng Z/ml urine would be less, this lower concentration of Z was not included as a standard component of the ELISA procedure.

A limit of detection for the ELISA procedure of 10 ng

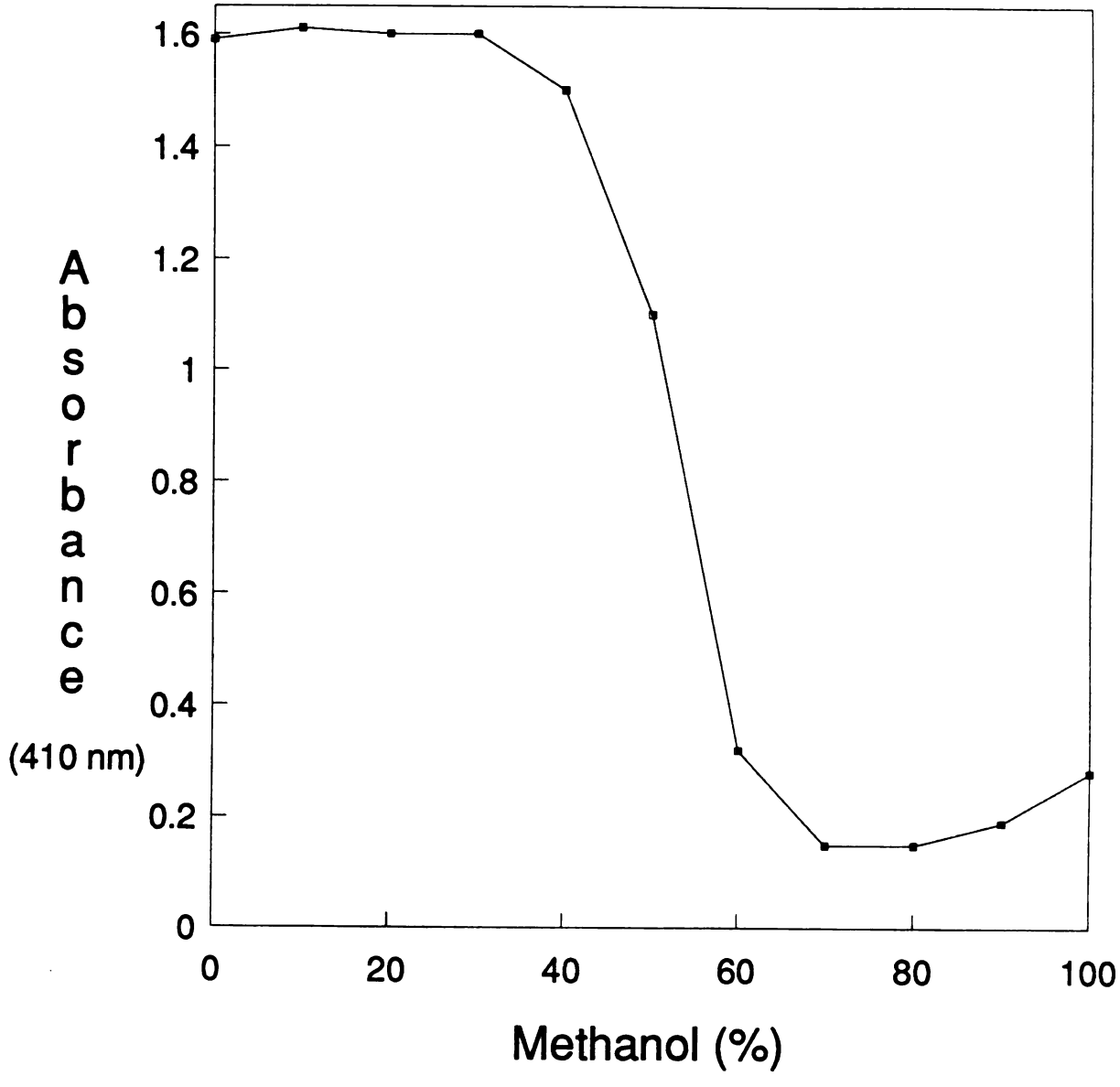


Figure 4. Effect of methanol on specific binding of zearalenone (Z)-horseradish peroxidase by monoclonal antibody. Methanol was added to microtiter wells coated with Z-specific monoclonal antibody. Methanol was removed after 5 min, and Z-horseradish peroxidase was added for 30 min at 37°C. Bound peroxidase was determined by a color reaction.

Z/ml urine compares favorably with the HPLC limit of detection of 4 and 10 ng/ml urine for Z and zearalenols, respectively (James et al., 1982), and with the 5 ng Z/ml urine detection limit reported with LC (Olsen et al., 1985b). Detection limit could be lowered by concentrating the sample.

Finally, total Z released by β-glucuronidase as a function of volume of naturally contaminated urine is linear in the range of 0 to 2600 ng Z released/assay (Figure 5). The correlation coefficient between optical density and concentration of Z was .997 with a slope of 128. These results validated the procedure for up to 2600 ng of Z released/ml urine.

The ELISA described here has advantages over HPLC or LC procedures on the basis of simplicity of extraction, rapid throughput time and low expense. In addition, the ELISA procedure is precise; coefficient of variation from triplicate estimates of each point in five randomly chosen standard curves (10 to 500 ng Z/ml) averaged 5.95%. Between assay coefficients of variation for samples in lower and upper ends of the standard curve were 10.9% (n=18) and 8.8%

The ELISA procedure has a disadvantage in that it cannot distinguish between Z and Z metabolites. The MAb used in this ELISA crossreacted with α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalenol by 107, 29, 35, and 25%, respectively, as compared with Z (Dixon et al., 1987). Therefore, a concentration of "Z equivalents" is determined,

