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The influence of an orally administered antibiotic combination on the toxicity of warfarin to swine

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The influence of an orally administered antibiotic
combination on the toxicity of warfarin to swine

by

Gary Douglas Osweiler

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Veterinary Pathology
Major: Veterinary Pathology (Veterinary Toxicology)

Approved:

Signature was redacted for privacy.

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For the Graduate College

Iowa State University
Ames, Iowa

1973

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INTRODUCTION

Hemorrhagic diseases in swine have occurred sporadically in the past, and in 1969 and 1970 outbreaks of unusually large proportions were observed in Iowa and surrounding states. The syndrome was characterized by acute onset with lethargy, anorexia, lameness, hemorrhagic diarrhea, and death. Gross lesions in swine dying of porcine hemorrhagic disease were anemia, hemarthrosis, subcutaneous hematoma, intermuscular hemorrhages, and gastrointestinal bleeding. The blood characteristically failed to coagulate for as long as 12 to 72 hours. Preliminary clinical-pathologic examinations disclosed anemia and elevated prothrombin times, while platelet numbers and liver function tests were normal.

Prominent epizootiological features of the hemorrhagic disease included high incidence in weanling swine consuming pelleted feeds which contained antibiotic-sulfonamide drug combinations. Furthermore, hemorrhagic manifestations were ameliorated by administration of vitamin K. Mycotic cultures from suspect diets revealed several species of Aspergilli, but aflatoxin was not isolated from the feeds.

Experimental reproduction of the disease was accomplished by feeding suspected rations to swine under controlled conditions. Examination of coagulation factor activity revealed a hemostatic deficiency pattern similar to field cases of hemorrhagic disease and characteristic of warfarin poisoning or vitamin K deficiency.

Hemorrhage-inducing feeds were determined chemically to contain no warfarin or aflatoxin. However, compounds of molecular weight intermediate between aflatoxin and warfarin were isolated. The possibility of a natu-

rally occurring mycotoxin with anti-vitamin K activity similar to dicoumarol was suggested.

Research and clinical reports from human and veterinary medicine reflect conflicting evidence regarding the role of oral antibiotics in the production or exacerbation of vitamin K deficiency. Some investigators felt that antibiotic feeding could reduce vitamin K synthesis by intestinal microflora to a dangerously low level when prothrombin reserve was partially depleted by other conditions. Vitamin K requirements in young antibiotic-fed swine were not well established. Since warfarin poisoning could cause a clinical and pathological condition similar to this hemorrhagic disease, it seemed reasonable that an antibiotic influence upon warfarin poisoning could be related to the potential role of antibiotic feeding in spontaneous porcine hemorrhage. Evidence from the literature also indicated that warfarin can interact with or be influenced by many drugs, including antibiotics and sulfonamides. The activities of drug interaction, plasma protein binding, or enzyme induction had not been observed for warfarin and antibiotics in swine.

An experimental study was undertaken to determine the relationship of antibacterial feeding to exacerbation or alteration of vitamin K responsive hemorrhaging in swine. Specific objectives of the study were as follows:

1. Develop the use of warfarin as a model for induced hemorrhage in swine in order to simulate potential naturally occurring hypoprothrombinemia.

2. Determine a minimum clinically toxic dosage of warfarin in swine.

3. More fully characterize the clinical, hematologic, and pathologic changes in swine exposed to warfarin with comparison to other hemorrhagic diseases of swine.

4. Investigate the effect of normal feeding levels and high levels of oral antibiotics and sulfonamides on standard measures of blood coagulation activity.

5. Demonstrate whether any potential detrimental effect on blood coagulation occurs in swine exposed to a vitamin K antagonist (warfarin) and antibacterials concurrently.

6. Measure changes in plasma levels of warfarin when sulfonamide is used as a potential enzyme inducer.

REVIEW OF LITERATURE

Field and Experimental Porcine Hemorrhagic Diseases

In the period from October, 1967, to April, 1971, 28 instances of a porcine hemorrhagic condition were reported to the Iowa Veterinary Diagnostic Laboratory. The majority of these were accompanied by submission of porcine tissues or animals for examination. The incidence, clinical signs, lesions, and hematologic studies of those swine were reported by Osweiler et al. (1970). The disease was characterized by either acute onset of lameness and subcutaneous swelling or by high mortality in barrows from post-castration hemorrhage.

Similar outbreaks were described in Nebraska by Fritschen et al. (1970) and in Missouri by Muhrer et al. (1970). In the incidences described, the hemorrhage appeared to be caused by a clotting mechanism defect similar to hemophilia or dicoumarol poisoning. Osweiler et al. (1970) reproduced the disease in weanling swine by feeding the diet collected from a farm with affected swine. The condition was characterized by prolonged clotting time, elevated One-Stage Prothrombin Time (OSPT), and prolonged Activated Partial Thromboplastin Time (APTT).¹ A probable deficiency of Factor VII or Factor X was indicated. The hematologic and clotting factor changes were suggestive of vitamin K deficiency or warfarin poisoning as described by Deykin (1970a).

Deykin (1970a) reviewed the functions of vitamin K in the synthesis of procoagulants. He stated that Factors II, VII, IX, and X are dependent

¹Clinical Pathology Laboratory, McFarland Clinic, Ames, Iowa.

upon vitamin K activity for their synthesis. Furthermore, at high doses of warfarin or dicoumarol, synthesis of the same procoagulants ceases.

Rowsell (1968) stated that coagulation factor activity in swine is identical to that in man for standard test systems when considering Factors V, VII, and X and that coagulation factor activity for Factor IX is slightly increased for swine.

Didisheim et al. (1959) studied the effects of various sources of brain tissue thromboplastin upon different species of animals in studying coagulation factors and prothrombin time. The pattern of coagulation factor deficiencies observed in human patients with warfarin overdosage, animals with vitamin K deficiency, and the swine from field cases reported by Osweiler et al. (1970) were similar.

Porcine hemorrhage was reproduced by Muhrer et al. (1970) and by Fritschen et al. (1970) by feeding suspect diets from field cases. The clinical signs reported by Osweiler et al. (1970) and by Fritschen et al. (1970) were halted by administration of menadione sodium bisulfite therapy at 2 gm/ton of feed.

Epizootiological factors considered important in the onset of this condition were as follows:

1. Swine involved were primarily of weanling age and were consuming increasing amounts of feed.
2. Nearly all of the rations were pelleted complete feed.
3. Antibiotics were added to the feed with tylosin and sulfamethazine combinations most often used.

Related Diseases Characterized by Hemorrhage

Numerous outbreaks of disease with hemorrhagic manifestations have been reported in domestic animals. Schofield (1924) reported on a disease of cattle resembling hemorrhagic septicemia and blackleg. Subsequent studies revealed that the disease was associated with molded sweetclover hay. The mold was not toxic alone but only when associated with sweetclover. Link (1944) confirmed the association of sweetclover disease with molded, improperly cured hay. He isolated the toxic principle, later named dicoumarol, and elucidated its structure as 3,3'-methylene bis (4-hydroxycoumarin). Application of dicoumarol to rodent control and human thromboembolic cardiovascular disease resulted from these early findings according to Levine (1970).

A moldy corn toxicosis characterized by hematmata and intramuscular, renal, and hepatic hemorrhage was reported by Albright et al. (1964). However, hepatic lesions and lack of response to vitamin K were features of this disease.

Intestinal hemorrhage in swine was reported by Jones (1967) and characterized as an acute disease with extensive hemorrhage into the lumen of the small intestine in pigs 2 to 5 months in age. The disease was believed to be a hypersensitivity reaction or vitamin E deficiency. A hemorrhagic bowel syndrome was reported by O'Neill (1970) and by Van Ulsen (1971). Both workers discounted the possibility of infectious agents, most specifically Clostridium perfringens. Van Ulsen (1971) suggested a possible relationship to Scopoletin (7-hydroxy-6-methoxy-coumarin) which is found in various plants throughout nature.

Muhrer et al. (1942) studied an apparent hemophiliac-like disease in a strain of experiment station swine. In his work no dietary relationship was shown, and the condition was ascribed to a probable inherited Factor VIII deficiency.

The role of fungal toxins has been suspect in instances of hemorrhagic disease. Hesselstine (1969) reviewed the literature regarding mycotoxicoses in animals. Specific lesions of hemorrhage and elevated prothrombin time were noted only for a toxin from corn invaded by Chaetomium globosum. Christensen et al. (1966) reported that swine and rats were not susceptible to the Chaetomium toxin. Bamburg et al. (1968) isolated toxic spiroepoxy compounds from Fusarium tricinctum and observed internal hemorrhages caused by feeding the toxin to rats. Speer (1971) reviewed some conditions and possible toxins associated with mycotoxicosis and hemorrhage in swine. He indicated that field shelling of corn, recently increased in the Midwest, enhanced its vulnerability to storage fungal growth. Speer (1971) noted that a scirpenol-like compound was isolated from swine feeds associated with hemorrhagic disease reported by Osweiler et al. (1970). Bababunmi and Bassir (1969) determined an effect of aflatoxin B₁ on blood coagulation in the rat. The extrinsic system of coagulation was found defective in a pattern similar to changes induced by warfarin.

Matschiner and Doisy (1962) found a tendency for vitamin A to increase the clinical signs of vitamin K deficiency in rats as measured by a decreased prothrombin activity.

Natural and experimental warfarin poisoning in swine has been studied in a limited manner. Warfarin poisoning in swine was reported by Frazier (1966). The disease was characterized by lameness, weakness, normal appe-

tite, and hemorrhages on the pelvic musculature and under the renal capsule. McGirr and Papworth (1955) studied warfarin poisoning in various species including the pig. Swine were found susceptible to warfarin administered at 0.37 mg/kg body weight for six days. Symptoms and lesions were variable but included inappetence, hematomata, lameness, and bloody feces. Articular and intramuscular hemorrhage were the most common lesions.

Mushett and Seeler (1947) studied the effect of sulfaquinoxaline on rats after removal of the gastrointestinal tract and found suppression of prothrombin activity within 24 hours after administration of the drug. Sulfanilamide and sulfamerazine failed to produce hypoprothrombinemia within four weeks. When the surgically treated rats were not given sulfaquinoxaline, the body stores of prothrombin appeared sufficient for several days. The authors suggested that these results indicated a direct effect of sulfaquinoxaline on the prothrombin complex. They also noted that sulfonamides, by nature of their antibacterial activity, might indirectly cause vitamin K deficiency by curtailing synthesis of vitamin K in the intestine of animals fed vitamin K-deficient diets.

Griminger (1957) suggested that oral sulfaquinoxaline-induced hypoprothrombinemia could be reversed by vitamin K. Griminger and Donis (1960) demonstrated that vitamin K₁ would effectively reverse sulfaquinoxaline-induced hypoprothrombinemia of poultry. Menadione sodium bisulfite was found by Griminger (1965) to be as effective as vitamin K₁ in reversing the hypoprothrombinemic effect of sulfadimethoxine. Nelson and Norris (1959) found that in poultry menadione sodium bisulfite corrected clotting time but not hemorrhages.

Dempsey and Sanford (1960) and Sadek et al. (1955) observed hemorrhages in poultry when various levels of antibiotics were fed. Buck (1969) reviewed hemorrhagic diseases of poultry and concluded that the two probable factors involved are hypoprothrombinemia and thrombocytopenia. He cited evidence indicating that stress factors including drugs or antibiotics may be directly or indirectly implicated.

Forgacs et al. (1958), Hinshaw (1956), Schumaier et al. (1961), and Forgacs and Carll (1955) reported on possible relationships of mycotoxins to hemorrhagic disease in poultry.

Vitamin K Deficiency in Livestock and Poultry

Induced hypoprothrombinemia in animals and man is generally regarded as a result of vitamin K deficiency from several possible causes. Finkle (1961), Rowsell (1968), and Deykin (1970a) stated that vitamin K is responsible for hepatic synthesis of clotting factors II (Prothrombin), VII (Proconvertin), IX (Plasma Thromboplastin Component), and X (Stuart-Prower Factor). Thus, any condition interfering with synthesis, absorption, or utilization of vitamin K will be manifest as a decrease in clotting factor activity. Green (1966) listed possible causes of vitamin K deficiency, including sulfaquinoxaline intake, dicoumarol anticoagulant therapy, ulcerative colitis, sprue, obstructive jaundice, hepatic damage, and vitamin A excess.

Dam et al. (1937) found that rats, dogs, and guinea pigs were resistant to dietary vitamin K deficiency. Green (1966) stated that a practical method for inducing vitamin K deficiency in the rat was via supplementation

of the diet with sulfaquinoxaline or a similar drug. His rationale was that sulfonamides inhibit microbial synthesis of vitamin K.

Greaves (1939) created a surgical shunt of the bile duct to the colon in rats and produced hemorrhage in rats given a vitamin K-deficient diet. The rats continued to excrete fecal vitamin K even when fed a vitamin K-deficient ration. Cover et al. (1955) cited evidence that avitaminosis K in the chick was caused by a diet low in vitamin K combined with prevention of coprophagy. This view is supported by Deykin (1970a) who extensively reviewed the anticoagulant literature and concluded that the two primary sources of vitamin K are diet and intestinal microflora.

Porter (1946) ascribed vitamin K potencies to various bacteria. In relation to an alfalfa standard, Escherichia coli was 3 times as potent for vitamin K as was alfalfa, while Bacillus subtilis was 38 times more effective than alfalfa as a vitamin K source. Almquist et al. (1938) also demonstrated an antihemorrhagic factor in E. coli and B. subtilis. Goodwin (1963) and Isler and Wiss (1959) felt that the bacterial synthesis of vitamin K depended on the composition of intestinal bacterial flora. Castle (1967) and Almquist et al. (1938) expressed the traditional view that in man vitamin K is ordinarily obtained from bacterial action in the intestine, and little is required from the diet. Barnes et al. (1959) experimentally demonstrated that rats resist dietary vitamin K deficiency by consuming their own feces. Coprophagy prevention for two weeks resulted in a rise in prothrombin time. Mameesh and Johnson (1959) also found that coprophagy prevention in rats on a purified diet greatly exacerbated vitamin K deficiency. They suggested that vitamin K was not absorbed from the lower intestine or colon but must be ingested and absorbed at higher enteric

sites. Scott (1966) stated that the most important factor modifying the dietary vitamin K requirement is the synthesis of vitamin K by gastrointestinal microflora. Gustafsson et al. (1962) fed E. coli and Sarcina-like micrococci to vitamin K-deficient germ-free rats and reversed vitamin K deficiency within from 24 to 48 hours.

The role of antibiotics in vitamin K deficiency has been of interest in various species. Schendel and Johnson (1962) studied the vitamin K requirements of young swine. Pigs fed a purified diet deficient in vitamin K developed signs of hypoprothrombinemia, which could be prevented by administration of menadione sodium diphosphate at 5 μ g/kg body weight daily. The use of sulfathiazole at 5 gm/kg of dry feed or oxytetracycline at 60 mg/kg of dry feed did not conclusively influence the onset of vitamin K deficiency in the pig. Griminger (1965) found that in chicks fed low levels of vitamin K the inclusion of tetracycline caused a significant increase in severity of hemorrhages in poultry suffering from hemorrhagic disease. Similar effects were obtained for zinc bacitracin and oxytetracycline.

Haden (1957) described a human patient on heavy post-operative antibiotic therapy who developed an elevated prothrombin time and Factor VII deficiency after 12 days of treatment. Response to vitamin K₁ therapy was good. Kabins (1972) concluded that tetracycline, ampicillin, and other oral antibiotics could cause suppression of intestinal synthesis of vitamin K₁ resulting in the hypoprothrombinemia of vitamin K deficiency.

Koch-Weser and Sellers (1971b) extensively reviewed work in the area of drug interactions and concluded that the contribution of bacterial vitamin K synthesis is important when dietary intake of vitamin K is restricted.

They postulated that the hypoprothrombinemic state in patients on coumarins might be potentiated by antibiotic-induced reduction of microfloral synthesis of vitamin K. This could happen even though the antibiotic does not increase the prothrombin time of normal subjects.

In man, potentiation of drug-induced anticoagulant effect has been shown for chloramphenicol, chlortetracycline, neomycin, and sulfonamides. Koch-Weser and Sellers (1971a) cited evidence that chloramphenicol retards the transformation of bishydroxycoumarin, while neomycin results in decreased absorption of vitamin K. In man, they found no evidence of sulfonamides decreasing intestinal bacterial synthesis of vitamin K. Kabins (1972) reviewed evidence that sulfonamides affect plasma binding of oral anticoagulants and enhance their efficacy.

Antibiotic Toxicity in Swine

Numerous studies of antibiotics in swine rations have been conducted. Of these, very few have involved a study of the coagulation system itself. Anderson et al. (1965) reported on the toxicologic properties of tylosin. Rats were given 10,000 parts per million tylosin in the feed for two years with no significant changes in growth, viscera, or hematology. Dogs were able to tolerate 400 mg/kg/day for 2 years with no visceral damage. Studies of the hematologic changes in dogs were not reported. Mayrose et al. (1966) studied the effects of tylosin on swine reproduction. They fed tylosin at 220 mg/kg of food for 1 week pre-mating to 3 weeks after mating. The pigs were weaned with a greater weaning weight than littermate controls. No effect on blood components or hemostasis was indicated. Jordan and Waitt (1963) administered 500 mg of tylosin per day to

sows for 5 days pre-partum until 2 weeks post-partum. A higher percentage of pigs from tylosin-fed sows survived, but no report was given on the hematologic effects of tylosin. Melliere et al. (1968) fed sulfamethazine alone and sulfamethazine in combination with tylosin and observed an increase in gain significant to the 0.05 level. No data on hematology was obtained. Conrad and Beeson (1960) observed the effects of chlortetracycline and oxytetracycline at high levels in a protein supplement for growing and finishing swine. They noted increased feed intake and gain and increased average daily gain at ranges of 50 to 250 gm/ton. No effect on blood coagulation or other hematologic factors was observed. Hays and Speer (1960) studied the effect of spiramycin on growth and feed utilization in swine and compared its effect to aureomycin and oxytetracycline. No report on hemostatic effects nor hematologic evaluation was given.

There appears to be a relative lack of studies of the coagulation factors and hemostatic function mechanisms in swine, specifically with regard both to vitamin K deficiency and the effects of antibiotics on the production of vitamin K by intestinal bacteria. The studies of Schendel and Johnson (1962) appear to be the only specific work regarding antibiotics and their effect on vitamin K deficiency in young swine.

