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DEVELOPMENT AND EVALUATION OF SUSTAINED-ACTION PARENTERAL PREPARATIONS OF SELENIUM AND VITAMIN E

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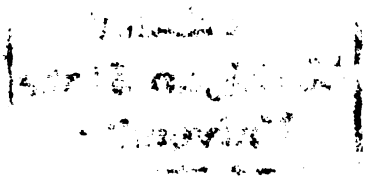

DEVELOPMENT AND EVALUATION OF SUSTAINED-ACTION PARENTERAL PREPARATIONS OF SELENIUM AND VITAMIN E

BY

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ABSTRACT

DEVELOPMENT AND EVALUATION OF SUSTAINED-ACTION PARENTERAL PREPARATIONS OF SELENIUM AND VITAMIN E

By

Cynthia Rogers Brockway

Four experiments were conducted in an attempt to improve on currently available selenium and vitamin E parenteral products. A single administration of barium selenate in an oil-based vehicle supported plasma selenium levels and plasma glutathione peroxidase activities longer than sodium selenite in a water-based vehicle. More of the selenium from the sodium selenite preparation was excreted during the first ten days post-injection than of the selenium from the barium selenate preparation. Improved preparations of barium selenate using both oil- and water-based vehicles were equally effective for extending the efficacy period of a single injection to 120 days post-injection while limiting selenium excretion during the first ten days post-injection. The incorporation of alpha-tocopherol or alpha-tocopheryl acetate into a water-based preparation with barium selenate did not significantly alter the efficacy period. Repeated injections of a formulation of barium selenate and alpha-tocopherol did not elicit a selenium toxicity or induce an anaphylactic reaction.

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Introduction

Selenium (Se) and vitamin E have been found to play an important role in animal and human nutrition. By 1940 vitamin E deficiency had been linked to fetal death and resorption in rats, nutritional encephalomalacia in chicks and nutritional muscular dystrophy (NMD) in guinea pigs, rabbits and ducks. Work in 1957 by Schwartz and Folz demonstrated the essentiality of selenium and its link to vitamin E. Selenium deficiency results in microangiopathy, NMD, edema, hemorrhage and necrosis of the liver, impaired immune response, impaired reproduction and sudden death (Trapp et al.,1970). The biochemical roles of selenium and vitamin E are similar and both act to spare the other, but the overlap is not complete.

Oxygen is necessary for aerobic life. Certain normal cellular reactions produce oxygen by-products. These are superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radicals (HO'). These free radicals attack cellular and subcellular membranes. Various pathways are used in living systems to deal with these radicals. One utilizes selenium as the enzyme glutathione peroxidase (GSH-Px) and another utilizes vitamin E.

Rotruck et al. (1973) showed that Se is an integral component of GSH-Px which catalyzes the reaction:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2\,H_2O$$

where GSH is reduced glutathione. Activity is not limited to hydrogen peroxide. The enzyme has an important role in converting hydroperoxides of lipids to the corresponding alcohols (Lawrence et al., 1974). GSH-Px is a tetramer with each subunit containing selenocysteine as part of the active site of the enzyme (Forstrom et al.,1984). Enzyme activity can be measured in most tissues of the body, blood being the most often evaluated. Because of inter-laboratory variation in the assay method used, comparisons of absolute activities should be made with care (Langlands et al., 1981.) To evaluate only the activity of the selenium dependent enzyme, hydrogen peroxide should be used as the substrate (Prohaska and Ganther, 1977). Another factor is incubation temperature which is important in any enzyme assay but is not often reported.

There is a direct correlation between blood GSH-Px, blood Se levels and Se intake in sheep and cattle when diets are low to adequate in Se (Thompson et al., 1976). Pigs and rhesus monkeys show little or no correlation between enzyme activity and blood Se when dietary Se is held constant (Thompson et al., 1976; Beilstein, 1983). Humans show a strong correlation between enzyme activity and blood Se when dietary Se is low but this disappears when adequate to high Se diets are consumed (Thompson et al., 1976; Schrauzer and White, 1978).