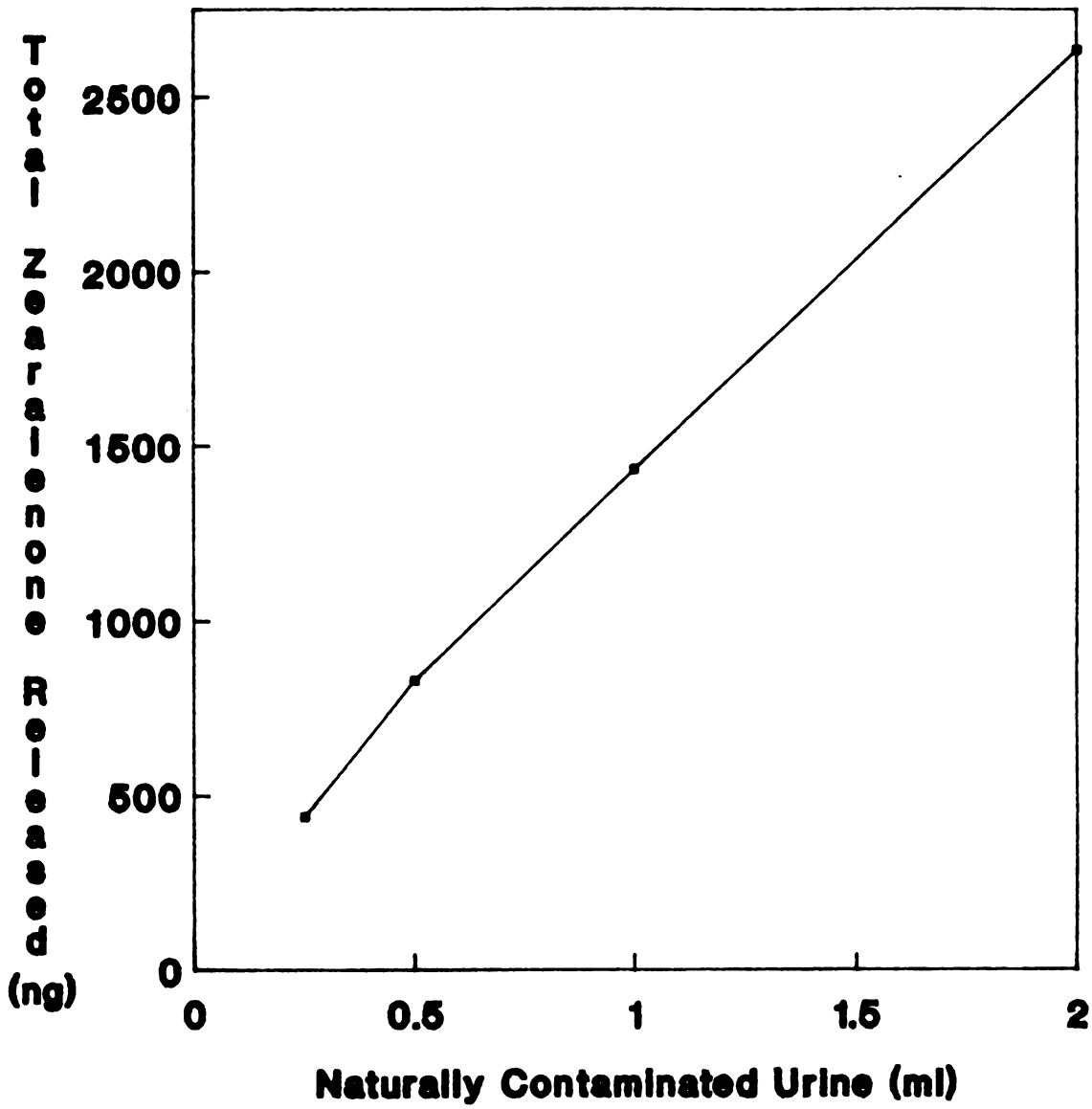


Figure 5. Linear release of zearalenone (Z) with increasing volumes of naturally contaminated urine. Urine was incubated with 3000 units of β-glucuronidase at 37°C for 16 h. Correlation coefficient was .997.

with no information given on relative concentrations of Z metabolites. This is a significant problem when this procedure is used to estimate effect of treatment on metabolism of Z.

IV. ACTIVE IMMUNIZATION OF SWINE AGAINST ZEARALENONE

Introduction

Zearalenone is an estrogenic mycotoxin produced in feedstuffs by several species of Fusarium (Neish et al., 1982). It is suspected that Z caused what early literature details as porcine hyperestrogenism following ingestion of moldy corn (Buxton, 1927; McNutt et al., 1928). However, it was not until 1962 that Stob isolated and crystallized Z. Subsequently, it was proven that upon diffusion of Z into sensitive target cells, low concentrations of this toxin cause an estrogenic response (Kiang et al., 1978; Katzenellenbogan et al., 1979) which creates numerous reproductive problems in swine (Edwards et al., 1987a,b; Young and King, 1986a). Although a number of approaches for preventing Z mycotoxicosis in swine have been investigated (Young et al., 1988; Green et al., 1988), current methods for controlling this problem are inadequate. In some cases, immunization against estrogen neutralizes biological activity of estrogen in vivo (Cowchuck et al., 1972). Since estrogen and Z have the same mechanism of action (Katzenellenbogan et al., 1979), I hypothesized that antibodies specific to Z would reduce estrogenic effects of Z in vivo. Therefore, our approach to reducing estrogenic effects of Z in swine was to actively immunize against Z.

Materials and Methods

All inorganic chemicals and organic solvents were reagent grade quality or better. N-N'-dicyclohexylcarbodiimide (DCCD), N-hydroxysuccinamide, egg albumin (ovalbumin, grade II), bovine serum albumin (BSA; fraction V, fatty acid free), 2,2'-azinobis(3 ethylbenzthiazoline sulfonic acid), hydrogen peroxide, Freunds complete adjuvant (FCA) and Freunds incomplete adjuvant (FICA) were purchased from Sigma Chemical Co. (St Louis, MO). Carboxymethoxylamine hemihydrochloride and demethyl formamide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Peroxidase conjugated rabbit antiswine IgG was purchased from Cappel Worthington (Malvern, PA). Z was provided as a gift from International Minerals and Chemical Corporation (Terre Haute, IN).

To prepare immunogen, Z was converted to Z-6'-carboxymethoxylamine (Z-oxime; Thouvenot and Morfin, 1983) and subsequently conjugated to BSA by the N-hydroxysuccinimide procedure (Kohen et al., 1980). Z-BSA conjugate was lyophilized and stored at -21^{o}C. Conversion of Z to Z-oxime appeared to be 100% as estimated with thin layer chromatography (TLC). Molar ratio of Z:BSA was approximately 50:1 as determined spectrophotometrically (Thouvenot and Morfin, 1983).