Drug Effects on Coumarin Anticoagulants

Drugs may alter the effect of coumarin anticoagulants. Koch-Weser and Sellers (1971a) summarized the ways in which coumarin and drug interactions may be influenced as follows:

1. The bioavailability of vitamin K may vary as in decreased bacterial synthesis of vitamin K.

2. The metabolic fate of coumarins may be modified by such things as absorption, protein binding, biotransformation, and excretion.
3. Prothrombin complex synthesis or catabolism may be altered.
4. The receptor affinity for coumarins at their site of action may be influenced.
5. Nonprothrombin-dependent hemostatic mechanisms may be changed.

Deykin (1970b) reviewed the literature concerning therapy with the anticoagulant drug warfarin and concluded that drugs can influence the response of patients to warfarin. A drug may displace warfarin from its binding site and increase the concentration of free warfarin, or the affinity of receptor sites for coumarin or warfarin may be changed. There may also be competition between drugs for degradation sites on the endoplasmic reticulum, suppression of degradative enzymes by an administered drug, or increases in enzymes associated with the endoplasmic reticulum. Changes in the protein synthesis mechanism and impaired thrombocyte function are other possible drug effects.

Conney (1969) in reviewing drug metabolism and therapeutics observed that chronic administration of a drug can enhance the metabolism of other drugs or increase its own metabolism after a period of continued administration. He stated that induction of microsomal liver enzymes, especially in the endoplasmic reticulum, can change the effect of a specific drug when multiple agents are administered.

Coumarin drugs are metabolized at widely varying rates. Conney (1969) and Solomon (1969) studied variations in the metabolism of coumarin anticoagulant drugs. They reported that there may be a genetic difference in the ability of subjects to metabolize warfarin. Solomon (1969) found that

the metabolism of warfarin and bishydroxycoumarin is extensively carried on by hepatic microsomal enzymes.

Nagashima and Levy (1969) studied the comparative pharmacokinetics of coumarin anticoagulants in the rat, dog, monkey, and man by following the half-life for plasma warfarin. They concluded that warfarin and bishydroxycoumarin are probably subject to the same major biotransformation pathways and that the half-life varies widely among species. They found further that warfarin concentrations in plasma decline exponentially with time in each species. However, there was a variation in the way individual species handled increased doses of warfarin. Conney (1969) stated that the acute toxicity of warfarin and bishydroxycoumarin in animals was reduced when phenobarbital was used as an inducing drug.

Koch-Weser and Sellers (1971a) reported that coumarins are hydroxylated by hepatic microsomal mixed-function oxidases. Several ring-hydroxylated inactive metabolites of warfarin may be found in urine, and 7 fluorescent chromatographically-separate compounds have been detected in the urine of warfarin-treated subjects. Hypoprothrombinemia was not influenced by the hydroxylated form in rats. Four prime hydroxycoumarin was one-fourth as potent as warfarin for the production of hypoprothrombinemia in the rat. These authors stated that in man the metabolites of warfarin have a negligible effect on prothrombin status. Ullrich and Staudinger (1968) confirmed that metabolism of warfarin in the rat involved transformation to 6, 7, and 8-hydroxy warfarin by liver microsomes and that NADPH and molecular oxygen were required. They also found that the metabolites of warfarin are pharmacologically inactive.

Koch-Weser and Sellers (1971a) concluded that individual differences in coumarin half-life reflect differences in the rate of biotransformation of the coumarin drug. Thus, the degree of induction by a drug is accurately reflected by the change in half-life of a coumarin provided no other metabolic interaction is present. About one week of exposure to an inducing agent may be required for maximal induction in most cases. When the inducer is withdrawn, several weeks are needed to return to normal status as compared to controls.

The binding of coumarin drugs to plasma proteins may be a significant factor in the response of animals to doses of anticoagulant coumarin drugs. Koch-Weser and Sellers (1971a) reviewed the plasma binding of warfarin and other coumarin drugs. They found that coumarins are bound to plasma proteins and become pharmacologically inactive, protected from biotransformation and excretion, and excreted only when the unbound drug is available in the plasma in equilibrium with its site of action. Only unbound drug is susceptible to glomerular filtration. The interaction of warfarin with plasma albumins and globulins largely partitions the warfarin into the plasma, and binding to plasma proteins involves approximately 97 percent of the total drug in the plasma. Albumin binding is readily reversible, and the total plasma-bound reservoir is gradually released as biotransformation lowers the concentration of free drug.

Koch-Weser and Sellers (1971a) found no drugs which enhance coumarin binding. They reviewed the mechanism by which drugs displace coumarin from binding sites in the plasma and thereby potentiate its hypoprothrombinemic action. Displacing drugs may either compete with coumarin for the same binding sites on albumin or bind to albumin at sites remote from coumarin

action but which induce tertiary structural changes in the albumin precluding or decreasing coumarin binding. Drugs or metabolites which displace coumarin are generally accumulated to high levels in the plasma. Most of the important displacing drugs are acidic compounds with an ionized acidic group at physiological pH. Because all coumarins are highly bound, displacement of even a small amount of coumarin causes a large increase in free drug concentration, thus markedly enhancing toxicity. Administration of strongly displacing drugs to individuals continuously exposed to coumarins may markedly increase concentration of free coumarin and cause severe hypoprothrombinemia and bleeding.

Increased displacement of warfarin makes a greater amount of drug available for hepatic biotransformation and glomerular filtration. This shortens the plasma half-life, and excretion may exceed intake, with the result that plasma concentration declines. The effect of high initial free coumarin concentrations may be offset by an increased rate of coumarin removal.

A number of drugs are known to increase the displacement of warfarin from the plasma binding sites. Aggeler et al. (1967) reported that phenylbutazone administration significantly decreased the plasma concentration and biological half-life of warfarin, but the anticoagulant effect was increased markedly. They felt that phenylbutazone displaced warfarin from human plasma albumin binding and acted to increase the effect of warfarin by making greater quantities of free warfarin available to drug metabolizing enzymes or to biologic action sites.

Sher (1971) reviewed the literature and tabulated an extensive list of drugs associated with enzyme induction and drug interactions. Included

among these were drugs which caused displacement of warfarin from plasma albumin binding sites. Both phenylbutazone and tolbutamide were found to decrease the binding of coumarins to plasma proteins. The potentiation of coumarin by tolbutamide in dogs was followed by decreased sensitivity to anticoagulant associated with very low levels of plasma coumarin. This latter stage was probably due to a stimulatory effect of tolbutamide on warfarin metabolism. Solomon et al. (1968) found that phenylbutazone decreased plasma warfarin half-life by binding to the same site on plasma protein. Sher (1971) included sulfonamides among the drugs listed as causing displacement of warfarin from plasma binding sites.

Sher (1971) listed drugs which antagonize the action of warfarin via their effect on hepatic microsomal enzyme induction. Included among these were phenobarbital and pentobarbital. Ullrich and Staudinger (1968) also found that pre-treatment of rats with phenobarbital increased the rate of 3-hydroxylation reactions of warfarin. Welch et al. (1969) studied the interaction of drugs with bishydroxycoumarin in dogs. They found that phenobarbital caused a decrease in prothrombin time and plasma bishydroxycoumarin concentration after 9 days on phenobarbital. When phenobarbital was removed, none was detectable in the blood after 3 days, but the depression of prothrombin time and bishydroxycoumarin concentration continued for 6 to 10 days. Kabins (1972) reviewed evidence for increased warfarin metabolism after griseofulvin administration resulted in hepatic enzyme stimulation.

Stowe (1965), reviewing the metabolism and excretion of sulfonamides, stated that phenylbutazone competes with sulfonamides and with warfarin for plasma binding sites. He found that the main metabolic site for sulfo-

namides is the liver. Acetylation is the most important form of metabolism, and acetylated derivatives are therapeutically inactive and less soluble. A secondary pathway involves oxidation of the benzene ring of sulfonamides to a quinone structure which is then reduced to a hydroxylated form. The phenolic group formed is conjugated, either with sulfate or with glucuronic acid. Both of these are therapeutically inactive.

Dalgaard-Mikkelsen et al. (1953) studied the distribution of sulfathiazole and sulfamethazine in swine. They found high concentrations of sulfonamides in the kidney and liver. The degree of acetylation for sulfamethazine was greater than for sulfathiazole in each of the tissues. Stowe (1965) stated that protein binding is a major factor in the distribution of sulfonamides in the body fluids and tissues. Loss of the unbound fraction results in resupply from the bound portion. Thus, for both sulfonamides and warfarin, hepatic metabolism and plasma albumin binding appear to play a major role in the transformation, biologic activity, and excretion of these drugs.

Robinson et al. (1971) investigated the interaction of warfarin and nonsystemic gastrointestinal drugs. They found that concomitant administration of a second drug would alter the absorption of an anticoagulant. This could occur when intestinal pH or motility, drug solubility, and anticoagulant binding to the drug itself were changed as warfarin entered the small intestine and was rapidly converted to its anionic form. Thus, consideration of enzyme induction effects must also take into account the possible diminished absorption of warfarin due to complexing in the intestinal lumen.

MATERIALS AND METHODS

Experimental Swine

Eighty-eight crossbred pigs ranging from 12.1 to 26.0 pounds in initial weight were utilized. Swine were obtained from the Iowa State University Swine Nutrition herd and from 2 commercial swine herds. All controls and principals were confinement reared, weaned within the week prior to procurement, and prevented access to green forage, pasture, hay, or alfalfa meal. Animals were confined for seven days to accustom them to the new surroundings and ration. All swine were housed in concrete-floored pens which were cleaned and washed daily.

To ensure complete consumption of the ration, feed was limited to 6 percent of body weight daily in each pen. After selection of pigs for various trials, the animals were weighed and placed in the appropriate pens.

Experimental Ration

During the acclimation period, the experimental basic ration was made available to the swine. This ration was free of antibiotics until a time appropriate to each trial. The ration was a standard mixture.¹ The formula for this feed was as follows:

Ingredients

Ground Yellow Corn	78.25 lbs
50% Soybean Oil Meal	18.50 lbs
Vitamin Premix	0.50 lb

¹Swine Grower Ration, Swine Nutrition, Iowa State University, Ames, Iowa.

Calcium Carbonate	0.90 lb
Dicalcium Phosphate	1.25 lbs
Iodized Salt	0.50 lb
Trace Mineral Mix	<u>0.10 lb</u>
	100.00 lbs

Calculated Analysis

Protein	16.14%
Calcium	0.72%
Phosphorus	0.54%
Vitamin A	1532 I.U.
Vitamin D ₂	300 I.U.
Riboflavin	2.6 mg/lb
Calcium Pantothenate	6.8 mg/lb
Niacin	18.2 mg/lb
Choline Chloride	407.0 mg/lb
Vitamin B _{1 2}	10.0 mcg/lb

Experimental Drugs

Antibacterial drugs for the feeding trials were supplied by a commercial company.¹ The drugs used were tylosin phosphate at 100 grams (low level) and 1,000 grams (high level) per ton of feed and sulfamethazine sodium at 100 grams (low level) and 300 grams (high level) per ton of feed. The antibiotics were used in combination and were mixed in the feed supply at appropriate levels for the various trials.

¹Tylan[®] and Tylan-sulfa[®], Eli Lilly and Company, Greenfield, Indiana.

Warfarin (3-[a-acetylbenzyl]-4-hydroxycoumarin) was obtained as the pure reagent-grade compound.¹ It was dissolved in ethanol and added to cellulose powder² to make a slurry. The mixture was evaporated at room temperature to leave a dry powder containing 1.0 percent warfarin on a dry-weight basis. This preparation was stored in glass in a darkened cabinet at room temperature.

Clinical and Sampling Methods

All swine were observed twice daily for clinical signs of warfarin poisoning. Clinical response was recorded daily. When animals were judged to be beyond humane maintenance, euthanasia was performed.

Blood samples were collected throughout the experiment utilizing an ocular bleeding technique described by Huhn et al. (1969). Blood was collected at 1:10 dilution in 0.1 molar sodium oxalate and immersed in an ice bath immediately after collection. Plasma was separated from erythrocytes by centrifugation and removed to cold storage (4° C) within 30 minutes after collection. All laboratory procedures for coagulation factor activity were performed within 1 hour or less after collection, as recommended by Quick (1966).

In those swine judged moribund, a blood sample was taken immediately before euthanasia.

¹K & K Biochemicals, Plainview, N.Y. 11803.

²Brinkman Instruments, Westbury, N.Y. 11590.

Laboratory Procedures

Blood coagulation time was determined using the Lee and White technique described by Benjamin (1961). The One-Stage Prothrombin Time (OSPT) developed by Quick (1966) and the Activated Partial Thromboplastin Time (APTT) described by Coles (1967) were used to assess the activity of vitamin K-dependent or warfarin-sensitive coagulation factors.

Correction tests utilizing barium sulfate-adsorbed plasma and aged serum were conducted by the technique of Sirridge (1967). Blood for this technique was collected from normal swine.¹ Normal plasma and the correcting reagents were examined for OSPT and APTT activity and stored in 3-ml aliquots. Correction tests were conducted using a fibrometer to detect initial clot formation.²

Total protein and fibrinogen levels were determined with a protein refractometer according to the technique outlined by Benjamin (1961).

Smears for differential leukocyte counts were made from fresh undiluted blood and evaluated by counting proportions of different white cells in 100 total leukocytes. Preparation and staining was according to Schalm (1965).

Microhematocrit tubes were used as described by Benjamin (1961) to measure packed cell volume. Clot retraction was assessed by observation of the clotted blood sample in glass tubes at 2 and 24 hours after collection.

¹ Meat Science Laboratory, Iowa State University, Ames, Iowa.

² BBL Fibrometer, BIOQUEST, Cockeysville, Md. 21030.

Necropsy and Collection of Tissues

Swine dying of hemorrhage were necropsied as soon as possible after death. Any pigs found moribund were stunned with electrical shock and euthanized by exsanguination. Where possible, blood was collected for analytical procedures.

Tissues for histopathologic examination were collected and immersed in 10 percent buffered formalin. Portions of the brain, liver, kidney, lung, pancreas, stomach, intestine, colon, body lymph nodes, muscle, and subcutaneous tissue were taken.

Histologic Procedures

Tissues were fixed in 10 percent formalin, embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin.

Statistical Evaluation

Swine were selected from herds of origin either at random or for litter differences. Assignment of pigs to pens and treatments was at random using a table of random numbers from Snedecor and Cochran (1967).

Statistical treatment of data from laboratory procedures was by analysis of variance. F-values were determined from computed sums of squares and mean squares for litter, treatment, day, and day by treatment interaction.¹ Statistical significance was assigned to F-values according to Snedecor and Cochran (1967). Where missing data were encountered, a weighted analysis was computed.

¹Statistical Laboratory, Iowa State University, Ames, Iowa.

Experimental Design

Trial 1: Establishment of a warfarin dose-response curve

Ten weanling swine were selected and assigned at random to various peroral daily doses of warfarin on a milligrams per pound (mg/lb) of body weight basis. The levels were as follows:

<u>Number of pigs</u>	<u>Daily warfarin dose in mg/lb body weight</u>
1	0.00
2	0.05
2	0.10
2	0.15
1	0.20
1	0.30
1	0.50

Pre-trial blood samples were taken and additional samples were drawn at 3-day intervals thereafter until completion of the trial. Pigs were observed for 14 days if death did not occur sooner. Measurements made included clotting time, One-Stage Prothrombin Time (OSPT), Activated Partial Thromboplastin Time (APTT), packed cell volume (PCV), and differential leukocyte count.

Trial 2: Determination of a subclinical dose level of warfarin

Four swine were given warfarin daily at levels of 0.0075 mg/lb for each of 2 animals and 0.0125 mg/lb for each of 2 animals. Similar clinical and laboratory procedures were conducted as for Trial 1. An attempt was made to select the dose of warfarin causing slight increase in coagulation time but no clinical hemorrhage. The trial was continued for 12 days with

samples taken before and at 3, 6, and 11 days after warfarin feeding started.

Trial 3: The effect of high and low levels of tylosin-sulfamethazine combination on pigs fed subclinical levels of warfarin

Thirty-two swine were used in this trial. Four pigs were selected at random from each of 8 litters. Pigs of the same litter were assigned to pens of 2 each and each pen assigned to one of 4 treatments in a split plot design.

The 4 treatment combinations in this experiment were as follows:

<u>Number of swine</u>	<u>Treatment</u>
8	Warfarin + Low Level Tylosin-Sulfamethazine
8	Warfarin + High Level Tylosin-Sulfamethazine
8	Control + Low Level Tylosin-Sulfamethazine
8	Control + High Level Tylosin-Sulfamethazine

Warfarin at 0.0075 mg/lb body weight was fed daily to individual swine on a body weight basis which was redetermined weekly. Warfarin was mixed with a small amount of feed and hand fed to insure that consumption was complete.

Parameters measured in this trial were coagulation time, OSPT, APTT, PCV, total protein, fibrinogen, and differential leukocyte count. One pig from each litter was euthanized and necropsy and histologic examinations conducted at the termination of the trial. Pre-trial and once-weekly blood samples were taken during the 28-day experiment.

Trial 4: The effect of high feed levels of tylosin-sulfamethazine on clinical warfarin poisoning

Changes in coagulation time, OSPT, APTT, clot retraction, and PCV were evaluated. Sixteen swine selected to account for random litter differences were utilized. Four pigs were selected at random from each source litter. The daily dosage of 0.025 mg warfarin per pound of body weight was mixed in a portion of the ration and hand fed individually to assure consumption of the dose. Blood was collected immediately before the trial and at 4, 7, 11, 14, and 21 days thereafter.

Clinical response, necropsy lesions, and histopathologic findings were recorded where appropriate to the affected animals.

Trial 5: The effect of high levels of tylosin-sulfamethazine on clinical warfarin poisoning

The number of swine, experimental design, clinical effects, necropsy findings, and hematologic parameters were similar to Trial 4. However, litter control was not obtained, so these 16 animals were selected at random from a large group of recently weaned pigs.

Fifty milligrams of 1 percent warfarin was mixed in each pound of feed. This permitted a warfarin concentration of 0.5 mg/lb of feed. Pigs were fed at 5 percent of body weight giving a resultant dose of 0.025 mg/lb warfarin daily to each animal. Pigs in each pen were divided from one another when feeding was carried out. At intervals of 3 days, the weight and appropriate feed allotment were redetermined.