The activity of GSH-Px varies within blood fractions. The distribution of GSH-Px between ovine erythrocytes and plasma is 99:1 (Lawrence et al., 1974) and in bovines is

49:1 (Koller et al., 1984a). In swine the ratio is 26:1 (Zhang et al., 1986). Typical activity levels cover a wide range. Reported values vary from 6-36 IU/g of hemoglobin in cattle to 77-179 IU/g of hemoglobin in sheep (Thompson et al., 1976).

Species differences have been noted with regard to stability of the enzyme during storage. Zhang et al. (1986) demonstrated a substantial effect of storage on enzyme activity in porcine plasma. Storage at 4 C produced an 18 % decrease in activity over a 24 hour period. Storage at -20 C resulted in a 9 % loss. Samples stored 56 days showed 33 % and 43% of the original activity, respectively. Koller et al. (1984a) reported that GSH-Px activity in bovine plasma does not show appreciable loss during storage at 4 C for 7 days.

Vitamin E plays a role similar to that of Se and may act as a free oxygen radical scavenger. McCay and King (1980) proposed that H_2O_2 which escapes GSH-Px action may react with superoxide to form hydroxyl radicals. These radicals can then initiate peroxidation of membrane lipids or can be trapped by vitamin E. The subcellular location of vitamin E has yet to be confirmed, but it is generally considered to be membrane bound. Vitamin E has been detected in all body tissues analysed with adipose tissue, liver and muscle as the major storage locations.

Vitamin E is the common name for alpha-tocopherol and its acetate and succinate esters (Fernholz, 1938; USP

XX). One USP unit is equivalent to 1 mg dl-alpha-tocopheryl acetate (all-rac). One mg dl-alpha-tocopherol (all-rac) is equivalent to 1.1 USP units. Tocopheryl esters are more stable than the free alcohol. There are eight potential stereoisomers of alpha-tocopheryl acetate, each of which have different biopotencies (Weiser, 1982). Studies by Machlin et al. (1982) and Weiser (1982) comparing the stereoisomers determined that RRR-alpha-tocopheryl acetate has the highest biopotency of all isolated isomers. The all-rac-alpha-tocopheryl acetate mixture showed a biopotency 31% greater than was expected from calculated values, indicating a synergistic relationship between isomers. The general mode of action of the vitamin is through a series of ring and chain rearrangements which terminate the peroxidation cycle. Tocopherol is converted to a tocopheryl radical which can be regenerated, or can be further metabolised to a tocopheryl quinone, an irreversible process in vivo. Turnover of vitamin E is low, and it is difficult to deplete an adult animal so the former pathway seems most utilized in vivo.

Nutritional requirements for selenium and vitamin E have been established. Lambs have been shown to develop NMD when consuming diets containing less than 0.05 ppm Se (NRC, 1985). Acute clinical signs of the disease were prevented by increasing the diet Se level to 0.06 ppm or higher. In 1974 the US FDA approved Se additions at 0.1 ppm for swine diets. Further research by Mahan and Moxon

(1978) and Meyer et al. (1981) led to the approval of the addition of 0.3 ppm to swine starter and prestarter diets. The current NRC requirement is set at 0.1 to 0.15 ppm for later growth and production for swine (NRC 1979; Piatkowski et al., 1979). For sheep, the US FDA has approved the use of supplemental Se for ewes. Added Se can not exceed 0.1 mg/kg diet (Federal Register, 1978).

The recommended dietary vitamin E level varies with the Se content of the diet. When Se is adequate, 10 to 15 USP units of vitamin E per kilogram diet are sufficient to prevent deficiency signs in pigs fed grain-soybean meal diets (NRC, 1979).Estimates of the vitamin E requirement for sheep varies from 10 to 60 mg/kg diet when Se values exceed 0.05 mg/kg. This requirement varies according to age and reproductive status (NRC, 1984). Pregnant ewes and young lambs have higher requirements than older, non-gravid animals.