Twenty four crossbred pigs were anesthetized, ovariectomized (OVX) and allowed 7 wks to recover and grow. Twelve OVX pigs (˜46kg) were then immunized with 5 mg Z-BSA

conjugate in 4 ml FCA:saline (1:1) and a booster administered 4 wk later with 2 mg conjugate in 4 ml FICA:saline (1:1). Immunization was achieved with 30 to 40 subcutaneous/intradermal injections 10 to 30 cm ventral to the dorsal midline (along the loin). Blood was collected by venipuncture at 2,4,6,8, and 10 wk as well as before and after dietary challenge with Z. Serum was purified with 35% ammonium sulfate precipitation (Hebert et al., 1973) and stored at -21^{o}C until analysis.

Antibody titer was determined using a procedure modified from Pestka et al. (1985). Z-oxime was conjugated to ovalbumin by the N-hydroxysuccinimide ester method of Kohen et al. (1980). Z-ovalbumin conjugate (Z-oval; 10 ug/ml) was added (1:500) to 1% ovalbumin-water and 100 μl aliquotted to wells in a microtiter plate (Immunolon Removawells, Dynatech Laboratories, Alexandria VA). Plates were incubated for a least 12 h at 4^{o}C or at least 2 h at 37^{o}C. Plates were washed three times with 300 μl of .025% (v/v) Tween 20 in .01 M sodium phosphate buffer (PBS-Tween; pH 7.5). Serial dilutions of antiserum in .1 M PBS were incubated, 50 μl/well, with 50 μl 1% methanol or 50 μl 1% methanol with excess Z 1 h at 37^{o}C. Inclusion of excess Z minimizes specific binding of antibody to Z-ovalbumin. Plates were washed four times with PBS-Tween. Bound antibody was detected by addition of peroxidase-conjugated rabbit antiporcine IgG, diluted 1:500 in 1% ovalbumin-water for 30 min at 37^{o}C. Plates were washed five times with PBS-Tween and bound enzyme determined

colorimetrically by the procedure of Warner et al (1986). The titration curve for antibodies specific for Z was then determined by difference between total and nonspecific binding of porcine antibody. Antibody titer was defined as the intersection of ELISA antibody titration curve with .1 absorbance units. The method of determining antibody titer is depicted graphically in Figure 6.

To determine if feeding Z reduced detectable titer in immunized pigs, a comparison between detectable titer for those gilts fed Z or no Z was made using linear regression and a t-statistic (Gill, 1978). Titers were compared at a common dilution of 10^5 in two ELISA plates loaded and incubated in succession. Final optical density of samples common to both plates were not different. Since color produced varied with Z-specific antibody, difference in color was representive of difference in relative titer.

At wk 10, antibody to Z was detectable after a dilution of 10^7. An experiment was then performed to determine efficacy of active immunization of swine against Z. Immunized (I) or nonimmunized (NI) pigs received Z (Z) or did not receive Z (NZ). Those treatment groups receiving Z contained eight pigs each and those receiving no Z contained three. Z was dissolved in 100% ethanol, and aliquotted by volume (equivalent to 150 ug Z/kg BW) onto 200 g of basal diet. After ethanol evaporation, 200 g meals were fed once daily for 3 d following a 12 h feed withdrawal.