Trial 6: The effect of injectable sulfamethazine on warfarin poisoning

Clinical and necropsy procedures were similar to Trials 3, 4, and 5. OSPT was used as the indicator of changes in the coagulation system. Oxa-

lated plasma saved for determination of warfarin levels was frozen and stored at -20° C.

Swine were dosed individually with the appropriate 0.025 mg/lb level of warfarin. Blood samples for warfarin and OSPT determinations were drawn pre-trial and at days 5, 7, 9, 12, 14, 16, 19, 21, and 26 of the trial.

Sulfamethazine sodium¹ at 250 mg/ml was administered parenterally to 4 of the 8 pigs fed warfarin. Each pig received 25 mg/lb body weight of sulfamethazine sodium. Administration of sulfamethazine was begun 5 days prior to warfarin feeding and was continued for 26 days. Warfarin administration began day 5 of the trial and was terminated at day 19.

Trial 7: The effect of sulfamethazine on plasma levels of warfarin

Two swine were fed a single dose of warfarin at 1.25 mg/lb body weight. This high dose was chosen in order to assure analytical detection of warfarin in plasma and to permit quantitative measurement of the decrease in plasma warfarin with time. One of these swine was given daily injections of sulfamethazine sodium at 25 mg/lb body weight for 5 days prior to and throughout the trial. Blood samples were drawn before and at 0.5, 1.5, 2.5, and 4.5 days after warfarin administration. Plasma samples were examined for OSPT and for quantitative presence of warfarin.

Estimation of Plasma Warfarin

Four ml portions of exalated plasma were extracted with 20 ml 1,2-ethylene dichloride² (EDC) according to the procedure described by Welling et al. (1970). The EDC was separated by centrifugation at 200 rpm

¹ Sulmet[®], American Cyanamid, Princeton, N.J. 08540.

² Eastman Organic Chemicals, Rochester, N.Y. 14650.

for 20 minutes. The amount of EDC recovered was measured and that quantity evaporated to dryness with nitrogen. The sides of the container were rinsed with acetone and dried with nitrogen. The residue was dissolved in acetone and spotted for thin-layer chromatography as outlined by Lewis et al. (1970). The locus of blue fluorescence for warfarin was marked and the spot cut out and immersed in 2 ml acetone for 20 minutes. The concentration of warfarin was determined fluorometrically¹ as described by Lewis et al. (1970).

The calculation of warfarin concentration in plasma was as follows:

1.
$$\frac{\text{sample } \Delta \text{ fluorescence} - \text{blank } \Delta \text{ fluorescence}}{\text{standard fluorescence} - \text{blank fluorescence}} \times \frac{\mu\text{g warfarin}}{\text{ml standard}} = \mu\text{g warfarin}$$
2.
$$\frac{\mu\text{g warfarin}}{\text{ml plasma}} \times \frac{\text{ml EDC used}}{20 \text{ ml}} = \frac{\mu\text{g warfarin}}{\text{ml plasma}}$$

Change in fluorescence after acidification of the sample is signified by the symbol Δ fluorescence. Results calculated were plotted versus time.

¹ Aminco-Bowman Spectrophotofluorometer, American Instrument Company, Inc., Silver Spring, Md.

RESULTS

Clinical Signs and Gross Lesions in Swine Fed Warfarin

Clinical signs associated with warfarin poisoning were observed in Trials 1, 2, 4, 5, 6, and 7. The clinical signs in warfarin-poisoned swine were lethargy, lameness, subcutaneous swellings, soft fluctuating articular enlargements, epistaxis, melena, ocular and auricular hemorrhage, recumbency, anemia, and sudden death. The number and degree of clinical signs varied slightly in the various trials.

Trial 1

The effects in swine of different levels of warfarin is summarized in Table 1. The term traumatic hemorrhage refers to ocular hemorrhage as a result of the bleeding technique employed. Traumatic hemorrhage was also observed in the ear where metal identification tags had been placed. Dried and fresh blood covered the facial and lateral cervical areas of affected pigs. These swine consumed water frequently, and water in the drinking pans became blood tinged. The sclera of affected pigs became extremely pale, and the skin of white animals lost the normal pink color.

Hematomata, articular swellings, epistaxis, and melena were observed. A wide range of clinical signs including lameness, stiffness, lethargy, recumbency, dark tarry feces, and anorexia was noted in these pigs.

Only one animal recovered after being clinically affected by spontaneous bleeding. One pig at the 0.05 mg/lb level of warfarin appeared to be recovering from clinical hemorrhage but died suddenly. Pulmonary edema was observed at necropsy. Both hemolytic and smooth colony types of Escherichia

Table 1. Clinical response of swine to various levels of warfarin

Pig number	Warfarin dose in mg/lb	Days on trial															
		1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1251	0.05	0 ^a	0	0	h ^b	h	0	h	0	h	H ^c	H ^e	H	d ^d			
1256	0.05	0	0	0	h	h	h	h	H	0	0	D ^e					
1252	0.10	0	0	0	H	H	d										
1257	0.10	0	0	0	H	H	H	D									
1253	0.15	0	D														
1258	0.15	0	0	0	H	H	H	H	h	0	0	0	0	0	0	0	0
1254	0.20	0	0	H	D												
1255	0.30	0	0	H	H	d											
1260	0.50	0	0	H	H	h ^f	H	D									
1259	0.00	0	0	0	0	-	0	0	0	0	0	0	0	H	h	D	

^aNo clinical signs of hemorrhage.

^bTraumatic hemorrhage.

^cSpontaneous hemorrhage.

^dEuthanasia due to severe hemorrhage.

^eDeath.

^fBegan administration of warfarin at 0.025 mg/lb body weight.

coli were isolated from the intestine, and a diagnosis of edema disease was made.

Pig 9 was changed from the control diet to one of warfarin at 0.025 mg/lb body weight on day 7 of the trial. After 8 days at that level of warfarin, lameness occurred in the left pelvic limb, and the animal died after 10 days of warfarin administration.

Lesions of warfarin poisoning were found in 8 of 10 swine in Trial 1. Pigs 6 and 8 did not have gross lesions of hemorrhage. The type and distribution of lesions found in 8 swine with warfarin poisoning are summarized in Table 2. The most frequent lesions were anemia, hematmata, and articular hemorrhage.

Trial 2

Of 4 pigs given warfarin at 0.0075 mg/lb body weight and 0.0125 mg/lb body weight, none had clinical signs or lesions of warfarin poisoning. One pig developed diarrhea on day 12 of the trial, and this persisted throughout the experiment. Gross lesions of hemorrhage were not found in swine from Trial 2.

Trial 3

Pigs in this trial received warfarin at 0.0075 mg/lb and levels of tylosin and sulfamethazine as high as 1000 and 300 grams per ton of feed, respectively. Neither clinical signs nor lesions of warfarin poisoning developed during the 21 days of the experiment nor during the 7-day observation period after drugs were withdrawn. Appetite and general appearance were good. One pig from each of the 8 litters was necropsied, and no gross lesions were detected.

Table 2. Gross lesions in swine fed various levels of warfarin

Pig number	Warfarin dose in mg/lb	Gross lesions						
		Anemia	Colonic hemorrhage	Intra-muscular hemorrhage	Hematomata	Ocular hemorrhage	CNS hemorrhage	Hemorrhage in other areas
1259	0.025	++ ^a			+++ ^b			++
1251	0.050	+ ^c		+++			++	
1252	0.100	++				+++		+
1257	0.100	+++		++		+++	+	
1253	0.150	+++	+++		+++		++	
1254	0.200	++	++		+++		++	+
1255	0.300	++			+++		++	+
1256	0.500	++	+				++	

^aMarked.

^bSevere.

^cModerate.

Trial 4

The clinical signs in swine given warfarin at 0.025 mg/lb body weight are presented in Table 3. In the treatment group with tylosin-sulfamethazine added, no clinical signs or deaths were noted during a 20-day observation period, while all swine in the control (warfarin only) group had died by day 14. Spontaneous hemorrhage was observed in a majority of the control pigs. Four swine from the tylosin-sulfamethazine group were necropsied, and mild lesions of hematoma were found in two pigs. The type and degree of hemorrhagic lesions found in Trial 4 are recorded in Table 4.

Trial 5

No adverse clinical signs were observed in swine fed tylosin-sulfamethazine in combination with warfarin. Of pigs fed warfarin alone, two had died of hemorrhage by day 17 while the remainder of the group was free of clinical signs throughout the 23-day observation period. Please note Table 4.

Trial 6

Four of the 8 swine receiving warfarin were injected with sulfamethazine sodium at 25 mg/lb. All swine given only warfarin had hemorrhagic lesions, and 3 of these 4 pigs died. Only 1 of the 4 pigs injected with sulfamethazine was affected with hemorrhages. The type and degree of clinical signs observed are summarized in Table 5.

Table 3. Clinical signs in swine fed warfarin at 0.025 mg/lb in presence or absence of antibacterial combination^a

Treatment	Pig number	Days on trial																
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Antibacterial	751	0 ^b	0	0	0	0	0	0	0	0	0	0	0	0	0	H ^c	H	0
	752	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	753	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	754	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	755	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	756	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	757	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	758	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No Antibacterial	759	0	0	0	D ^d													
	760	0	0	0	0	0	h ^e	h	h	h	D							
	761	H	H	H	d													
	762	D																
	763	0	0	0	0	0	H	H	H	H	D							
	764	0	H	H	H	H	H	H	H	D								
	765	H	H	h	H	H	H	D ^f										
766	0	0	0	0	0	h	d ^f											

^aTylosin phosphate, 1000 grams/ton feed and sulfamethazine sodium, 300 grams/ton feed.

^bNo clinical signs of hemorrhage.

^cSpontaneous hemorrhage.

^dDeath.

^eTraumatic hemorrhage.

^fEuthanasia due to severe hemorrhage.

Table 4. Gross lesions in swine fed warfarin at 0.025 mg/lb in presence or absence of an antibacterial

Treatment	Pig number	Anemia	Colonic hemorrhage	Hematomas	Intra-muscular hemorrhage	Ocular hemorrhage	Hemarthrosis	Thoracic hemorrhage	CNS hemorrhage	Epistaxis
Trial 4 (No antibacterial)	759	++ ^a	+++ ^b							
	760		+ ^c	+++	++	++	++			
	761	+		++	++	+	+			
	762	+	++	+++	++					
	763	++		+++	++				++	
	764	++	+	+++	++					
	765	++	+++					+		
	766	++		+++	++			+		
Trial 5 (No antibacterial)	1608	++	+++							+
	1613	++				++			+	
Trial 6 (No antibacterial)	1681	++	+			+		+		
	1684	++	+++					+		
	1686	++				+				
Trial 6 (Antibacterial present) ^d	1687			+++	+		+			

^aMarked.

^bMassive.

^cModerate.

^dSulfamethazine sodium, parenteral at 25 mg/lb body weight.

Table 5. Clinical signs in swine fed warfarin at 0.025 mg/lb in presence or absence of parenteral sulfamethazine sodium

Treatment	Pig number	Days on trial												
		0	5 ^a	14	15	16	17	18	19 ^b	20	21	23	26	33
Sulfamethazine 25 mg/lb/day	1682	0 ^c	0	h ^d	0	0	0	0	0	0	0	0	0	0
	1683	0	0	0	0	0	0	0	0	0	0	0	0	0
	1685	0	0	0	0	0 ^e	0	0	0	0	0	0	0	0
	1687	0	0	h	h	d ^e								
No Sulfamethazine	1681	0	0	H ^f	H	D ^g								
	1684	0	0	h	h	h	h	h	h	D				
	1686	0	0	h	h	h	D							
	1688	0	0	h	h	h	h	h	h	0	0	0	0	0

^aInitiation of feeding warfarin at 0.025 mg/lb body weight.

^bCessation of feeding warfarin.

^cNo clinical signs of hemorrhage.

^dTraumatic hemorrhage.

^eEuthanasia due to severe hemorrhage

^fSpontaneous hemorrhage.

^gDeath.

Trial 7

On the fourth day after receiving 1.25 mg/lb warfarin, the control pig died from hemorrhage caused by coagulation failure. The pig given sulfamethazine survived a similar warfarin dose with no ill effects.

Survival Rate and Scope of Lesions
in Warfarin-Poisoned Swine

The number of swine that had survived to days 7, 14, and 21 in all trials where warfarin at 0.025 mg/lb was administered either alone or in conjunction with tylosin-sulfamethazine combination can be found in Figure 1.

The incidence and type of lesions in 14 swine dead from warfarin poisoning are summarized in Figure 2. The occurrence of lesions in decreasing order of frequency were anemia, colonic hemorrhage, subcutaneous hematomata, intramuscular hemorrhage, ocular hemorrhage, hemarthrosis, intrathoracic hemorrhage, CNS hemorrhage, epistaxis, and hemorrhage in other areas.

Gross and Microscopic Lesions
in Warfarin-Poisoned Swine

Anemia in affected pigs apparently was caused by hemorrhage. Important sites of blood loss were from the eye and colon and into subcutaneous or intermuscular spaces. No gross inflammatory reaction was observed in the colon, periorbital tissue, or subcutaneous spaces.

Contents of the colon and ileum were often dark brown to black, tarry, and foul smelling. This characteristic colonic hemorrhage is seen in Figure 3.

Figure 1. Number of swine surviving warfarin administration (0.025 mg/lb) when tylosin-sulfamethazine was present or absent in the diet

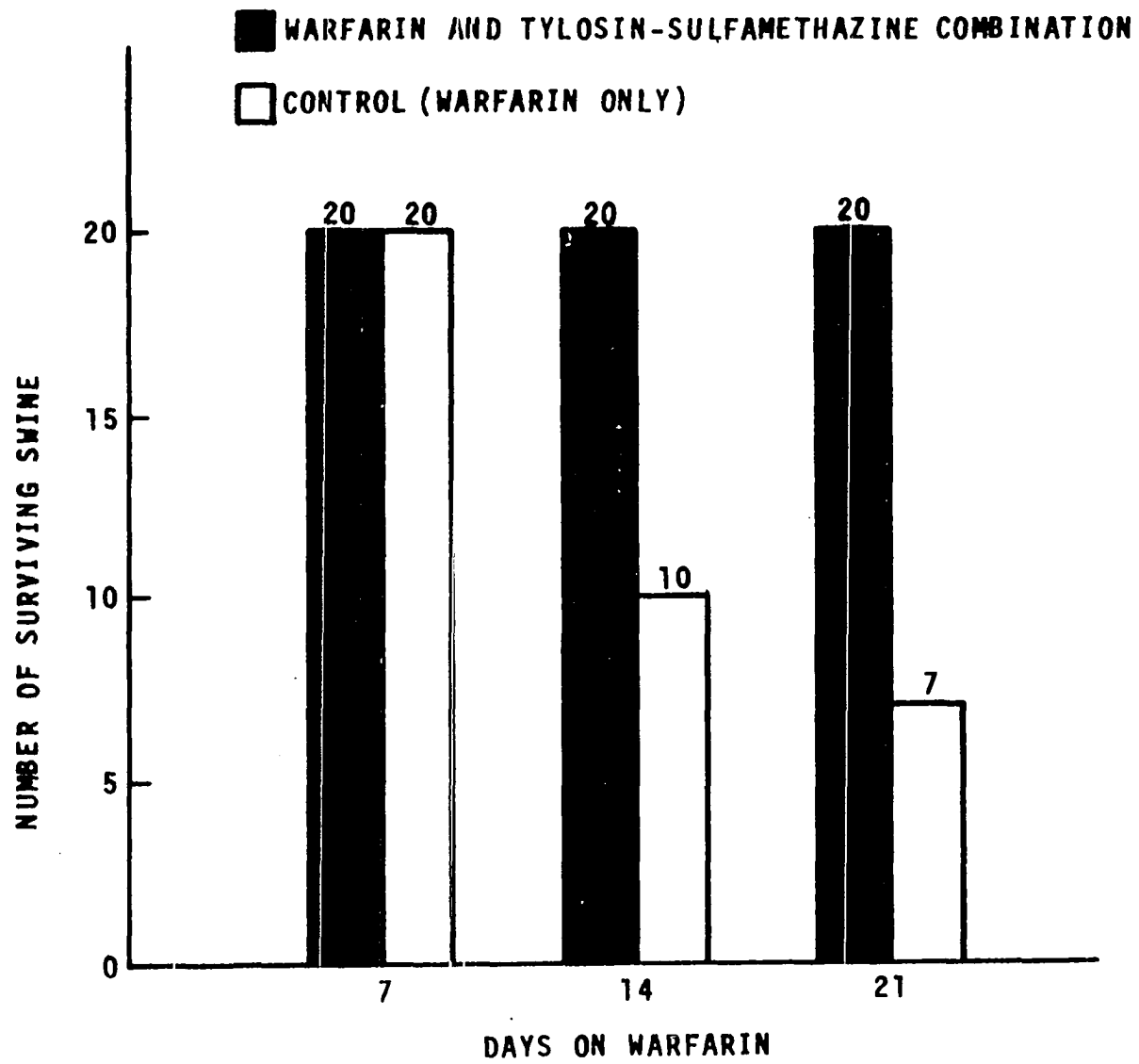


Figure 2. Incidence of specific hemorrhagic lesions in 14 swine dying from warfarin poisoning