The Se status of newborn animals can be a critical factor in their survival. Mahan et al. (1975) reported swine colostral Se values ranging from 0.043 ppm to 0.106 ppm and milk Se values of 0.013 to 0.029 ppm. The wide variation of these values can be attributed to differential supplementation (none or 0.1 ppm) of the gestation and lactation diets between sows. Loudenslager (1984) demonstrated a significant increase in sow colostrum and milk Se following supplementation of the sow's diet with sodium selenite. Gestation diets containing 0.18 mg Se/kg

and lactation diets containing 0.26 mg Se/kg produced a 33% to 50 % increase in colostral Se levels over those of sows fed basal diets containing 0.04 and 0.06 mg Se/kg diet, respectively. In cows there is a relative increase in milk Se levels by supplementing the diet with sodium selenite, but absolute increase seen is still below the requirement (Conrad and Moxon, 1979). Perry et al. (1977) reported bovine milk Se values comparable to those of swine. They confirmed that there was little or no change in Se content of cows milk despite supplementation. Low colostral Se concentrations and extremely low milk levels can cause a marked decline in Se status in the calf within eight weeks despite adequate supplies at birth.

Vitamin E is also a factor in neonate survival. Colostrums of many species have high vitamin E concentrations, but these decline rapidly in milk (Jagadeesan and Prema, 1980; Jansson et al., 1981; Malm et al., 1976). Loudenslager (1984) showed that a significant increase in colostral vitamin E could be achieved with dietary vitamin E supplementation, but the effect diminished by the end of the lactation period.

Koller et al. (1984b) demonstrated that the calf fetus can sequester enough Se from the dam to have whole blood Se concentrations up to 67% higher at birth than those of the dam. The effect of the dam's Se status on the newborn was confirmed by Weiss et al. (1983). Prepartum dietary supplementation of the dam elevated serum Se levels in the calf at birth. Kott et al. (1983) evaluated the effect of

Se and vitamin E injections of ewes on the survival of their lambs. Monthly injections of alpha-tocopheryl acetate and sodium selenite increased plasma Se levels in the ewes and significantly increased pre-weaning survival of the lambs. A single oral dosing of ewes with 30 mg Se as sodium selenite was not effective in preventing NMD in their lambs when the dose was given 80 days prepartum. Whanger et al. (1978a) determined that three subcutaneous injections with 5 mg Se as sodium selenite given to ewes at 90, 60 and 30 days pre-partum were able to prevent NMD from occurring in the lambs when ewes were maintained on Se-deficient diets. When injections were discontinued, blood Se levels in the ewes decreased to below normal within 30 days. This indicates little or no long-term storage of Se in tissues. When 30 mg sodium selenite in a peanut oil-beeswax vehicle to retard release was given subcutaneously to the ewes, their lambs showed no signs of NMD but had blood Se levels below those receiving dietary supplements of 0.1 ppm Se as sodium selenite (Kuttler et al., 1960).

Placental transfer of vitamin E is low in most species, confirming the need for elevated colostral vitamin E. Human infants, prior to nursing, have one-half the maternal level of plasma tocopherol, and pre-colostral rat pups show low circulating levels of vitamin E (Martinez et al., 1981; Pazak, 1983).

The onset of rumen function can also limit the utilization of Se supplied in milk. The developing

microflora accumulate available Se (Whanger, 1978b). Sequestering of available Se and the low Se content of milk combine to enhance the development of clinical signs of Se deficiency which may not have been present at birth. Adult ruminants may be able to use only a fraction of dietary selenium. A high percentage of dietary selenium can be converted in the rumen to elemental selenium which is then excreted in the feces (Cousins and Cairney, 1961; Butler and Peterson, 1963).