Three vaginal samples were biopsied per d on d 0 and 3

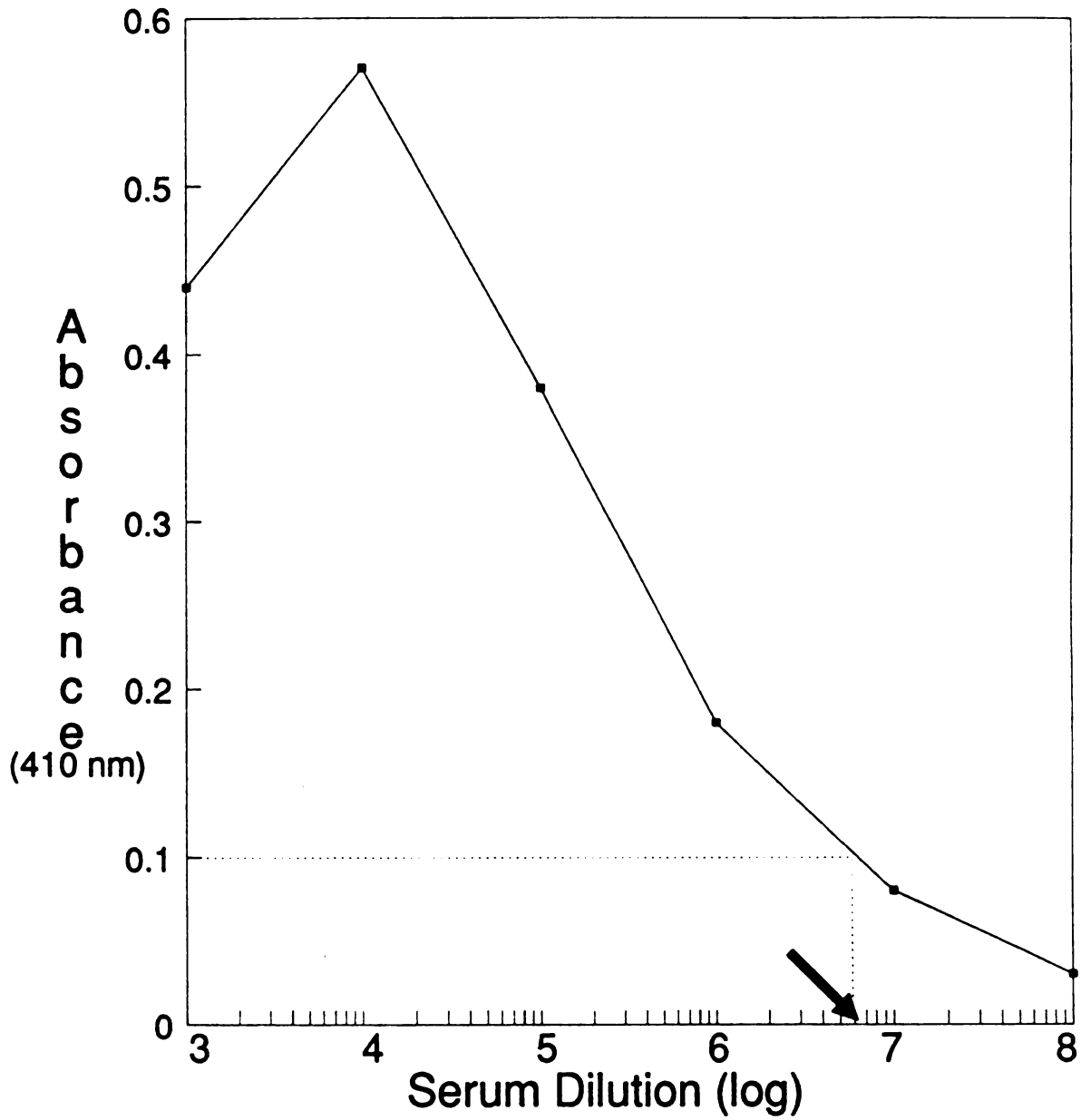


Figure 6. Method for determining antibody titer. Antibody titer was defined as the serum dilution (designated by arrow) where the titration curve for enzyme-linked immunosorbent assay intersected with .1 absorbance units. The titration curve was generated by serial dilution of antisera.

of the experiment. Tissues were collected, processed and analyzed according to MacDougald et al. (1987). Briefly, vaginal samples were fixed, sectioned, stained and examined histopathologically. The number of observations recorded per pig per d varied between 25 and 100. Mean height of vaginal epithelium was determined within pig and treatment. Response of VE to Z was quantified using linear regression and differences between treatments with t-statistics (Gill, 1978).

Urethral catheters were inserted and urine collected 1 h before and hourly for 8 h after the first feeding of Z. Urine volume was recorded and five 1.5 ml aliquots stored at -21^{O} C until analysis. Concentration of Z "equivalents" was determined by the method discussed in section IV. Briefly, conjugated Z was cleaved to free Z and glucuronic acid by incubation of urine with 3000 enzyme units β-glucuronidase/ml urine. Z was extracted from urine with ethyl acetate. Then the ethyl acetate-Z solution was dried to residue under nitrogen. Residue was resolubilized in 40% methanol and concentration of Z determined using direct competitive ELISA.

Results and Discussion

The production of antibody to Z after initial and booster immunizations is depicted graphically in Figure 7. Immunized pigs in this experiment had much greater antibody titers, and responded more rapidly to the booster immunogen than those pigs immunized by Pestka et al. (1985). This could be because we immunized with 30 to 40 subcutaneous/intradermal

injections in the loin area instead of five to six subcutaneous injections in the neck and rear flank as prescribed by Pestka et al. (1985). Differences in antibody titer could also be partially explained by procedures used to estimate titer. Antibody titer in our experiment was defined as the intersection of an ELISA antibody dilution series with .1 absorbance units (Figure 7). Pestka et al. (1985) defined antibody titer as the last well in an antibody dilution curve to yield visually distinct color. Our use of spectrophotometry and graphic techniques for determining titer allowed a higher and probably more accurate determination of the endpoint of antibody titration. In addition, production of antibody may have been greater in this experiment due to greater antigenicity of immunogen. The molar ratio of Z:BSA was 50:1 in our experiment, as compared to 20:1 in Pestka et al. (1985).

Ingestion of Z did not reduce detectable titer (Table 2). A decrease in detectable titer has been observed in similar experiments with digoxin (Schmidt and Butler, 1971) and aflatoxin B_1 (Ueno and Chu, 1978). From these results, I concluded that dose of Z was small relative to circulating concentrations of antibody. The cross-reactivity of the glucuronide form of Z with antibodies generated against Z has not been determined, however I assume that this cross-reactivity would be negligible. Since Olsen et al. (1985a) could not detect any free Z in serum 30 min after ingestion of 80 μg Z/kg BW (detection limit of .4 ng/ml), it is