SWINE AFFECTED FROM AMONG 14 EXAMINED

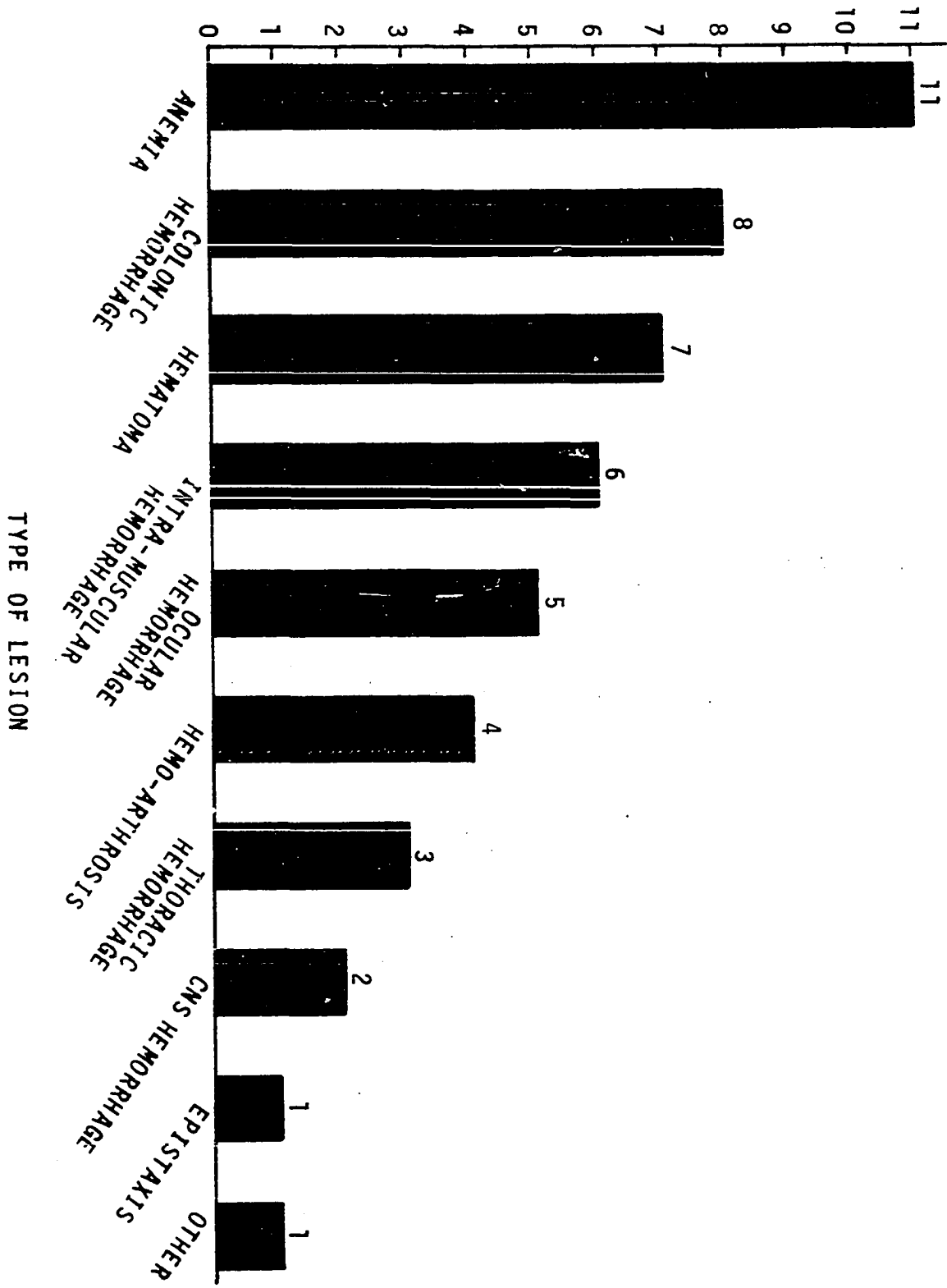
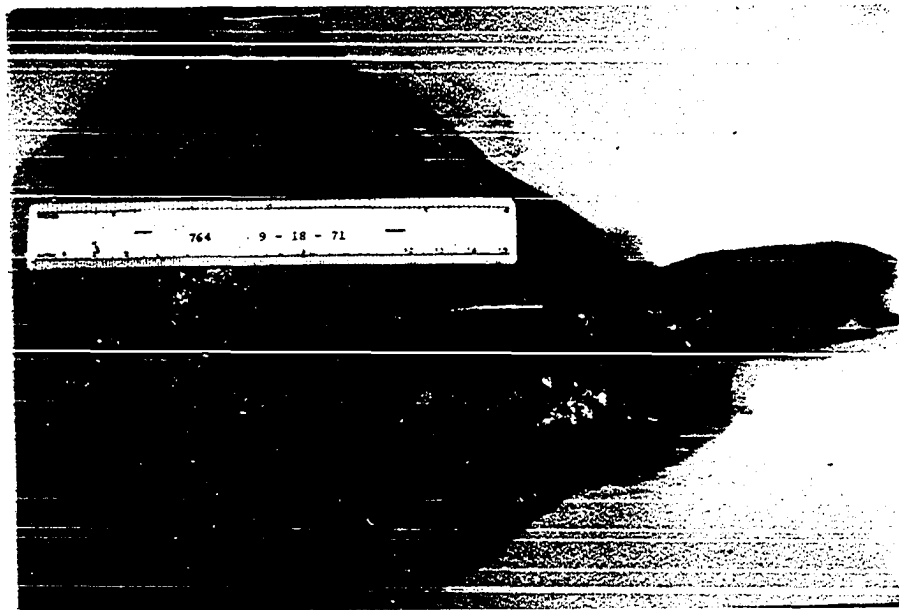


Figure 3. Marked colonic hemorrhage, blood-stained feces, lesser omental hemorrhage, and generalized anemia characteristic of swine with experimental warfarin toxicosis. The warfarin dosage was 0.20 mg/lb body weight

Figure 4. Subcutaneous and intermuscular hemorrhages in a pig fed warfarin at 0.10 mg/lb body weight



The intermuscular and subcutaneous hemorrhages were interspersed with small to moderate amounts of edema. Hemorrhages tended to follow along fascial planes and between major muscle bundles. Most frequent locations for hemorrhages were the submandibular area, subcutaneous inguinal area, and intermuscular spaces between the semitendinosus, semimembranosus, and gracilis muscles. Distribution of subcutaneous and muscular lesions may be found in Figures 4 and 5.

Hemorrhage in the subcutaneous periarticular tissue caused swelling around the tarsal-metatarsal articulations. Bleeding into the articular capsule and collection of free, unclotted blood in the articular space were observed.

Hemorrhage in the central nervous system (CNS) was subdural in nature over the parietal and occipital lobes of the cerebrum, cerebellum, and cervical spinal cord. Hemorrhage into CNS parenchyma was not observed. The gross lesion of cerebellar meningeal hemorrhage is shown in Figure 6.

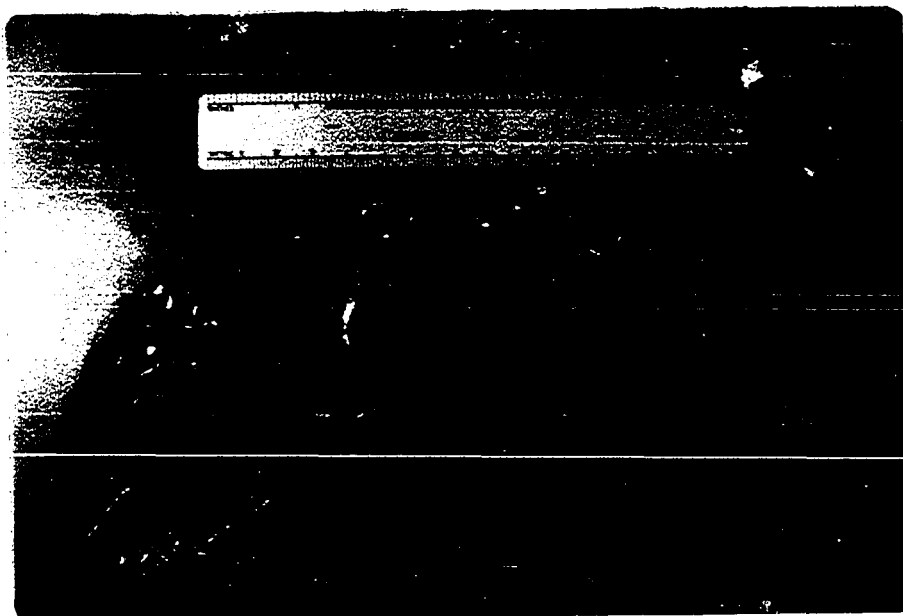
Tissues of warfarin-poisoned swine were examined microscopically for cellular and vascular changes. No evidence of a primary lesion due to warfarin was found by light microscopy. Mild centrolobular hepatic fatty change and passive congestion were occasionally observed. Degenerative or inflammatory vascular lesions in hemorrhagic areas were not found. Intermuscular edema and hemorrhage were observed microscopically.

Blood Coagulation Activity in Warfarin-Poisoned Swine

Affected swine had large and consistent changes in parameters of the coagulation system. Increases in clotting time, OSPT, and APTT were observed.

Figure 5. Large periarticular hematomata in a pig given at 0.025 mg/lb body weight

Figure 6. Hemorrhages in cerebellar subdural and arachnoid spaces in a pig with warfarin poisoning. The warfarin dosage was 0.10 mg/lb body weight



Trial 1

Results of clotting time, OSPT, and APTT are presented in Table 6 for pre-trial samples and for days 3, 6, and 9. Data presented are individual values where one animal was used or mean values when two animals were used. Figures 7, 8, and 9 depict mean values of clotting time, OSPT, and APTT, respectively, for pre-trial, three-day, and six-day samples. Coagulation activity times increased in approximately linear manner at day 6 for dosages of warfarin from 0.0125 mg/lb to 0.150 mg/lb. Small increases in OSPT and APTT were observed at most dosages when pre-trial values were compared to day 3 values.

Trial 2

Coagulation activity is included in Figures 7, 8, and 9 for blood samples from days 0, 3, and 6.

Trial 3

Mean values for clotting time, OSPT, and APTT are presented in Figures 10, 11, and 12. No animals were lost in this trial, so means represent a full complement of data. Marked differences in treatment means were not observed. Clotting time was elevated slightly at day 7 and then declined toward pre-trial levels. OSPT values followed a similar pattern of slight early rise followed by a decline. APTT values were erratic, and no trend could be detected.

Trial 4

Mean values for clotting time, OSPT, and APTT are presented in Figures 13, 14, and 15, respectively. Large and consistent rises occurred in these

Table 6. Blood coagulation parameters in swine fed warfarin

Pig num- ber	Warfarin dose in mg/lb	Days on trial											
		0			3			6			9		
		Clot ^a	OSPT ^b	APTT ^c	Clot	OSPT	APTT	Clot	OSPT	APTT	Clot	OSPT	APTT
1251	0.05	1.5	13.4	34.0	3.5	12.7	39.8	4.5	28.9	78.8	5.5	47.4	150.0
1252	0.10	1.5	12.4	29.1	3.0	10.8	37.4	19.5	63.7	152.7			
1253	0.15	1.0	12.3	32.4	4.0	12.2	49.9	17.5	68.9	165.7			
1254	0.20	0.5	12.1	33.7	1.0	11.1	43.1	26.0	150.0	240.9			
1255	0.30	1.0	11.5	32.9	3.5	11.5	53.2	42.5	248.5	580.6			
1256	0.05	1.0	12.8	37.1	2.5	12.3	61.2	12.5	34.0	172.2	9.0	81.1	150.0
1257	0.10	1.5	12.1	34.7	3.5	12.2	55.5	11.5	61.4	242.0			
1258	0.15	2.0	10.8	28.1	4.5	11.5	50.8	16.0	72.7	340.3			
1259	0.00	1.0	12.6	35.2	4.5	11.9	41.7	2.5	11.9	43.1	2.0	12.9	44.3
1260	0.50	1.0	12.0	39.1	4.0	11.9	53.7	32.5	211.9	360.0			

^a Clotting time in seconds.

^b One-stage prothrombin time in seconds.

^c Activated partial thromboplastin time in seconds.

Figure 7. Clotting time for swine fed graded doses of warfarin

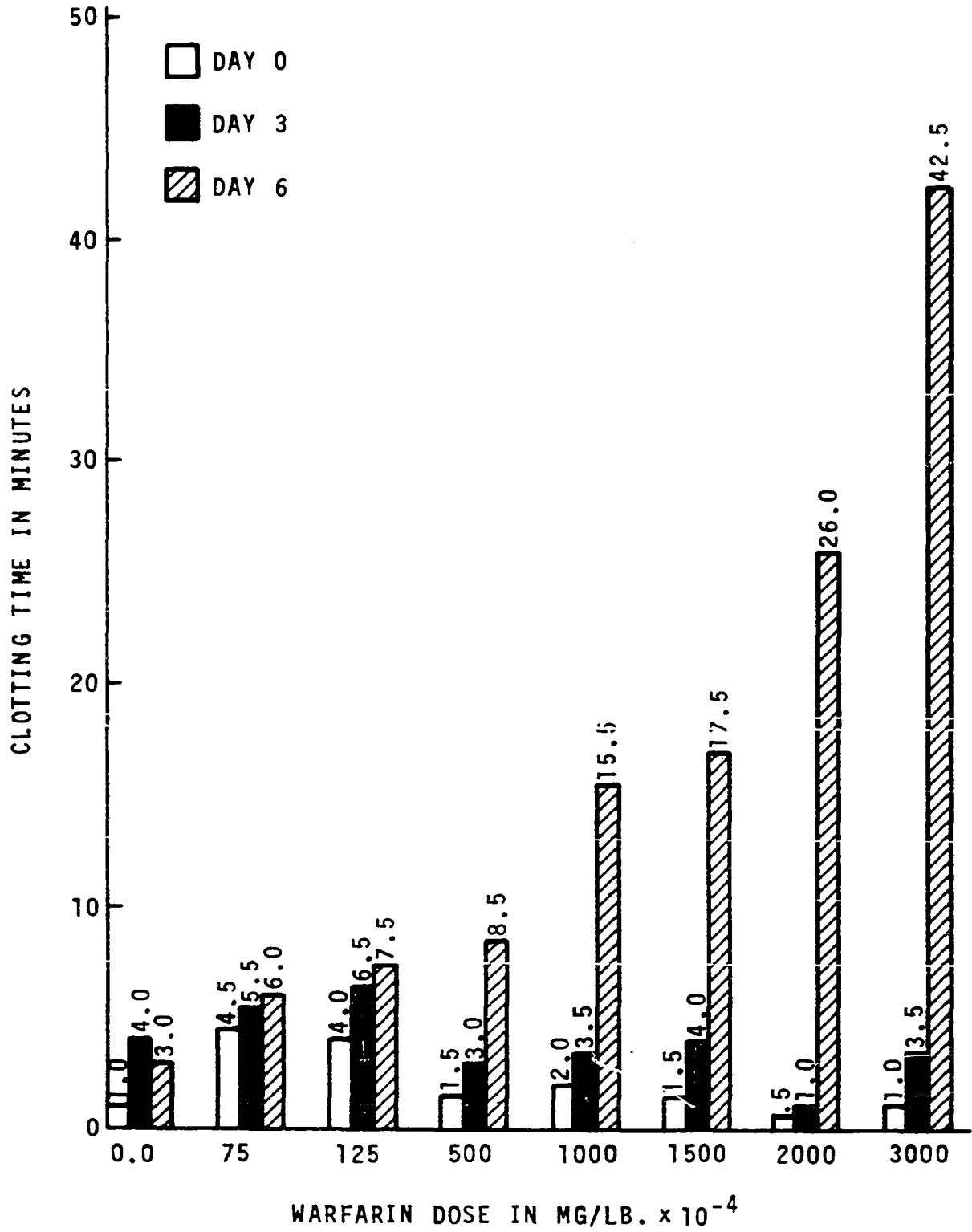


Figure 8. One-Stage Prothrombin Time in swine fed graded doses of warfarin

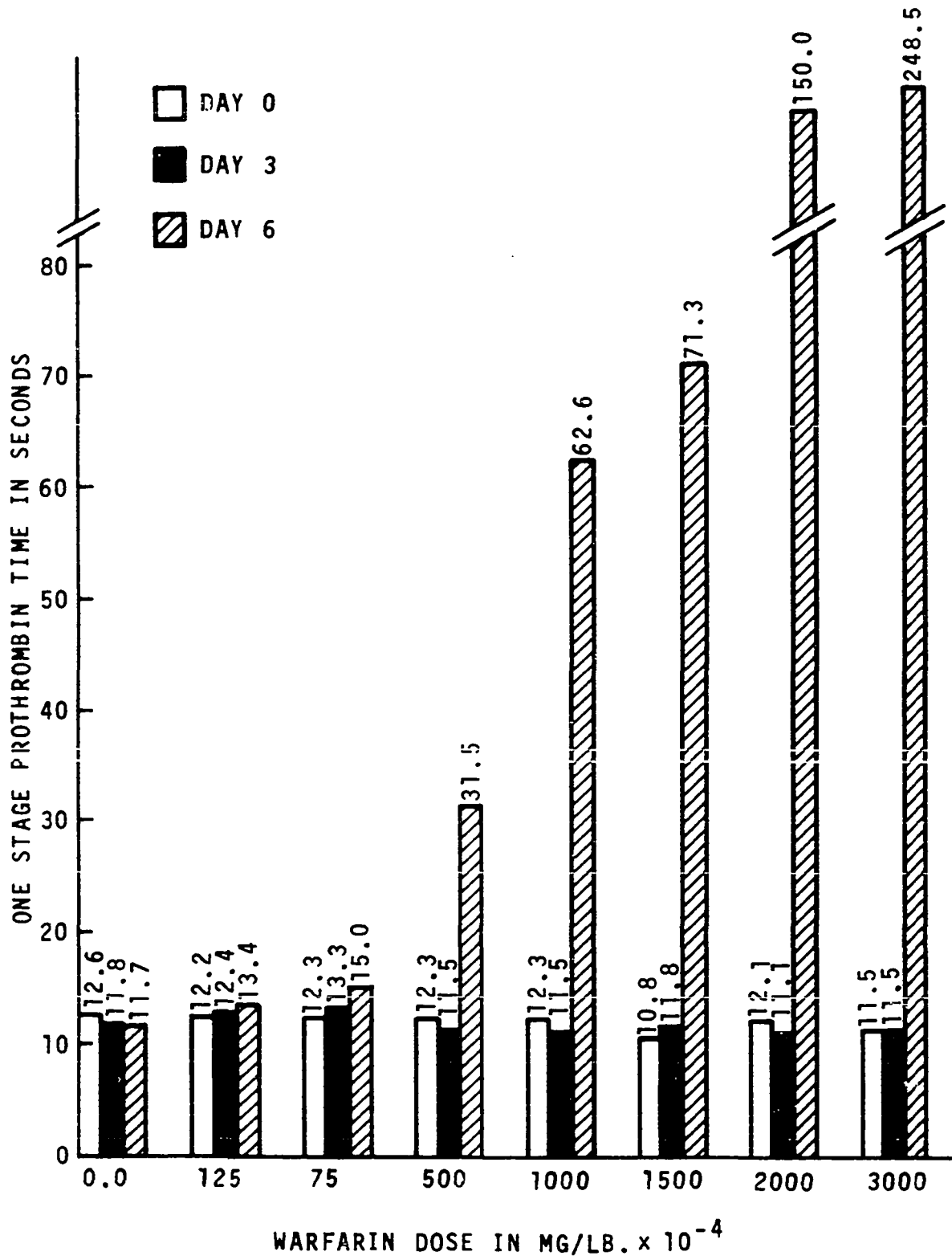


Figure 9. Activated Partial Thromboplastin Time in swine fed graded doses of warfarin