Selenium deficiency has been reported in unsupplemented grazing livestock in many parts of the world (McDonald, 1968). Muth and Allaway (1963) showed a direct correlation between low soil Se concentrations and the incidence of NMD in livestock. Wild animals from low Se areas have been shown to develop capture related myopathies as a result of overexertion during capture. The muscle lesions seen are similar to those of NMD. Lewis et al. (1977) reported acute capture myopathy in three North American elk (Cervus canadensis) and was able to link one case to a Se deficiency. Selenium and vitamin E deficiencies have been suspected as a factor in capture myopathies seen in Africa in most species of ungulates. Prophylactic treatment of these animals using currently available Se and vitamin E products given at the time of capture was not effective in reducing the myopathy. The product given has been suspected of containing too little vitamin E to be effective or that initiation of treatment was delayed excessively, allowing the myopathy to develop.

Dietary supplementation and injections of selenium and vitamin E have been effective in improving the immune response. Studies by Peplowski et al. (1980) showed parenteral treatment with Se and vitamin E increased humoral antibody titers in weanling swine antigenically challenged with sheep red blood cells. Two injections, one at weaning and a second two weeks later increased the response both in pigs on basal diets and those consuming supplemented diets.

Cases of NMD in domestic animals which are not responsive to currently available products have been reported. Maas et al. (1984) described cases of NMD in 2-4 week old lambs in which repeated parenteral treatment at the recommended dosage was required before a response was seen. Most cases did not become clinically normal until an additional parenteral vitamin E supplement was administered. Animals were subsequently diagnosed as adequate in Se but deficient in vitamin E before treatment was initiated.

The above situations are ones in which a parenteral Se and vitamin E supplement may prove useful. In many cases, immediate supplementation is desired either for prophylactic treatment in the case of capture myopathy or for sustained treatment in the case of the just weaned animal. The product should provide adequate vitamin E to alleviate a severe E deficiency and should simultaneously provide sufficient Se in a non-toxic form to counteract a

Se deficiency.

This study was undertaken to evaluate the effectiveness of parenteral products now in use and to improve on these products, if possible, with regard to duration of the effective period and the amount of Se and vitamin E supplied.

Experiment 1

Currently available Se and vitamin E products in the United States are composed of sodium selenite and alpha-tocopheryl acetate at dose levels ranging from 0.25 to 5 mg Se all with 68 IU vitamin E per ml[1]. In Europe, a selenium product is marketed utilizing barium selenate in an olagenous vehicle providing 50 mg Se per ml[2]. There is some question as to the effectiveness of sodium selenite as a long- lasting form of treatment. Barium selenate has been shown to be very effective in providing long term action. A reason for the sustained efficacy is the chemical nature of barium selenate.

Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$) is a white crystalline powder which is completely soluble in water. Barium selenate ($BaSeO_4$) is also a white powder but is only slightly soluble in water; 0.0118 g dissolves in 100 cc of cold water, 0.138 g in 100 cc of hot water. The solubility of the desired compound is a very important factor when designing a long acting parenteral. Slightly soluble salts of penicillin have been used extensively to prolong the protection given by one injection. Currently available products use procaine penicillin G as the active ingredient.

When designing a parenteral preparation, the choice of a vehicle is also important. Parenterals may take the

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1. Bo-Se, Burns Bio-Tech. Inc., Oakland, CA.
2. Deposel, Rycovet Ltd., Glasgow, Scotland.

form of solutions, emulsions or suspensions (Parrott, 1970). In a solution the desired compound is completely dissolved in the vehicle, resulting in a preparation which is clear to the naked eye. An emulsion is the result when a compound is dissolved in one phase that is then mixed with a second immiscible phase. When the first phase is a solid instead of a solution, a suspension is produced.

Water is the vehicle most desired for parenteral formulations. Sodium selenite in water forms a solution. Because barium selenate is relatively insoluble, a suspension must be made and water alone will not support a suspension. A non-aqueous vehicle can be used, both for added viscosity to keep the selenate from settling out during storage and to improve retention time in the animal injected.