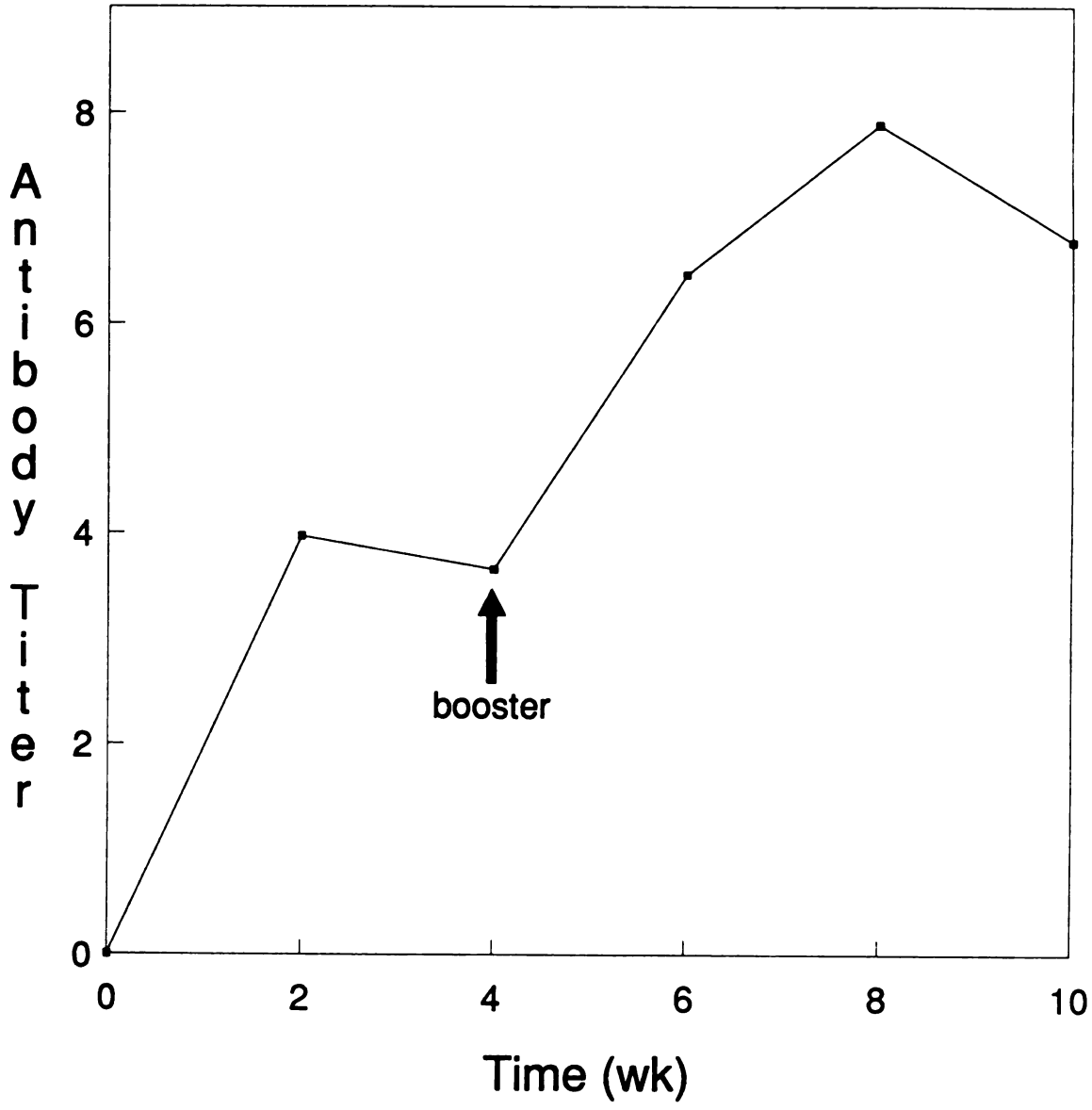


Figure 7. Production of antibody against zearalenone (Z). Pigs were immunized at wk 0 and wk 4 with a Z-bovine serum albumin conjugate. Antibody titers were expressed as log (1/ serum dilution). Additional procedures are in methods and materials.

reasonable that insufficient Z would be bound to antibody to cause a detectable decrease in detectable antibody.

Effect of immunization on height of VE is presented in Table 3. There was no difference in height of VE between immunized and nonimmunized gilts fed no Z. Both nonimmunized Z-treated and immunized Z-treated pigs had higher height of VE after 3 d than their respective controls fed no Z ($P<.01$). In addition, immunized Z-treated pigs had greater height of VE than nonimmunized Z-treated pigs after 3 ($P<.05$) and 10 d ($P<.005$). Therefore, immunization increased response of VE to Z for 3 d during treatment with Z, and this potentiated response was maintained for at least 7 d following withdrawal of dietary Z. In the preliminary experiment in which a dose response of VE to Z was observed, 150 μg Z/kg BW caused an increase in height of VE near the maximal response of VE to Z (Table 1). Therefore, the bioassay was relatively insensitive for detecting greater estrogenic effect, and immunization must have substantially increased estrogenic effect of Z on VE to be detected.

Effect of immunization on total excretion of Z equivalents in urine is in Table 4. In 8 h following ingestion of 150 μg Z/kg BW, immunized and nonimmunized pigs excreted approximately 18 and 7% of Z ingested, respectively. One immunized pig (no. 8) excreted 51% of ingested Z in this 8 h period. Since this was two standard deviations away from the mean excretion rate for immunized pigs, pig 8 was not included in statistical analysis.

TABLE 2: EFFECT OF DIETARY ZEARALENONE (Z) ON DETECTION OF ANTIBODIES SPECIFIC TO ZEARALENONE

Immunized gilts	Time after initial feeding of Z, d	
	0	3
	Antibody titer, absorbance units (414 nm)[a]	
Z[b]	.36[c]	.34[c]
NZ[e]	.44[d]	.42[d]

[a] Antibodies specific to zearalenone determined using indirect competitive ELISA.
[b] Zearalenone (150 µg/kg BW) meal fed daily for 3 d .
[cd] Slopes calculated between rows with same superscripts; slopes between rows with different superscripts not different.
[e] NZ = no zearalenone.

Therefore, mean excretion of Z was 13.6% of ingested dose in immunized pigs. Variance was heterogeneous between treatments for percentage of ingested dose excreted; however, coefficients of variation were similar, therefore, Lorhding's test was used to distinguish differences between treatments (Gill, 1978). Immunized gilts excreted a larger percentage of ingested Z than nonimmunized pigs ($P<.05$).

Effect of immunization on mean hourly excretion of free and total Z in urine is shown in Table 5. One nonimmunized pig excreted 1.1% of ingested Z as free Z. This was almost 9% of the total Z excreted by this pig in this period. This pig also excreted more total Z than any other nonimmunized pig. Only one immunized pig excreted a detectable amount of free Z, however this amount was negligable relative to Z ingested or total Z excreted. The total amount of free Z excreted in

TABLE 3: EFFECT OF IMMUNIZATION ON RESPONSE OF VAGINAL EPITHELIUM TO ZEARALENONE

Treatment[b]	Time after initial feeding of Z (d)[a]		
	0	3	10
	Height of vaginal epithelium (μm)[c]		
NI/NZ (3)[d]	24.2 (.3)	21.4 (3.2)[e]	23.8 (.2)
I/NZ (3)	23.0 (2.0)	25.0 (2.4)[g]	27.6 (2.7)
NI/Z (8)	21.0 (1.4)	64.4 (5.4)[fi]	44.0 (7.7)[k]
I/Z (8)	22.4 (7.0)	81.6 (4.9)[hj]	89.2 (11.4)[l]

[a] Zearalenone (150 μg/kg BW) meal fed for 3 d.
[b] NI = nonimmunized; I = immunized; NZ = no zearalenone and Z = zearalenone.
[c] mean with standard error of mean in parenthesis.
[d] number in parenthesis is number of pigs in treatment.
[e,f] means within column with different superscripts differ (P<.01).
[g,h] means within column with different superscripts differ (P<.05).
[i,j,k,l] means within column with different superscripts differ (P<.05).