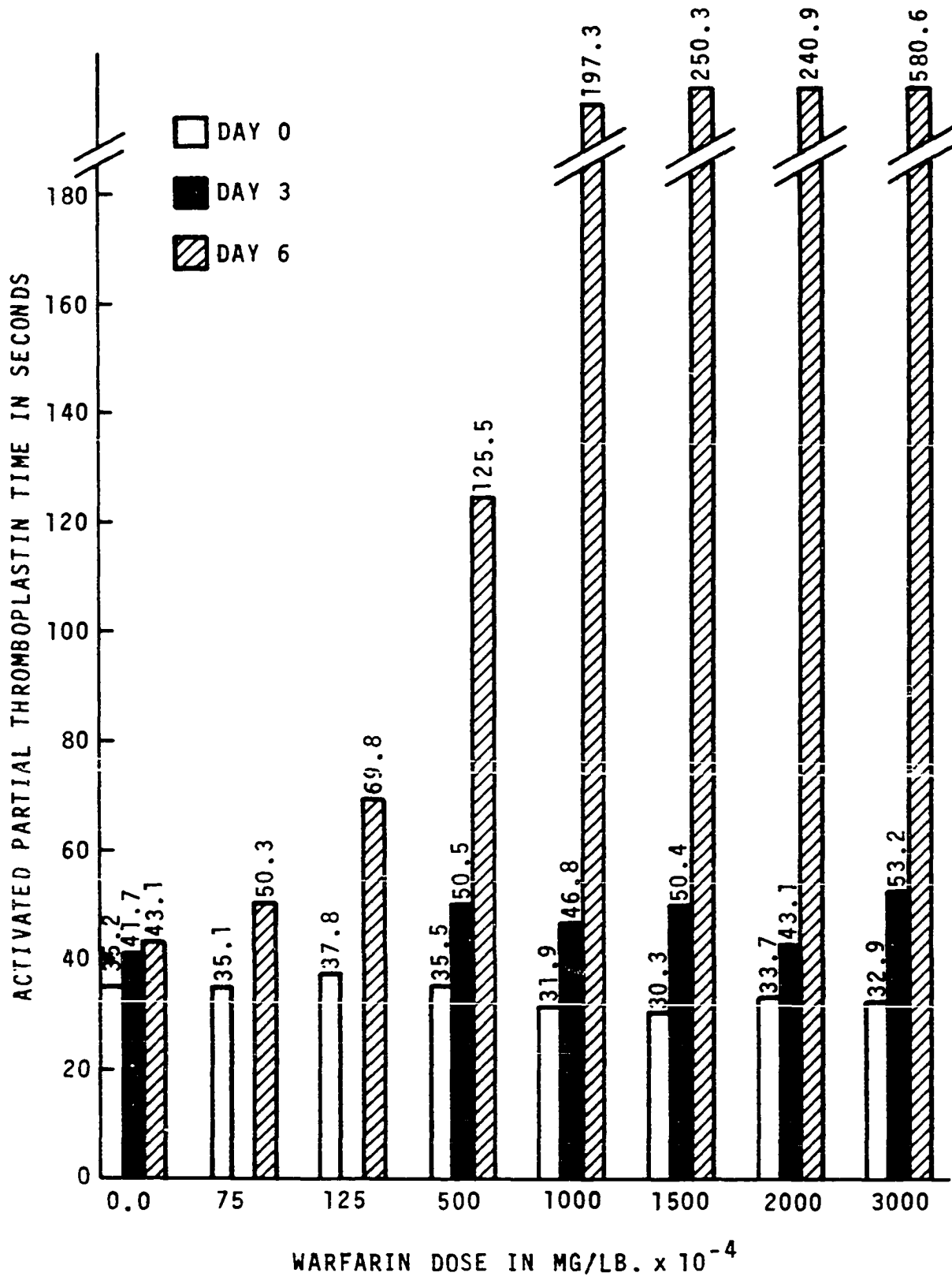


Figure 10. Mean clotting times for 32 swine fed tylosin-sulfamethazine combination with or without warfarin. The warfarin dosage was 0.0075 mg/lb body weight

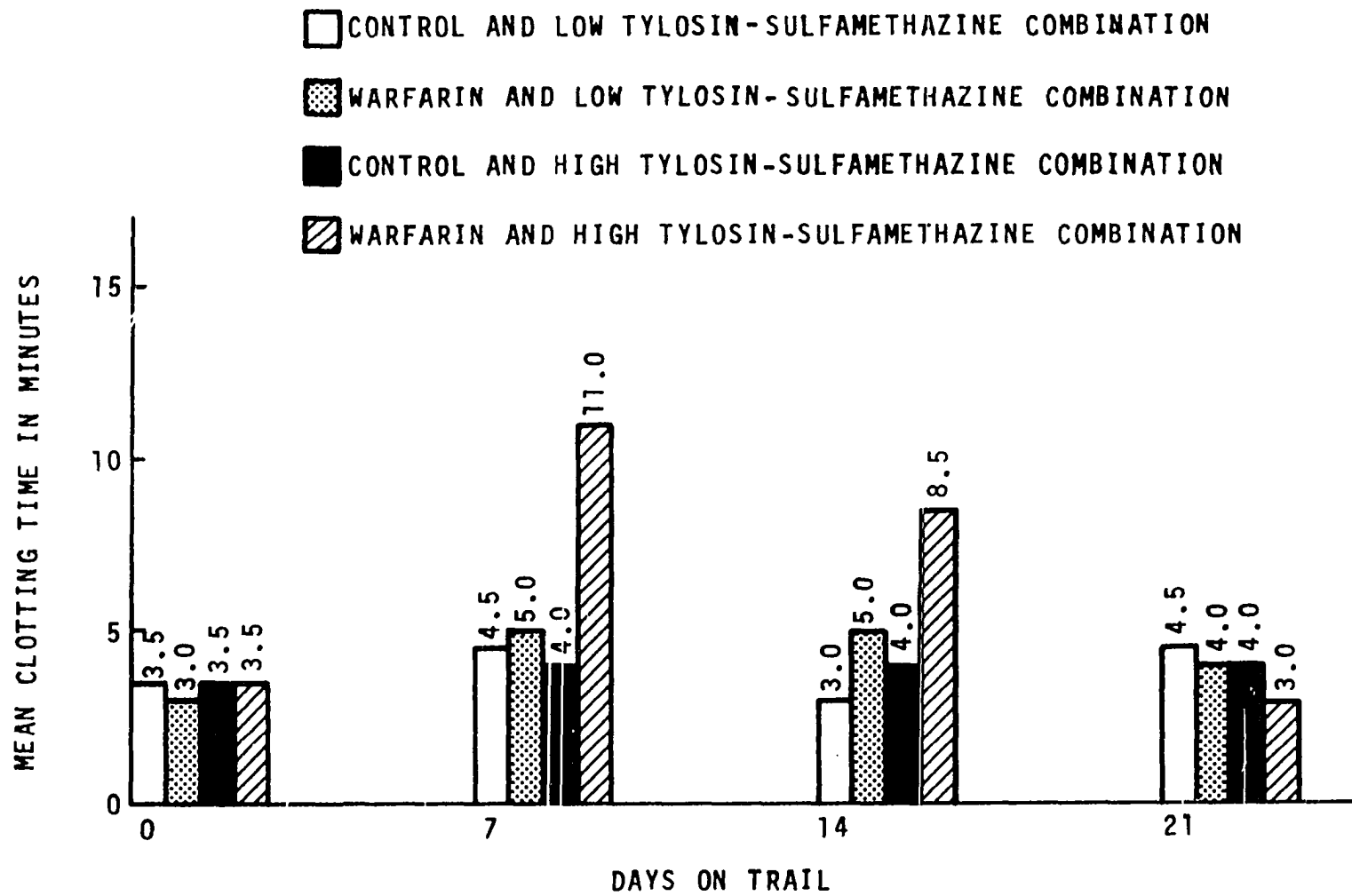


Figure 11. Mean One-Stage Prothrombin Times for 32 swine fed tylosin-sulfamethazine combination with or without warfarin. The warfarin dose was 0.0075 mg/lb body weight

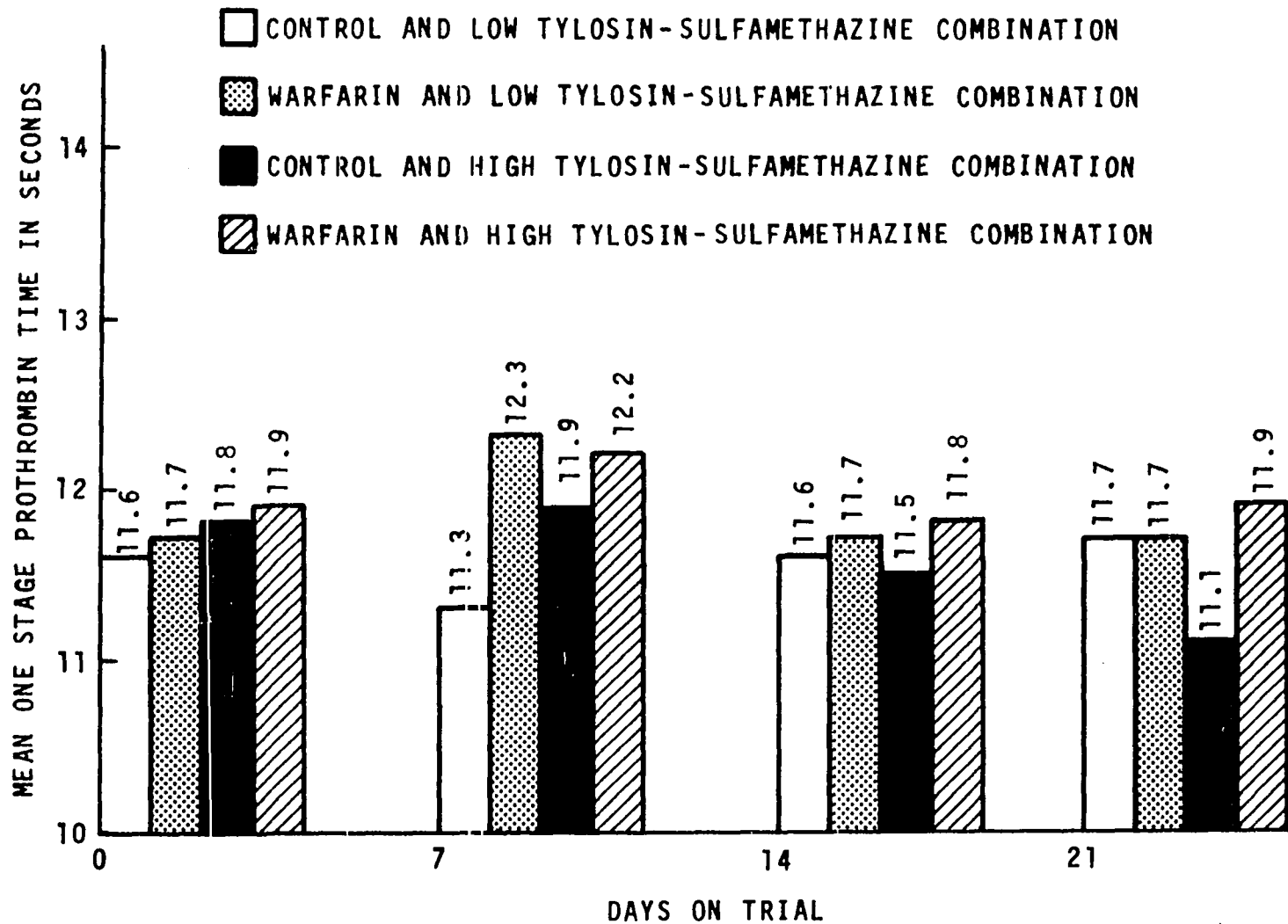


Figure 12. Mean Activated Partial Thromboplastin Time for 32 swine fed tylosin-sulfamethazine combination with or without warfarin. The warfarin dose was 0.0075 mg/lb body weight

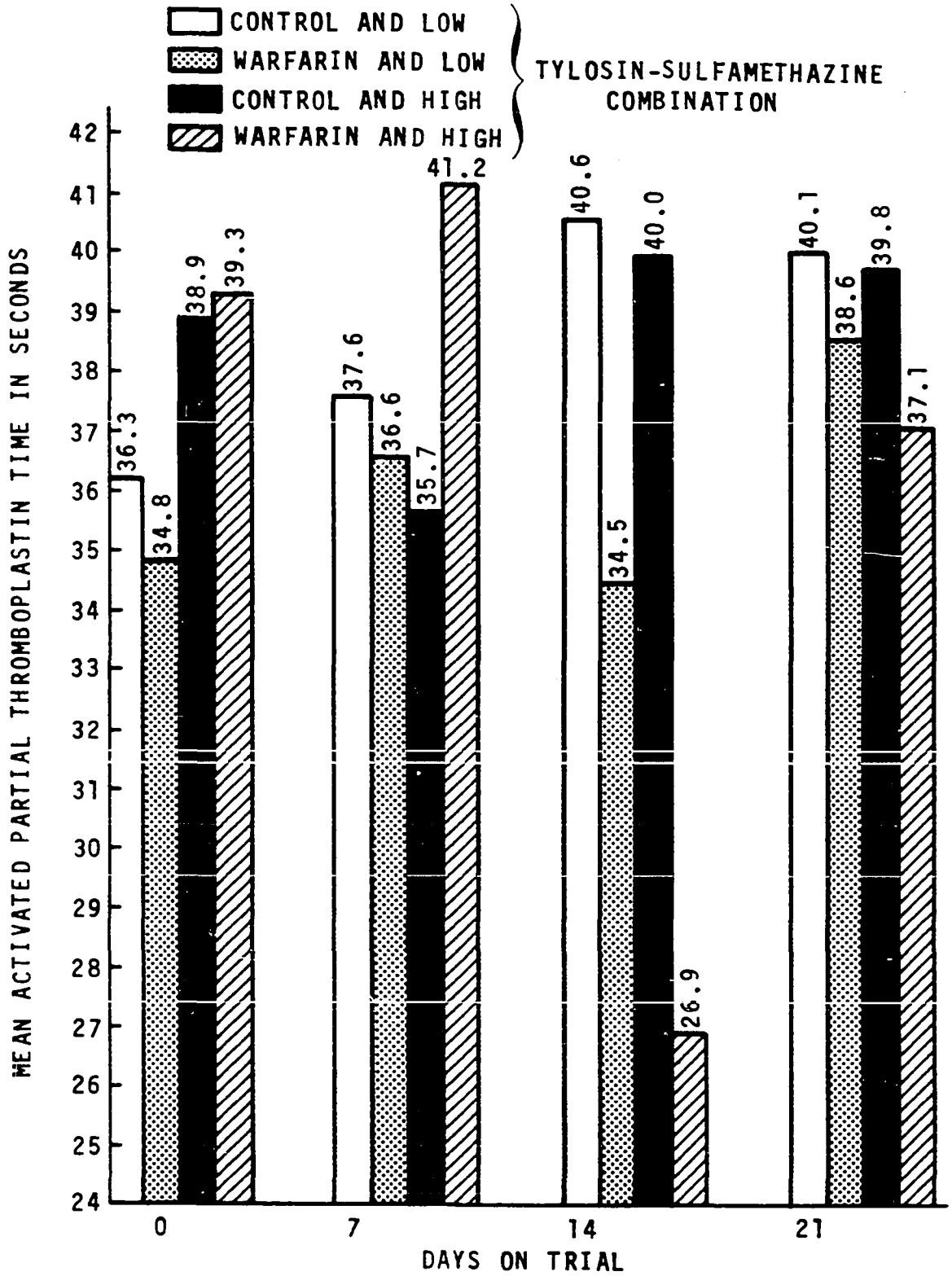


Figure 13. Mean clotting time for 16 swine fed warfarin with or without tylosin-sulfamethazine combination. The warfarin dosage was 0.025 mg/lb body weight

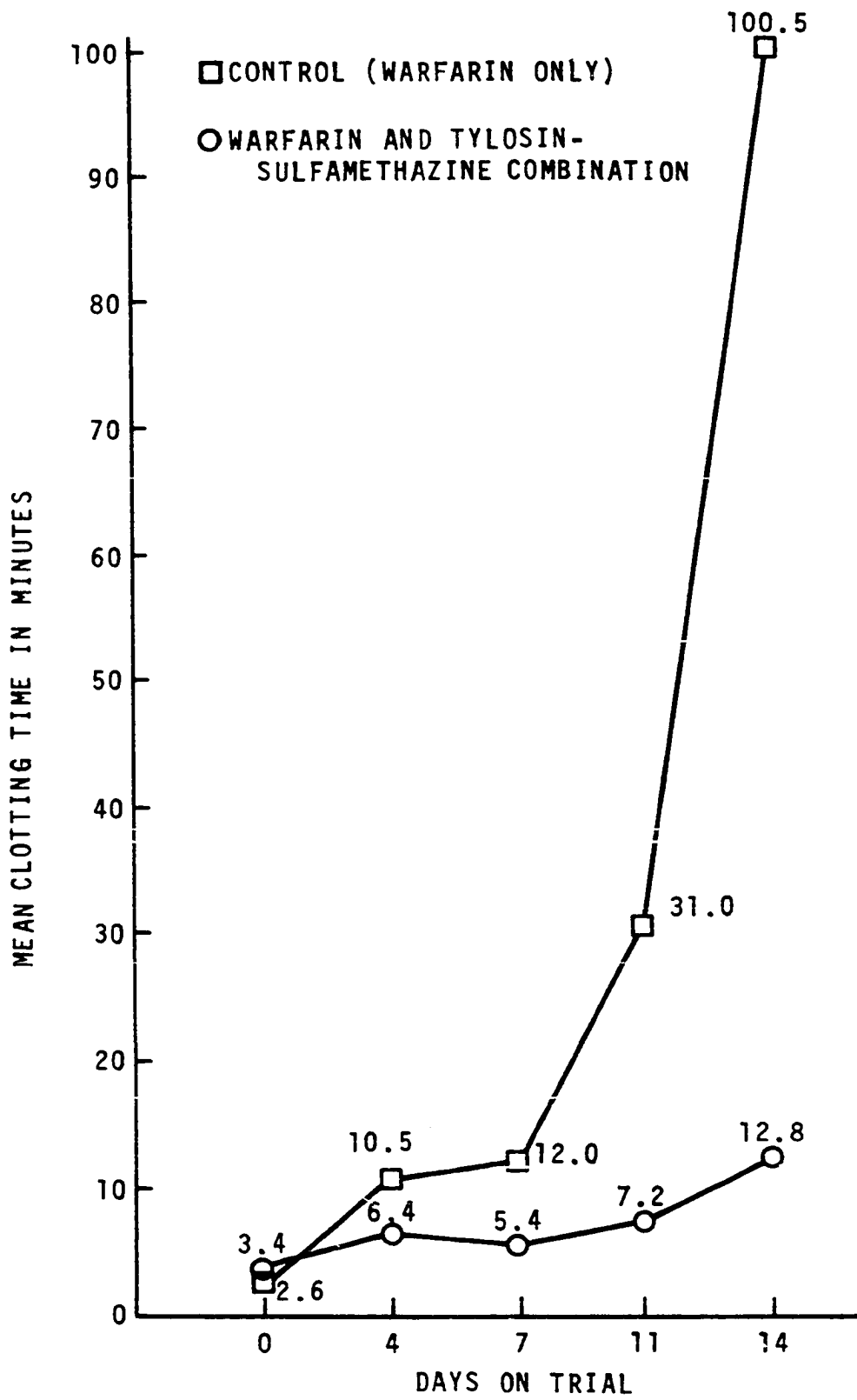


Figure 14. Mean One-Stage Prothrombin Times for 16 swine fed warfarin with or without tylosin-sulfamethazine combination. The warfarin dosage was 0.025 mg/lb body weight.