Among the non-aqueous vehicles currently used are ones based on fixed oils (Spiegel and Noseworthy, 1963). Oils are mixtures of esters of unsaturated fatty acids which are liquid at 20 C such as corn oil, cottonseed oil, peanut oil, or sesame oil. These are relatively non-toxic, the only precaution being concern about an anaphylactic reaction.

Oils alone are not sufficient absorption delaying vehicles. Early penicillin repository preparations used a peanut oil-beeswax formulation introduced by Romansky and Rittman (1945). This formulation was originally designed to delay the absorption of calcium penicillin G, a soluble

penicillin salt, and was able to maintain serum penicillin titers at effective levels for 48 hr post-injection as compared to less than 24 hr for a saline vehicle.

Muth et al. (1967) utilized a peanut oil-beeswax base as a vehicle for sodium selenite. Ewes were injected subcutaneously with 5 mg Se suspended in saline or in the oil vehicle. Their study showed no effect of the vehicle on Se retention over time but post-injection peaks differed significantly. Saline allowed whole blood Se levels three times those of the peanut oil-beeswax vehicle indicating slowed movement of the Se from the injection site.

Early work by Kuttler et al. (1960) compared barium selenate and sodium selenite, both in the Romansky-type vehicle, for their effects on serum Se levels in sheep. Both preparations were injected subcutaneously. The sodium selenite preparation produced a rapid increase in serum levels which peaked at 1 day post-injection. This peak was followed by a decrease in serum Se to a low level at 14 days. The barium selenate preparation produced a gradual rise in serum Se levels which reached a maximum plateau between 7 and 21 days post-injection. Serum Se decreased gradually to initial values by 140 days post-injection. The toxicity of sodium selenite was not attenuated by the vehicle. Dosages of sodium selenite at 1.8 mg (0.8 mg Se)/kg body weight (bwt) were lethal within 10 hr post-injection. Barium selenate was tested up to 23.8 mg (6.7 mg Se)/kg bwt. No toxic reaction was seen other than a

transient edema at the injection site.

The lesions seen in selenium toxicosis include fatty change and centrolobular necrosis in liver, congestion of the renal medulla, necrosis of lymphoid follicles, edema and hemorrhagic necrosis of pancreas, and serous atrophy of body fat. Depending on dose, fatal selenium toxicoses are characterized by vomiting, anorexia, weight loss, central nervous system depression, respiratory distress, coma and death (Herigstad et al., 1973.) Selenium salts differ in toxicity. Muth and Binns (1964) tested the toxicity of various Se compounds. The order of decreasing toxicity for non-organic compounds was sodium selenate, sodium selenite, selenium dioxide and elemental Se.

Cawley and McPhee (1984) tested the commercial barium selenate preparation for efficacy and safety with sheep. They determined the optimal dose level to be 1 mg Se/kg bwt. This level allowed sustained elevated whole blood GSH-Px activity for five months post-injection. The same authors reported efficacy periods of up to one year in cattle. Barium selenate did not produce toxicity signs when administered to lambs at doses of 266.2 mg (75 mg Se)/kg bwt.

In pigs, an injection of sodium selenite at 1.1 mg Se/kg bwt caused severe toxicity signs. Injection of 1.65 mg Se/kg bwt resulted in death 7 to 10 hr post-injection (Mahan and Moxon, 1979). Higher doses of sodium selenite caused death to occur sooner (Diehl et al., 1975).

The use of oil as the base for a vehicle presents practical problems. Sterilization of parenterals is accomplished by many methods (Brewer and Phillips, 1970). Combined wet heat and pressure sterilization using an autoclave is most common. Prolonged exposure to dry heat is a comparable method. Ultrafiltration also has been used as has exposure to germicidal gases. Ultraviolet light (UV) is recognized as effective for dry compounds and surface sterilization. No single procedure is appropriate for all substances. Oils cannot be sterilized under wet heat and pressure without breakdown occurring. Water is not suited to prolonged dry heat sterilization. Barium selenate decomposes when heated. Assembly of the parenteral products in this study combined the various methods which could be used. These will be described later.