TABLE 4: EFFECT OF IMMUNIZATION ON TOTAL EXCRETION OF ZEARALENONE EQUIVALENTS IN URINE

Pig Number	Pig Weight (kg)	Urine Excreted (ml)	Total Z Excreted (μg)[a]	Percentage Z Excreted
Immunized Pigs				
1	77.2	1178	2131.7	18.4
2	87.8	938	2773.8	21.1
3	65.0	606	1175.8	12.1
4	87.0	688	1191.5	9.1
5	80.4	580	1082.5	9.0
6	75.2	1182	1359.0	12.0
7	66.2	1690	1225.3	12.3
8	73.0	1690	5619.0	51.3
Mean	76.5	1069	2069.8	18.2[b]
Nonimmunized Pigs				
9	68.0	1493	571.6	5.6
10	91.0	1462	1246.1	9.1
11	84.8	3170	1485.2	11.7
12	120.0	1179	714.8	4.0
13	99.2	1831	783.9	5.3
14	69.0	1185	568.1	5.5
15	107.2	1906	1207.4	7.5
Mean	91.3	1746	939.6	7.0[c]

[a] Total excretion was the sum of 8 hourly excretions; hourly excretion was defined as concentration of "zearalenone equivalents" in hourly sample multiplied by volume of urine excreted per h.

[b,c] Means within a column with different superscripts differ ($P<.05$).

free form by immunized and nonimmunized pigs was negligable relative to Z excreted in the glucuronide form, therefore, Z was excreted mainly in the glucuronide form.

Immunized pigs excreted more conjugated Z in the 8 h following administration of Z than nonimmunized pigs. This was not due to immunized pigs excreting large quantities of Z in 1 h only. Mean hourly excretion of Z in urine was relatively consistent for both treatments in this period and appeared to be greater for immunized pigs than for nonimmunized pigs.

Percentage of Z excreted in 8 h by nonimmunized pigs (7.0%) was lower than that reported previously (15%) when a gilt was fed 80 μg Z/kg BW (Olsen et al., 1985b). However, mean hourly rates of excretion were similar between experiments. Nonimmunized pigs in this experiment excreted 117.5 μg/h and the gilt reported by Olsen et al. (1985b) excreted 96.6 μg/h. Therefore, at these concentrations of ingested Z, rate of excretion may be a function of maximal ability to metabolize and excrete Z, rather than a function of dietary intake of toxin.

The results from this experiment are difficult to interpret because immunization seemingly caused two opposite responses to occur. Estrogenic effect of Z on VE was potentiated with immunization, but excretion of Z equivalents was also increased. These results could have been achieved if immunization increased absorption of Z across the gastro-intestinal tract. The larger effective dose of Z would

TABLE 5: EFFECT OF IMMUNIZATION ON MEAN HOURLY EXCRETION OF FREE[a] AND TOTAL[b] ZEARLENONE EQUIVALENTS IN URINE

	Free Z, μg		Total Z, μg	
Time	Nonimmunized[c]	Immunized	Nonimmunized	Immunized
0	.0	.0	0.0	0.0
1	.3	.0	61.1	166.5
2	2.5	.0	81.9	180.5
3	3.3	.0	95.0	310.5
4	3.7	.0	138.6	326.1
5	2.4	.0	87.3	272.1
6	2.9	.0	173.7	256.4
7	2.3	.0	152.2	242.4
8	1.9	.0	149.9	281.9
Mean	2.7	.0	117.5	254.6

[a] Free Z determined by exclusion of β-glucuronidase from the ELISA procedure.

[b] Total Z determined by inclusion of β-glucuronidase in the ELISA procedure.

[c] Only one nonimmunized pig excreted detectable concentrations of free Z.

increase response of VE and could also increase the amount of Z conjugated to glucuronic acid and excreted from the body. This theory is not supported by my previous observation that rate of excretion of Z in nonimmunized gilts may be independent of dose (above 80 μg/kg BW). The increased excretion of total Z from immunized pigs could have been due to increased renal clearance of the glucuronide form of Z, however, I speculate that hepatic metabolism of Z was greater in immunized pigs, and resulted in increased conjugation of Z to glucuronic acid. If availability of Z to glucuronidation enzymes was increased, then availability of Z to reducing enzymes might also be increased. This would result in an increase in the biological activity of that Z retained within the body

It has been well documented that immunizing against a hapten will increase circulating concentrations of antigen (Adekunle and Chu, 1979; Butler, 1982), probably due to reduced distribution of hapten to peripheral tissues (Pardridge, 1981). I speculate that longer transit time through liver (5 sec versus 1 sec for brain), along with greater hepatic capillary membrane surface area, allowed for an increase in effective concentration of Z available for metabolism in the liver. Greater hepatic metabolism could result in increased conjugation of Z by UDP-glucosyltransferases, and increased Z excreted. In addition, more Z could have been metabolized by 3 α-dehydroxysteroid dehydrogenase to the more estrogenic form of Z, α-zearalenol.

α-Zearalenol has a much lower affinity than Z for antibodies generated against Z (Pestka et al., 1985), and therefore would become more widely distributed throughout peripheral tissues, including VE. Thus, total quantity of "estrogen equivalents" in VE would be increased simultaneously with an increase in excretion of urinary Z.

V. RECOMMENDATIONS

To more completely characterize effects of active immunization of swine against Z, an HPLC or LC procedure should be used to determine differences in excretion of urinary Z and Z metabolites. It would also be of value to correlate ELISA results for total urinary excretion of "Z equivalents" with total excretion of Z and Z metabolites from methods such as HPLC or LC.

We hypothesize that immunized pigs have increased hepatic conjugation of Z to glucuronic acid, thus these gilts would excrete a greater percentage of total toxin as Z, rather than α-zearalenol. If the concentrations of Z and Z metabolites were determined, I speculate that absolute urinary excretion of α-zearalenol would stay the same or decrease slightly. Normally, the major form of Z excreted by gilts in urine is α-zearalenol (Olsen et al., 1985b), however, immunized pigs probably excrete a higher proportion of Z.