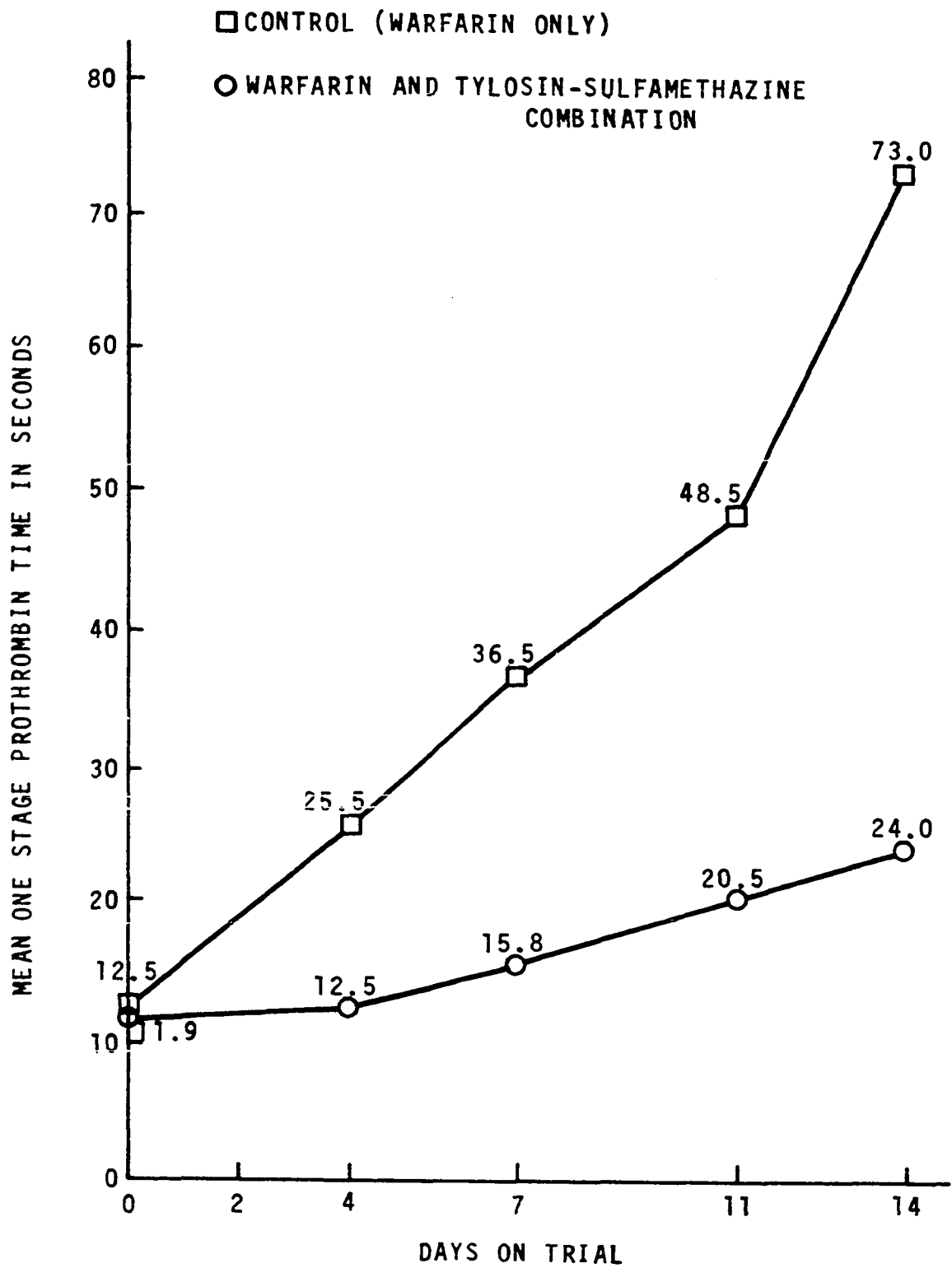
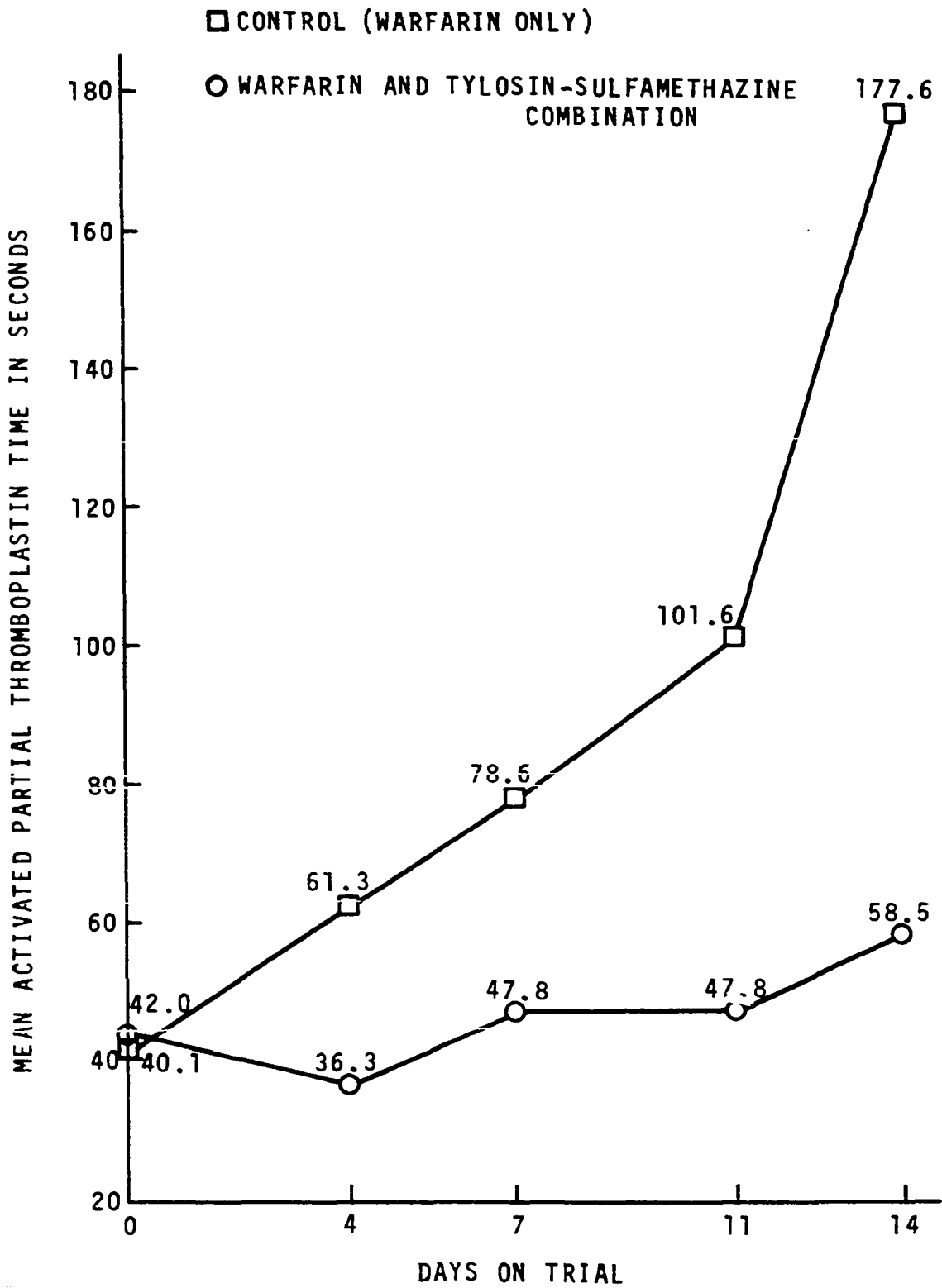


Figure 15. Mean Activated Partial Thromboplastin Time for 16 swine fed warfarin with or without tylosin-sulfamethazine combination. The warfarin dosage was 0.025 mg/lb body weight



parameters for the group receiving warfarin only. The rise in coagulation activity values was slower and of lesser magnitude in swine receiving tylosin-sulfamethazine in combination with warfarin.

Trial 5

Mean clotting and prothrombin times are plotted in Figures 16 and 17. Marked divergence in these values was observed, with the control (warfarin only) group having consistently higher values. During this trial, values for both treatments rose and then began to fall toward normal.

Trial 6

Mean OSPT values are shown in Figure 18 for swine receiving warfarin with parenteral sulfamethazine as well as those receiving only warfarin. There was marked elevation of OSPT in warfarin treated pigs when compared to those swine given warfarin with sulfamethazine. Upon withdrawal of warfarin, OSPT returned promptly to normal in both groups. One pig remained alive in the control group versus 3 pigs in the sulfamethazine group.

Trial 7

Prothrombin times are depicted in Figure 19 for each of 2 pigs fed a single dose of warfarin at 1.25 mg/lb body weight. The pig given warfarin without previous and concurrent exposure to parenteral sulfamethazine developed a markedly prolonged OSPT.

Hematologic Changes

Several hematologic parameters related to blood coagulation were evaluated. These included total protein, fibrinogen, packed cell volume, differential leukocyte count, and clot retraction. The results of hematologic

Figure 16. Mean clotting time for 16 swine fed warfarin with or without tylosin-sulfamethazine combination. The warfarin dosage was 0.025 mg/lb body weight

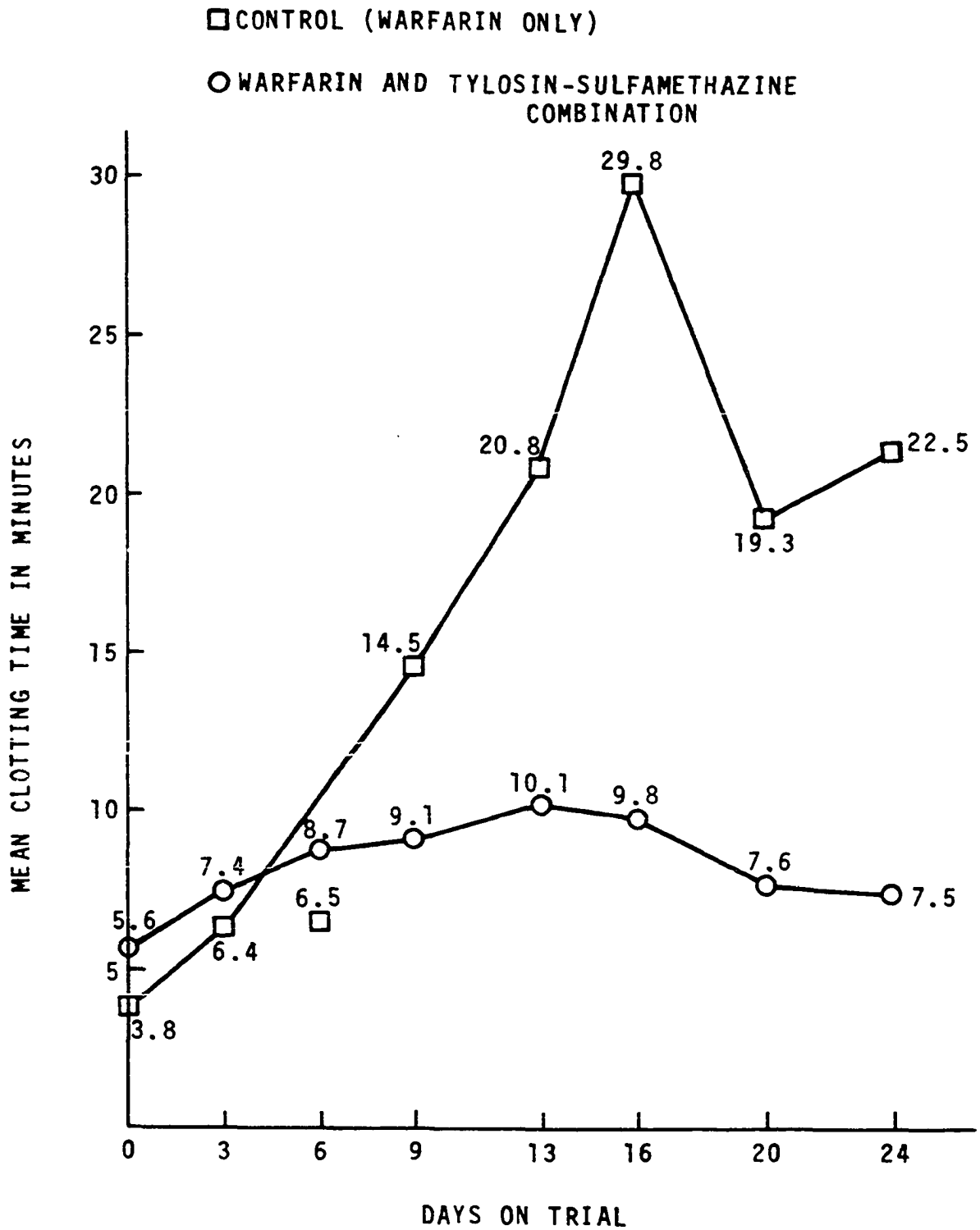


Figure 17. Mean One-Stage Prothrombin Time for 16 swine fed warfarin with or without tylosin-sulfamethazine combination. The warfarin dosage was 0.025 mg/lb body weight

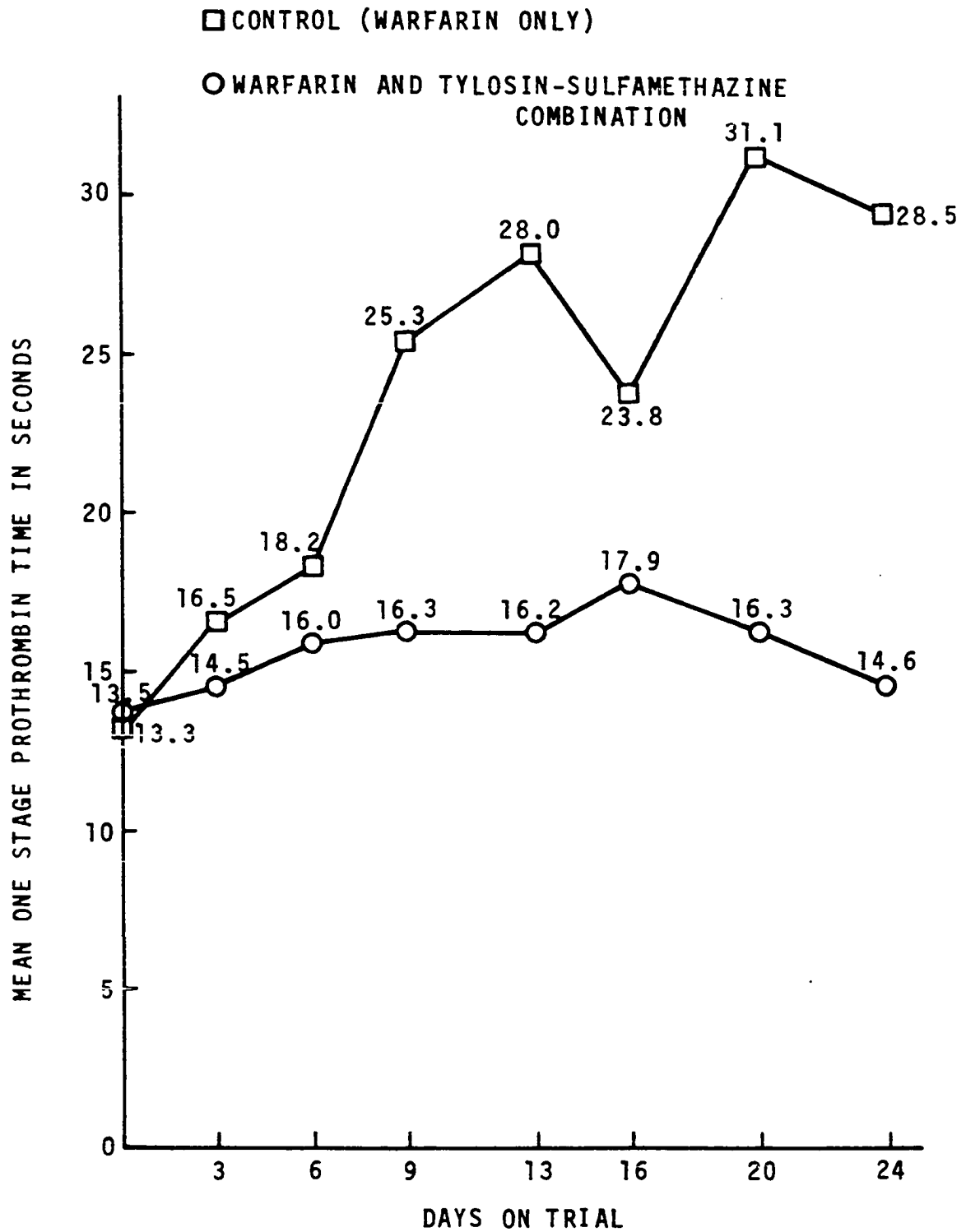


Figure 18. Mean One-Stage Prothrombin Time for 8 swine fed warfarin with or without parenteral sulfamethazine. The warfarin dosage was 0.025 mg/lb body weight