From speculations discussed previously on mechanism by which immunization against Z increased both urinary excretion and estrogenic effect on VE, it is recommended that active immunization of swine against both Z and α-zearalenol be investigated as a potential method of prophylaxis for Z mycotoxicosis. Antibodies highly specific for Z and α-

zearalenol might increase hepatic glucuronide conjugation and facilitate excretion of both molecules. Antibodies specific for α-zearalenol would decrease distribution throughout peripheral tissues and perhaps neutralize in vivo estrogenic effects.

Alternatively, ovariectomized gilts could be passively immunized with MAb generated by Dixon et al. (1987). Since this antibody exhibits strong affinity for both Z and α-zearalenol, it might neutralize the effect of dietary Z on VE observed with active immunization against Z.

Monitoring serum concentrations of free and conjugated Z equivalents would be essential to determining mechanism by which immunization affects distribution and metabolism of Z. An ELISA should be developed to monitor serum concentrations of free and conjugated Z equivalents. Alternatively, existing HPLC (Trenholm et al., 1981), LC (Olsen et al., 1985b) or radioimmunoassay (Thouvenot and Morfin, 1983) procedures could be used for determining serum concentrations of Z. Concentrations of Z equivalents in urine and serum should be monitored for 4 or 5 d to maximize information on metabolism of Z.

In addition, a few animals should be sacrificed and the effect of immunization on distribution of Z in tissues determined. This could be performed using an HPLC procedure developed for detection of Z and Z metabolites in rat liver (James et al., 1982) and kidney (Smith, 1982). Alternatively, the procedure of Mirocha et al. (1982) could be used, whereby

tritiated Z was administered to broilers and radioactivity in various tissues quantified. This experiment would determine if immunization caused a redistribution of toxin such that concentration of Z in sensitive target tissues was higher than in nonimmunized controls.

I recommend that other response variables be investigated in conjunction with height of VE. Another variable which responds to Z is secretion of LH. In ovariectomized monkeys, an oral dose of 400 μg Z/kg BW depressed serum concentrations of LH (Hobson et al., 1977). A depression in serum concentrations of LH has also been observed in gilts fed 108 mg of Z daily on postmating d 11 to 15 (Long and Diekman, 1986) and in prepubertal gilts fed 10 ppm Z for 25 d (Green et al., 1987). However, preliminary experiments should be performed to determine if significant alteration in circulating concentrations of LH would be observed in OVX gilts after ingestion of 150 μg Z/kg BW. It would probably be advantageous to use both mean serum concentrations of LH and height of VE as response variables when determining the estrogenic effect of Z. It is conceivable that a method which neutralizes estrogenic effects of Z in the pituitary gland might not reduce effects of Z in vagina. This has been observed with monkeys immunized against estrogen, although one monkey of six had a small, but consistently higher concentration of serum LH (Cowchock et al., 1972).

VI. APPENDIX A. MODIFICATION AND VALIDATION OF ELISA

An experiment was performed to evaluate validity of standard curve from Warner et al. (1986) over the time to load two microtiter plates (30 min). Standard Z concentrations (0 to 500 ng/ml) were made in 70% methanol, and an equal volume added of 1:500 solution of Z-HRP in 1% ovalbumin-water. Tubes were gently inverted to facilitate equal distribution of Z and Z-HRP. A standard curve was added to MAb-coated wells every 10 min. After the fourth standard curve, a 30 min incubation at $37^{o}C$ was started. Plates were washed four times with PBS-Tween and color was developed as before. Results are depicted graphically in Figure 2. We concluded that standard curves were not valid for determining an unknown concentration of Z in samples pipetted 10 min after starting the standard curve. We hypothesized that Z-HRP bound irreversibly to MAb, or that the equilibrium constant for Z-HRP was much larger than that for free Z. Potentially this could be due to protein-protein interactions (hydrophobic, Vander Waals or electrostatic forces) between HRP and MAb molecules.

To combat possible irreversible binding of Z-HRP to MAb-coated wells, procedures of Warner et al. (1986) and Dixon et al. (1987) were modified such that Z standard curves were added in 70% methanol every 10 min for 30 min. At this time,

an equal volume of Z-HRP (1:500 in 1% ovalbumin-water) was added to each well in a 96 well microtiter plate using an octapipette. This decreased the difference for Z-HRP addition from 30 min to 45 sec. Howeverk, optical density increased very slowly and no standard curve was observed. It was hypothesized that 70% methanol denatured MAb, and as a result, Z-peroxidase was only bound nonspecifically.

To quantify effects of methanol on specific binding of Z-HRP by MAb, concentrations of methanol from 0 to 100% were added to MAb-coated well for 5 min. Wells were washed three times with PBS-Tween, and Z-HRP (1:250 in 1% ovalbumin-water) added. Wells were incubated 30 min at 37^{o}C, washed 4 times and bound peroxidase determined colorimetrically as before. Results are illustrated in Figure 4. A precipitous decrease in optical density above 40% methanol was observed. From these data, I suggest that concentrations of methanol above 40% denatured MAb to various degrees, thus decreasing specific binding of Z-HRP. It was concluded that Z standards could be generated in 40% methanol without substantially decreasing optical density. A concentration of 40% methanol was chosen rather than the lower concentrations to maximize solubilization of Z. Of interest is that a slight rise in optical density is observed between 70 and 100% methanol. This experiment was repeated three times and this effect was noted each time.

Another experiment was performed with Z standards (in 40% methanol) added to MAb-coated wells every 10 min for 30

min. Then an equal volume of Z-HRP (1:500 in 1% ovalbumin) added in a period of 45 sec. Results are in Figure 3. Variation between standard curves has been reduced considerably. and I concluded that standard curves are valid for determination of unknown concentrations of Z in the same plate. Both methylene chloride and ethyl acetate extracted Z with minimal extraction difficulties. Ethyl acetate was chosen because it is less dense than water, thus separating to the more easily removed top layer.

To quantify validity of extraction procedures, Z (0 to 500 ng/ml) was added to urine or 40% methanol. Urine was extracted two or three times with 500 μl ethyl acetate. The upper layer was removed, dried to residue under nitrogen, and resolubilized in 40% methanol. Extracts and standard curve in 40% methanol were analyzed by the modified ELISA procedure. Results are in Figure 8. It was observed that extraction efficiency was similar with two or three extractions and that ~100% of Z was recovered as compared with direct addition of Z to 40% methanol. It was concluded that urine would be extracted two times with 500 μl ethyl acetate.