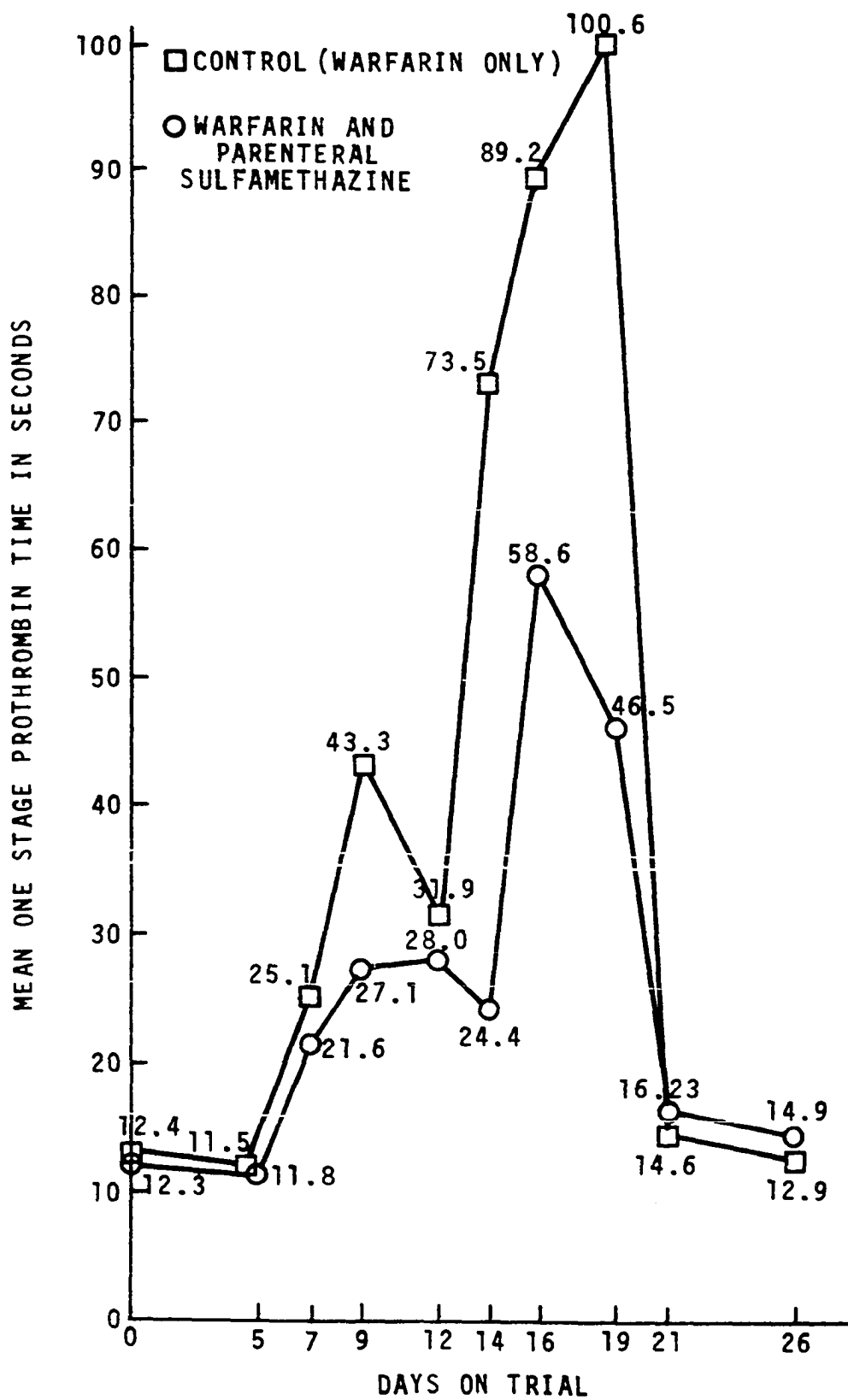
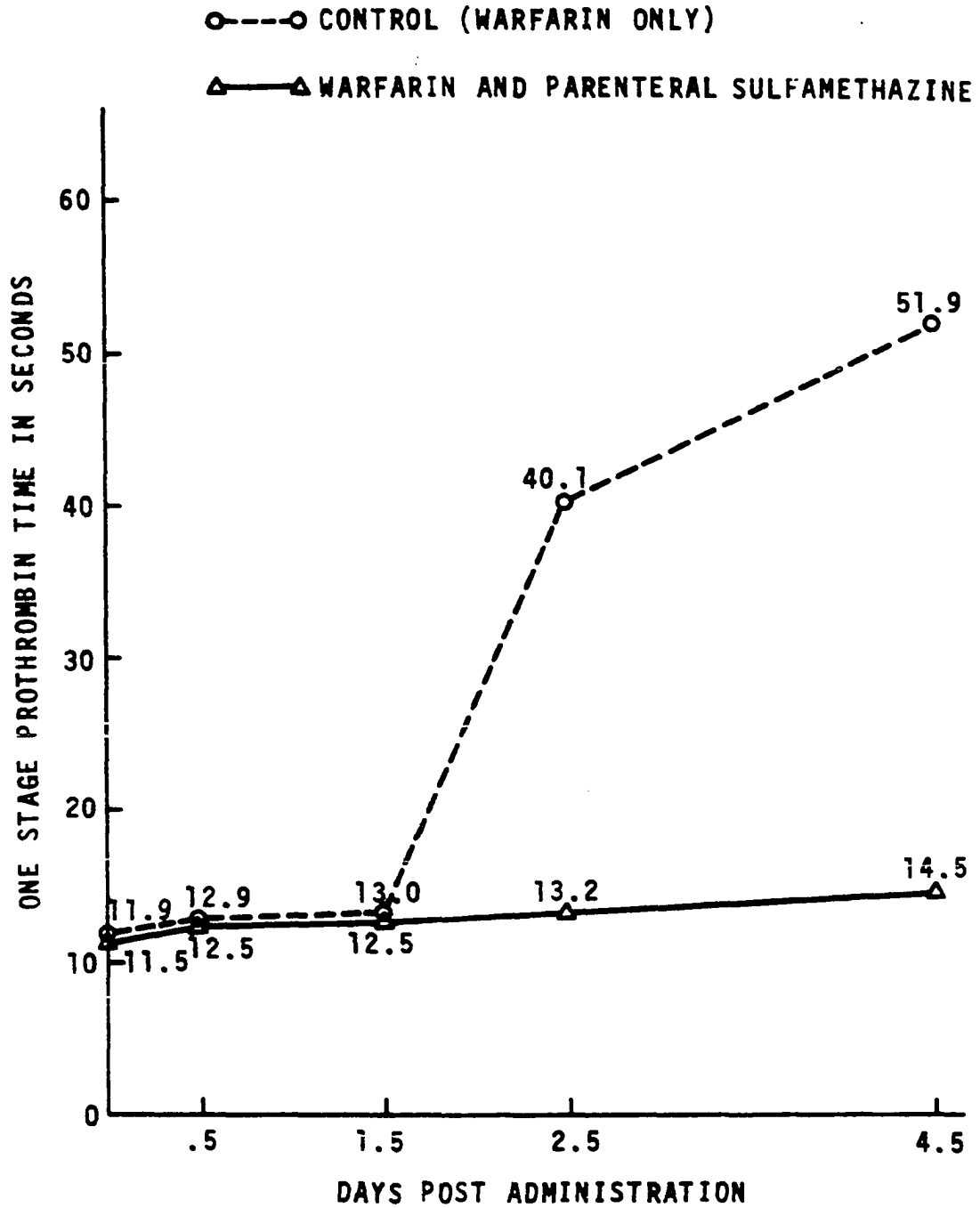


Figure 19. One-Stage Prothrombin Time for 2 swine fed warfarin with or without parenteral sulfamethazine. The warfarin dosage was 1.25 mg/lb body weight



studies in Trials 3 and 4 are shown in Tables 7 and 8 expressed as treatment means.

Trends away from control values were not observed for fibrinogen and total protein. A slight deviation in packed cell volume was noted in Trial 3. This change was not statistically significant. All blood samples had good clot retraction at 2 and 24 hours after coagulation occurred. Total protein and fibrinogen values were not depressed in any pigs. Based on data from these trials, the above parameters were not evaluated in subsequent trials.

Coagulation Factor Correction Studies

Plasma samples with prolonged OSPT and APTT from Trial 1 and Trial 4 were treated with both aged serum and adsorbed plasma. In every case, OSPT and APTT were corrected by addition of serum but not by adsorbed plasma. This was interpreted as deficiency of factors II, VII, IX, and X in warfarin-treated swine.

Statistical Treatment of Coagulation Activity Values

Tests of significance utilizing analysis of variance were conducted on coagulation data from Trials 3, 4, 5, and 6. Overall means, treatment means, and day means were computed for clotting time, OSPT, APTT, and PCV.

In Table 9 and 10, the statistical significance of parameters measured in antibacterial and warfarin trials is summarized. Table 11 compares correlation coefficients and their significance for Trials 3, 4, and 5.

Analysis of variance for Trial 3 was completed with no missing data. Results are presented in Table 9 for clotting time, OSPT, and APTT. F-

Table 7. Mean hematologic values for swine fed warfarin and antibacterial at two levels

Cells counted	Treatment							
	Low antibacterial ^a		High antibacterial ^b		Low antibacterial		High antibacterial	
	without warfarin		without warfarin		with warfarin		with warfarin	
	Day		Day		Day		Day	
	0	14	0	14	0	14	0	14
Segmented neutrophils (%)	17	28	23	27	24	34	29	30
Band neutrophils (%)	1	4	6	7	1	5	3	7
Lymphocytes (%)	77	66	69	64	72	58	66	70
Monocytes (%)	2	0	1	0	0	0	0	0
Basophils (%)	0	0	0	0	1	0	0	0
Eosinophils (%)	2	2	1	2	1	2	2	1
Nucleated erythrocytes (%)	0	2	1	2	1	2	1	1
Packed cell volume (%)	36	37	36	36	36	38	37	36

^aTylosin phosphate, 100 gm/ton feed and sulfamethazine sodium, 100 gm/ton feed.

^bTylosin phosphate, 1000 gm/ton feed and sulfamethazine sodium, 300 gm/ton feed.

Table 8. Mean hematologic values for swine fed warfarin with or without antibacterial^a

Cells counted	Treatment			
	Antibacterial and warfarin		Warfarin only	
	Day		Day	
	0	7	0	7
Segmented Neutrophils (%)	29	26	28	28
Band Neutrophils (%)	4	5	6	8
Lymphocytes (%)	65	67	64	63
Monocytes (%)	0	0	0	0
Basophils (%)	0	0	0	0
Eosinophils (%)	2	2	2	1
Nucleated Erythrocytes (%)	3	2	2	1
Packed Cell Volume (%)	31	33	31	31

^aTylosin phosphate, 1000 gm/ton feed and sulfamethazine sodium, 300 gm/ton feed.

values were regarded as significant ($P < .05$) according to the appropriate table in Snedecor and Cochran (1967).

Results of analysis of variance for clotting time, OSPT, and APTT in Trials 4 and 5 are presented in Table 10. Results are based on a weighted analysis computed to account for missing data caused by death of experimental swine.

Correlations between clotting time and OSPT, clotting time and APTT, and OSPT and APTT were computed and the results summarized in Table 11. Correlation coefficients for other parameters are included in Table 11.

Plasma Warfarin Levels in Control and Sulfamethazine Treated Swine

Results of analysis for plasma warfarin in 2 swine fed equal doses of warfarin on a body weight basis are plotted in Figure 20. The sulfa-

Table 9. Statistical significance of calculated F-values for swine fed warfarin or tylosin-sulfamethazine antibacterial

Source	Degrees freedom	F-value for each variable		
		Clot ^a	OSPT ^b	APTT ^c
Warfarin	1 ^d , 6 ^e	13.28*	13.57*	3.86
Antibacterial	1, 6	12.68*	1.06	0.00
Antibacterial X Warfarin	1, 6	10.30*	0.02	0.00
Day	1, 6	13.29*	4.37	1.60
Warfarin X Day	1, 6	12.05*	4.33	5.18
Antibacterial X Day	1, 6	7.34*	5.63	7.35*
Antibacterial X Warfarin X Day	1, 6	7.28*	8.94*	5.62

^aClotting Time.

^bOne-Stage Prothrombin Time.

^cActivated Partial Thromboplastin Time.

^dNumerator.

^eDenominator.

*Significant at $P < 0.05$.

methazine-free pig developed higher plasma warfarin levels, reached a greater peak of warfarin concentration, and maintained a more elevated plasma warfarin level than did the pig pre-treated with parenteral sulfamethazine.

Table 10. Statistical significance of calculated F-values for parameters studied in swine fed warfarin with or without tylosin-sulfamethazine antibacterial

Source	Trial number	Degrees freedom	F-value for each variable		
			Clot	OSPT	APTT
Antibacterial	4	1 ^a , 3 ^b	11.74*	25.66*	63.27**
	5	1, 6	14.88**	141.11**	--
Day	4	2, 12	89.87**	41.86**	33.79**
	5	1, 6	14.45**	22.12**	--
Antibiotic X Day	4	2, 12	30.70**	22.50**	20.23**
	5	7, 6	11.87**	17.84**	--

^aNumerator.

^bDenominator.

*Significant at $P < 0.05$.

**Significant at $P < 0.01$.

Table 11. Correlation coefficients for parameters studied in swine fed warfarin and tylosin-sulfamethazine antibacterial

Variable	Variable						Trial number
	Clotting time	Pro-thrombin time	Partial thrombo-plastin time	Total protein	Fibri-nogen	Packed cell volume	
Clotting time	1.000						3 ^a
	1.000						4 ^b
	1.000						5 ^c
Prothrombin time	0.153	1.000					3
	0.744**	1.000					4
	0.622**	1.000					5
Partial thrombo-plastin time	-0.074	0.069	1.000				3
	0.875**	0.922**	1.000				4
	0.725**	0.723**	1.000				5
Total protein	0.298**	-0.016	0.042	1.000			3
	--	--	--	--			4
	--	--	--	--			5
Fibrinogen	0.353**	-0.056	0.061	0.537**	1.000		3
	--	--	--	--	--		4
	--	--	--	--	--		5
Packed cell volume	0.157	-0.016	0.236*	0.186	0.090	1.000	3
	-0.842**	-0.697**	-0.820**	--	--		4
				--	--		5

^a96 degrees freedom.

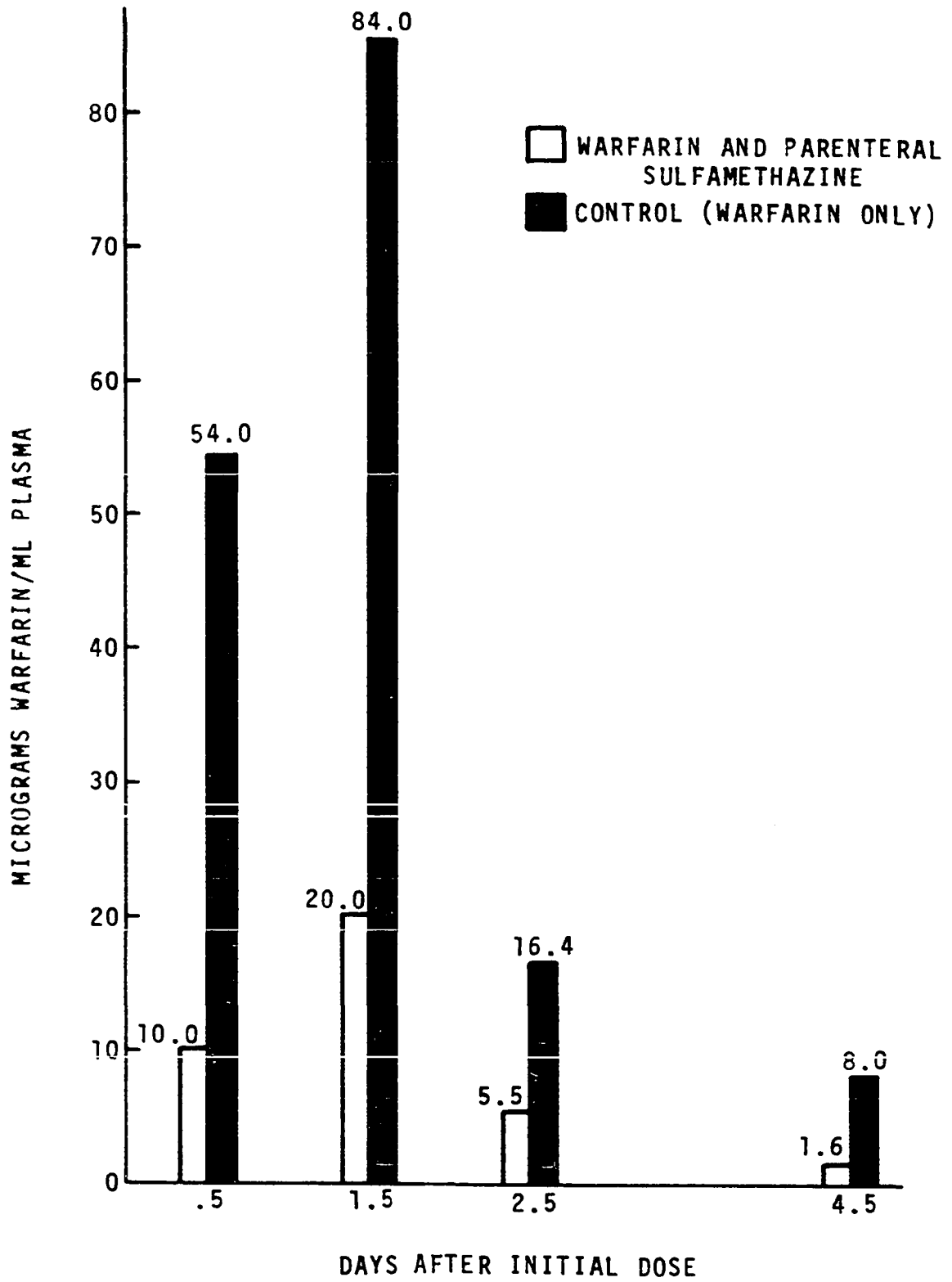
^b65 degrees freedom.

^c53 degrees freedom.

*Significant at $P < 0.05$.

**Significant at $P < 0.01$.

Figure 20. Warfarin concentration in plasma of two swine fed warfarin with or without parenteral sulfamethazine. The warfarin dosage was 1.25 mg/lb body weight



DISCUSSION

Clinical Response

The clinical response of swine to graded doses of warfarin in Trial 1 varied with the amount of drug consumed. While the trend is not strictly linear, there is a direct response to amount of warfarin administered. McGirr and Papworth (1955) demonstrated that swine fed warfarin at various doses tended to respond in 1 of 2 ways. Either there was a gradual onset of bleeding and hematoma or there was massive hemorrhage with associated acute death. Frazier (1966) reported swine accidentally poisoned by warfarin were affected by lameness and fecal hemorrhage but had near-normal appetites. Of the vitamin K-deficient swine studied by Schendel and Johnson (1962), acute onset and death from hemorrhage, as well as hemorrhage with recovery, were observed. Osweiler et al. (1970), in describing vitamin K-responsive bleeding in swine, included clinical histories of both chronic and acute hemorrhage.

Koch-Weser and Sellers (1971a), Rowsell (1968), and Deykin (1970a) reported that warfarin poisoning and vitamin K deficiency cause a similar depression in coagulation factors. Thus, the basis for the clinical course of warfarin poisoning, vitamin K deficiency, and porcine hemorrhagic disease appears similar.

The onset of clinical bleeding, especially ocular, subcutaneous, and intermuscular bleeding, was exacerbated by handling swine during blood sampling and weighing. Hemorrhage found at the sites of puncture by identification eartags was considered due to trauma. Although articular hemorrhages and hematomata were regarded as spontaneous, they were probably

initiated by trauma from articular movement or reaching over feeding pans. Signs of central nervous dysfunction seen in 2 swine were associated with diffuse subdural hemorrhage found at necropsy.

Lesions

Pale red blood of watery consistency was a characteristic lesion in warfarin-poisoned swine. No attempt was made to classify the type of anemia since acute blood loss appeared to be the cause. Furthermore, erythropoietic activity appeared normal as indicated by results of differential leukocyte counts and presence of nucleated erythrocytes and adequate platelet numbers. Hematomata were found in various parts of the body, particularly in areas of stress such as submandibular, lateral cervical, inguinal, and periarticular regions. These areas were most often in contact with feeding pans, concrete floors, and sidewalls of pens.

While the primary lesion observed was hemorrhage, edema was occasionally present. This could be caused by slowing and stagnation of blood flow through a hemorrhagic area or by blockage of lymphatics with erythrocytes.

Extravasated blood was often coagulated when hemorrhage was located in the subcutaneous or intermuscular spaces. Tissue damage caused by cellular anoxia may occur as a result of hemorrhage. Rowsell (1968) reported that Thromboplastin (Factor III) released from damaged tissues could initiate a more rapid coagulation.

No gross or microscopic lesions were found associated with colonic hemorrhage in experimental swine. Bleeding could result from peristaltic activity of the intestine and colon in a manner similar to the increased movement and resultant hemorrhage observed around joints. Ultrastructural

changes in capillary endothelium of rats treated with toxic doses of warfarin have been reported by Kahn et al. (1971). These changes were loss of ground substance and decreased organelle content. However, warfarin-induced capillary damage observed by light microscopy has not been reported.

The hemorrhagic lesions in warfarin poisoning have not been associated with decreased numbers of thrombocytes nor a decline in the levels of total protein and/or fibrinogen. Levine (1970) indicated a decrease in platelet adhesiveness which may be causative of minute capillary hemorrhages during warfarin poisoning.

The type and pattern of hemorrhage in affected swine could not be correlated with the amount of warfarin ingested nor the number of days for which the warfarin had been fed. The onset of hemorrhagic lesions and clinical signs appear more dependent upon the nature of traumatic or environmental conditions which initiate hemorrhage in a susceptible animal than upon total dosage.

Comparison of Tables 1 and 6 reveals that swine having prothrombin times on day 6 of less than 34 seconds did not exhibit spontaneous hemorrhage on that day. However, swine having 28.9-second and 34.0-second prothrombin times did have hemorrhage in response to the trauma of ocular bleeding. Further examination of Tables 1 and 6 reveals the day 12 values of prothrombin time for pigs 1258 and 1259 to be 19.9 and 16.4 seconds, respectively, at which time no hemorrhagic manifestations were noted. Comparison of Table 3 with Figure 14 discloses that on day 3 of Trial 4, the control (warfarin only) group swine had a mean prothrombin time of 25.5 seconds, but there was no associated clinical hemorrhage. The prothrombin time range in this group was from 18.1 seconds to 35.4 seconds. By day 5

of the trial, 3 swine in the control group had developed spontaneous hemorrhage or had died as a result of hemorrhage. These pigs had prothrombin times of 30.3 seconds, 33.4 seconds, and 42.4 seconds. Other swine in the group had prothrombin times as high as 51.9 seconds with no clinical evidence of hemorrhage.

From the clinical data relative to warfarin administration, it appears that approximately 5 days is needed after the administration of warfarin for clinical signs to appear. Langdell (1969) reported on the half-life of plasma coagulation factors depressed in man by warfarin administration. Factors II, VII, IX, and X have half-lives in man of 100 hours, 5 hours, 20-30 hours, and 20-30 hours, respectively. Deykin (1970a) stated that at chronic low doses, warfarin does not completely suppress procoagulant synthesis. Thus, the effect of low doses of warfarin depends on a balance between the rate of intake and the rate of degradation and excretion of warfarin. Nagashima et al. (1969) found that in most individuals the depression of production of Factors II, VII, IX, and X is a logarithmic function of plasma warfarin concentration. Since the acquired coagulation deficiency in warfarin poisoning is a multiple one, the clinical result will depend upon the relative rates of disappearance of each of the coagulation factors and upon the total effect of loss of these factors from the coagulation scheme.

The clinical response of swine to experimental warfarin administration was similar to the signs reported by Frazier (1966), McGirr and Papworth (1955), and Reihart and Reihart (1952) for uncomplicated warfarin poisoning. Results of primary clinical vitamin K deficiency in swine were not available for comparison. However, experimentally induced vitamin K defi-

ciency reported by Schendel and Johnson (1962) involved similar hemorrhagic lesions. Osweiler et al. (1970) and Fritschen et al. (1970) indicated that a vitamin K-responsive hemorrhagic disease of swine involved lesions similar to those reported for warfarin poisoning. No difference was detected between the lesions and signs reported for warfarin toxicosis and vitamin K deficiency.

Coagulation Factor Changes

Warfarin produced consistent changes in clotting time, OSPT, and APTT. This effect was exacerbated, both by increased dosage of warfarin (Trial 1) and by continued daily administration (Trials 4, 5, 6). Nagashima and Levy (1969) reported that the plasma half-life of warfarin in several species varied from 9.4 to 10.4 hours for the rat, 22 to 23 hours for the dog, and 29 to 43 hours for man. Furthermore, they determined that the effect of dosage varied with species. Their dogs had a plasma warfarin half-life independent of dosage while half-life declined slightly with increasing dosage in the rat but increased markedly with increasing dosage in the monkey. Although specific warfarin half-life studies have not been reported for swine, it would seem that an appropriate half-life in swine was allowing larger experimental doses of 0.025 mg/lb to accumulate while the smaller 0.0075 mg/lb doses used in Trial 3 were excreted at a rate approximately equaling or exceeding intake.

The parameters chosen for assessing clotting function were selected on the basis of availability of instrumentation, applicability of the technique, and suitability to assess the coagulation factors most affected by warfarin and vitamin K. Rowsell (1968) reported that the coagulation fac-

tor activity of swine is identical to that of man in standard test systems for Factors II, V, VII, and X. Porcine coagulation activity for Factor IX was slightly increased over man. For these reasons, commercial reagents were chosen for the tests employed giving the advantages of standardization and stability not found in prepared reagents.

Deykin (1970a) and Sirridge (1967) reviewed the coagulation factors depressed during warfarin poisoning or vitamin K deficiency. They reported that depression of Factors II, VII, IX, and X was associated with both situations. Quick (1966) and Thomson (1970) listed the factors measured by OSPT as fibrinogen, Factor II, Factor V, Factor VII, and Factor X. These factors are part of the extrinsic system of the blood coagulation scheme as described by Langdell (1969). The APTT was recommended by Thomson (1970) to measure the intrinsic system of the coagulation scheme. The APTT measures deficiencies in Factors II, V, VIII, IX, X, XI, and XII. It is more sensitive to abnormalities occurring at early stages of coagulation leading up to prothrombin activation. However, a minor clotting factor deficiency may be masked and compensated for by an elevation of one or more of other factors.

When the extrinsic system alone is deficient, a lack of Factor VII is most likely. If the APTT is prolonged in the presence of a normal OSPT, Factors VIII or IX are generally deficient. When both OSPT and APTT are increased, a multiple deficiency involving Factors II, V, VII, IX, or X is usually indicated.

The preparation of barium sulfate adsorbed plasma described by Langdell (1969) results in plasma deficient in Factors II, VII, IX, and X but rich in Factor V and Factor VIII. Thomson (1970) lists the factors

present in aged serum to include Factors VII, IX, X, XI, and XII. Owen et al. (1969) have devised a method for estimation of factor deficiencies by addition of adsorbed plasma and aged serum to plasmas with prolonged OSPT. This has been expanded to include use of the APTT¹ as follows:

<u>Original time</u>		<u>APTT</u>		<u>OSPT</u>		<u>Deficiency indicated</u>
<u>APTT</u>	<u>OSPT</u>	<u>Plasma reagent</u>	<u>Serum reagent</u>	<u>Plasma reagent</u>	<u>Serum reagent</u>	
A ²	N ³	C ⁴	NC ⁵	-- ⁶	--	VIII
A	N	C	C	--	--	XI, XII
A	N	NC	C	--	--	IX
A	A	C	NC	C	NC	V
A	A	NC	C	NC	C	X
A	A	NC	NC	NC	NC	II
N	A	--	--	NC	C	VII

By the above scheme, a deficiency of Factors VII and X as well as probable deficiency in Factors II and IX was suspected in Trials 1 and 4. This agrees with findings of Deykin (1970a), Sirridge (1967), and Owen et al. (1969) relative to warfarin therapy and vitamin K deficiency in man.

No significant effect upon OSPT or APTT was found related to tylosin-sulfamethazine treatments used in Trial 3. A significant change in clotting time was noted in Trial 3 for the tylosin-sulfamethazine treated swine. The change in clotting time was not in agreement with the insignificant

¹Dade Division, American Hospital Supply, Miami, Florida 33152.

²Abnormal time.

³Normal time.

⁴Corrected time.

⁵Not corrected time.

⁶Not applicable.

changes reported for OSPT and APTT in the same trial. Nor is the change in clotting time observed on the high tylosin-sulfamethazine with warfarin treatment during days 7 and 14 of the trial associated with decreases in clot retraction or plasma fibrinogen level. Messersmith et al. (1967) studying combinations of sulfamethazine, aureomycin, and penicillin reported a decrease in clotting time with increased days on sulfamethazine.

Some difficulties are encountered in the use of clotting time as a quantitative measure of the coagulation system. Rowsell (1968) suggested that tissue trauma with inclusion of Factor III in the sample would promote clotting at a more rapid rate. Didisheim et al. (1959) studied the coagulation systems of several mammalian species and concluded that clotting time appeared quite dependent upon variations in technique. Experimental technique in Trial 3, including bleeding procedure and oxalate dilution, was investigated with no indication of technical or procedural variation. Neither available literature nor subsequent clotting time findings were suggestive of the mechanism for increased clotting time in Trial 3.

A significant elevation in OSPT and APTT was observed for those swine fed warfarin at a level of 0.0075 mg/lb of body weight in Trial 3. This was the effect expected from warfarin fed at low levels. However, the times did not approach the levels of Trial 1 where clinical hemorrhage was produced. The results of Trial 3, when swine were fed 2 levels of tylosin-sulfamethazine combination with or without warfarin, support the conclusions of Griminger (1957), Udall (1965), Deykin (1970a), and Koch-Weser and Sellers (1971a) that in the presence of a normal diet, oral antibiotics do not enhance or contribute to clinical hypoprothrombinemia. Furthermore, no evidence was found that 2 standard laboratory coagulation tests

were prolonged by antibiotic. If the increased clotting time of the group fed warfarin with high tylosin-sulfamethazine is considered in light of normal OSPT and APTT, then some nonvitamin K dependent clotting function may be involved. The marked and consistent changes in clotting time, OSPT, and APTT, seen in swine given only warfarin at 0.025 mg/lb, are evidence for a protective or competitive effect of tylosin-sulfamethazine against warfarin.

Antibiotics or antibiotic combinations have not been previously reported as antagonistic to warfarin. Kabins (1972), Koch-Weser and Sellers (1971b), and Sher (1971) reported that drugs inhibiting the action of coumarins in man included barbiturates, glutethimide, griseofulvin, and heptabarbital. These drugs act either by enhancing coumarin metabolism or by inhibiting coumarin absorption.

Robinson et al. (1971) reported that nonsystemic gastrointestinal drugs could interact with warfarin to form unabsorbable complexes. When warfarin ($K_{p_a} 5.6$) entered the small intestine, it was rapidly converted to the anionic form which was bound to cholestyramine given orally. Koch-Weser and Sellers (1971b) reported that cholestyramine binding of bile salts may lower the absorption of vitamin K and enhance coumarin action. Thus, the net effect of a drug such as cholestyramine must be elevated relative to both warfarin binding and vitamin K absorption.

In Trial 6, warfarin was administered orally with or without the concomitant parenteral administration of sulfamethazine. The results indicate that the anti-hypoprothrombinemic effect observed from the oral tylosin-sulfamethazine combination was also seen when sulfamethazine was given

parenterally. This would suggest that an intestinal antibacterial-warfarin complex is not involved in the antagonistic mechanism.

Stowe (1965) reported that sulfonamides are bound to plasma albumin after absorption. Koch-Weser and Sellers (1971b) and Kabins (1972) concluded that sulfonamides increase the action of coumarin anticoagulants by displacement of warfarin from plasma binding sites. This effect has not specifically been reported for sulfamethazine. Welch et al. (1969) found that phenylbutazone and tolbutamide competed with warfarin for binding sites on human plasma albumin. Further studies indicated that long-term pre-treatment of dogs with phenylbutazone, tolbutamide, or phenobarbital markedly increased the tolerance of the animals to warfarin. This pre-treatment parallels the prior administration of oral tylosin-sulfamethazine or parenteral sulfamethazine employed in experimental warfarin administration. This effect is similar to the response described by Koch-Weser and Sellers (1971a) for hepatic microsomal enzyme inducers.

Stowe (1965) indicated that sulfonamides are metabolized both by acetylation and oxidation. Furthermore, the proportion of acetylation versus oxidation varies with species and with type of sulfonamide. Thus, it seems appropriate that sulfamethazine may act to antagonize the toxicity of warfarin by initially increasing the proportion of unbound warfarin in plasma making it more rapidly available for metabolism and excretion. Secondly, a microsomal enzyme induction effect resulting from prior sulfamethazine administration may increase the rate of warfarin metabolism to inactive metabolites similar to those found by Ullrich and Staudinger (1968).

The trial involving feeding of warfarin to swine in a single dose of 1.25 mg/lb of body weight was designed to detect differences in the excretion of plasma warfarin for a control versus a sulfamethazine-treated animal. The results indicate a high and prolonged plasma warfarin level for the control animal. This correlates with the markedly elevated prothrombin time, clinical hemorrhage, and death in the control animal while the sulfamethazine pig remained clinically healthy with near-normal prothrombin time.

Due to the highly significant correlations among OSPT, APTT, and clotting times, only OSPT or OSPT and clotting time were used subsequent to Trial 4 to assess the progress of coagulation factor deficiency. For the pig, prothrombin time appears to be a reliable and simple technique for screening potential coumarin poisoning in swine.

SUMMARY

Pertinent literature on naturally occurring hemorrhagic diseases in poultry and livestock were reviewed and compared to recent outbreaks of a hemorrhagic syndrome in swine. Epizootiological, clinical, and hematologic data from porcine hemorrhagic disease suggested vitamin K-responsive hypoprothrombinemia.

Warfarin was administered daily to 88 weanling pigs, either at a predetermined subclinical level or at a clinically toxic level. The toxicity of warfarin was compared in swine receiving tylosin and sulfamethazine antibacterial combination versus those receiving warfarin only.

Toxicosis was induced in weanling swine fed warfarin daily at 0.025 mg/lb body weight. Approximately 5 days were required for clinical poisoning to occur at that level of warfarin. Characteristic clinical signs are lameness, anorexia, subcutaneous hematoma, melena, and periarticular enlargement.

Administration of warfarin at 0.005 and 0.0075 mg/lb did not cause clinical toxicosis, while 0.0125 mg/lb produced significant increases in One-Stage Prothrombin Time (OSPT) and Activated Partial Thromboplastin Time (APTT). When tylosin-sulfamethazine antibacterial combination was fed at normal and high levels concurrently with warfarin at 0.0075 mg/lb body weight, no elevation of clotting time, OSPT, or APTT was observed due to antibacterial action. The antibacterial combination fed alone did not produce changes in clotting time, OSPT, APTT, fibrinogen, total protein, differential leucocyte count, or packed cell volume.

When toxicosis was induced by feeding warfarin at 0.025 mg/lb body weight, a significant protective effect was observed both from tylosin-sulfamethazine given orally and sulfamethazine administered parenterally.

Of two pigs given warfarin at 1.25 mg/lb body weight, the pig treated daily with parenteral sulfamethazine had lower plasma warfarin levels and returned more quickly toward normal than did the animal receiving only warfarin. This supports the hypothesis that swine are protected from warfarin poisoning due to increased drug metabolism or excretion stimulated by sulfamethazine.

Increases in clotting time, OSPT, and APTT correlate well with one another, and the OSPT would be a suitable laboratory procedure for screening potential natural vitamin K antagonists similar to dicoumarol or warfarin. At prothrombin times approaching 30 seconds, severe clinical hemorrhage may be expected.

These studies largely dispel the theory that antibiotics are detrimental to the vitamin K or prothrombin complex status of swine. Furthermore, evidence was obtained to support the conclusion that tylosin-sulfamethazine or sulfamethazine protect or ameliorate clinical hypoprothrombinemia in swine exposed to warfarin.

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