An experiment was designed to validate release of Z from glucuronic acid with β-glucuronidase. Aliquots of naturally contaminated urine (500 μl) collected 5 h after feeding 150 μg Z/kg BW were incubated with 500 μl of .2 M sodium acetate and 10 μl β-glucuronidase solution (3000 units enzyme activity in 10 μl .2M sodium acetate buffer, pH 5.5) for 0, 8, 16, 24, or 30 h. Samples were extracted two times with

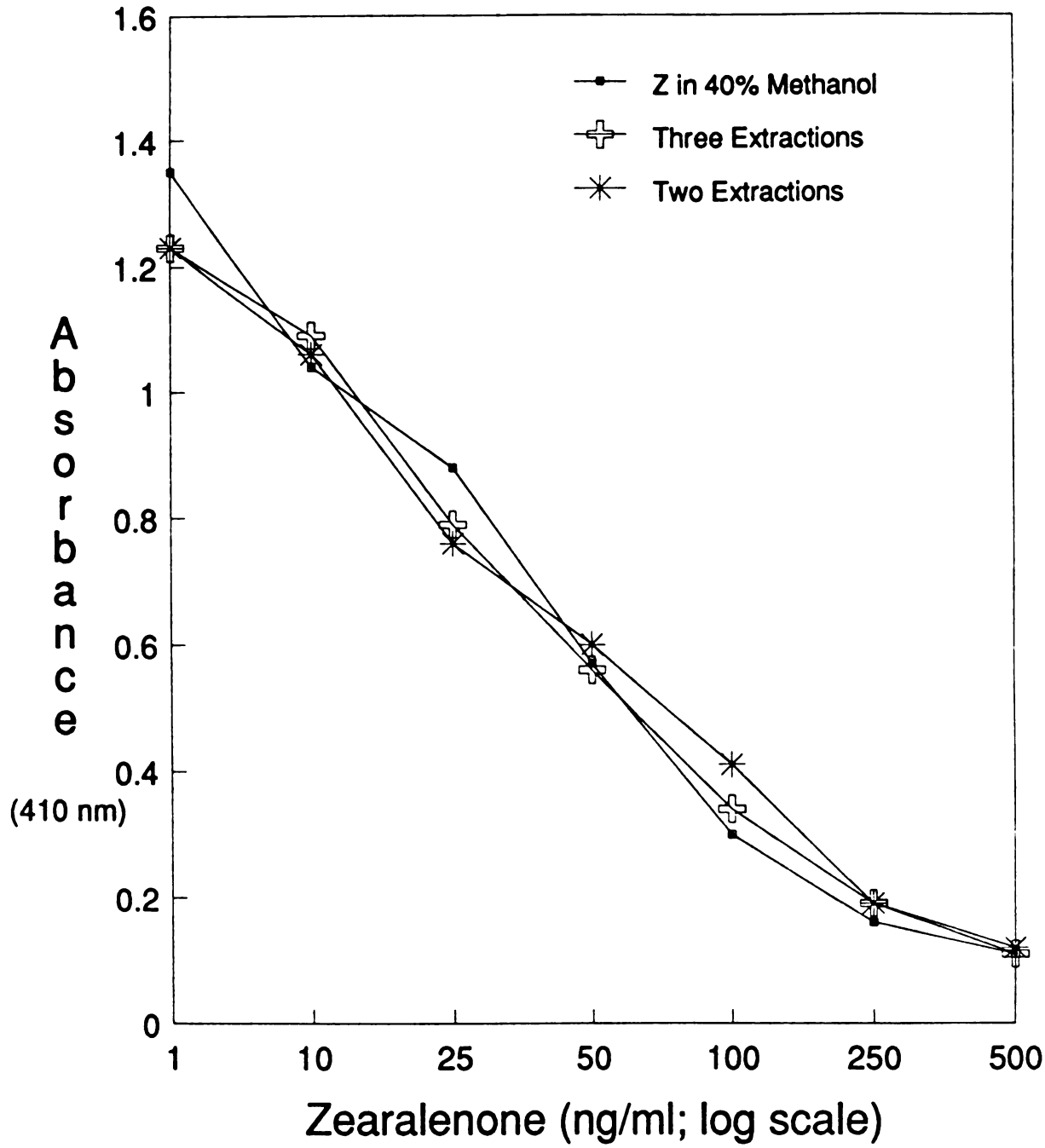


Figure 8. Efficacy of extraction procedure for zearalenone (Z) in urine. Two versus three extractions of crystalline Z from urine were compared to a standard curve (Z in 40% methanol). Zearalenone was added to urine or 40% methanol to make final concentrations of 1 to 500 ng/ml. Extraction of Z from urine was made with .5 ml ethyl acetate. Addition procedures are reported in methods and materials.

(n=20), respectively.

ethyl acetate and concentration of Z determined as before. Results are in Table 5. Uniform release of Z was observed at all time points.

Another experiment was performed to validate linear release of Z with increasing concentrations of Z. Two aliquots of .25, .5, 1, or 2 ml naturally contaminated urine was incubated for 18 h with 3000 enzyme units of β-glucuronidase as before. Z was extracted and concentration of Z determined as before. Results are in Figure 5. A linear release of Z was observed with increasing total Z. The correlation coefficent was .997 and the slope was 128.

An experiment was conducted to validate precision of estimates in different regions of the standard curve. A sample was diluted 1.67 to 50 fold and concentration of Z determined. Results were multiplied by their dilution factors to transform data back to an undiluted basis. Mean of estimates were 240.1 ng/ml, coefficient of variation was 5.2% and standard error of mean was 4.28. It was concluded that the area of standard curve in this range (4 to 140 ng/ml) was valid for estimating unknown concentrations of Z.

TABLE 6: EFFECT OF TIME ON RELEASE OF ZEARALENONE FROM URINE NATURALLY CONTAMINATED WITH ZEARALENONE

Time (h)	Z released, ng/ml[a]
0	19.7
8	129.7
16	120.5
24	125.8
32	132.6

[a] Coefficient of variation for zearalenone released at 8, 16, 24, 32 h was 4.1%.

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