For the preliminary study sodium selenite in a water-based vehicle was compared to barium selenate in an oil-based vehicle.

Materials and Methods

A. Formulation of barium selenate

Barium selenate was prepared by oxidizing sodium selenite with 30% hydrogen peroxide, adjusting the pH to 10 by addition of sodium hydroxide and precipitating barium selenate by addition of barium chloride. The resulting barium selenate was filtered, washed with water and acetone and air dried. Analysis by ionic coupled argon plasma atomic emission determined the compound to be 27.6% Se and

48.9% barium. Theoretical values for barium selenate are 28.2 and 49.0% Se and barium (Ba), respectively.

B. Assembly of the parenteral preparations

Sodium selenite was dissolved in physiological saline made with distilled deionized water using 0.6 mg sodium selenite per ml. The pH was adjusted to 7 and aliquots of the solution were placed in acid-washed vials, sealed and autoclaved 15 min at 121 C and 12 kg/cm^2.

Barium selenate was measured into sterile 10 ml glass vials under UV light. Two sterile glass mixing beads were added and the vials sealed. Peanut oil (No. 15408, Eastman Kodak Co., Rochester, NY 14650) with 2% beeswax added (w:w; National Formulary grade, Fisher Scientific Co., Fair Lawn, NJ 07410) was heated until a uniform mixture was obtained. Aliquots were placed in acid-washed vials which were sealed and sterilized by dry heat (160 C for 2 hr). After the peanut oil-beeswax mixture cooled, it was withdrawn into a sterile syringe and, under UV light, was introduced into the opened vial containing the barium selenate. The vial was then resealed, the contents mixed thoroughly, and then stored at 4 C until used. Concentration was set at 17.5 mg barium selenate/ml.

C. Pigs

Twelve crossbred pigs, four from each of three litters were housed in individual stainless steel collection cages and were removed twice daily and placed in individual stainless steel feeding cages containing feed cups. Pigs

were fed an amount of finely ground corn-soy diet (Table 1) equivalent to 2% of bwt twice daily. The diet was mixed with an equivalent amount of water to form a slurry to ensure rapid consumption. Movement of pigs between feeding and collection cages was rapid to prevent urination and defecation outside the collection cages. The diet was unsupplemented with Se and by analysis contained 0.04 ppm Se. Vitamin E was added to the diet at 50 USP/kg as dl-alpha-tocopheryl acetate to prevent confounding the study with a vitamin E deficiency. This diet was utilized throughout the experiment.

A seven-day adjustment period was allowed prior to injection. A 24-hr urine and feces collection was taken the day before injection.

At the time of injection, pigs were blocked by litter and assigned to one of four treatments. The treatments were injections of saline, sodium selenite in saline, peanut oil-beeswax, and barium selenate in peanut oil-beeswax. The saline and sodium selenite preparations were given intramuscularly in the right thigh at 1.0 ml/5 kg bwt (0.055 mg Se/kg bwt). Barium selenate and the peanut oil-beeswax vehicle were given subcutaneously in the right flank at 1.0 ml/5 kg bwt (1.0 mg Se/kg bwt).

Pigs were weighed, an initial blood sample was drawn from the anterior vena cava and the injection given. The amount of feed was increased at this time to compensate for weight gained during the adjustment period. Subsequent

Table 1. Experiment 1 Diet

Ingredient	% Composition
Corn	66.00
Soybean meal (44% crude protein)	30.00
Calcium carbonate	1.00
Mono-di-calcium phosphate	1.50
Salt	.30
MSU vitamin-trace mineral premix (a)	.75
ASP-250 (b)	.25
Vitamin E premix (c)	.01
L-lysine HCl	.19
	100.00

a) Vitamin concentrations (per kg): retinyl acetate, 660,000 USP units; cholecalciferol, 132,000 USP units; riboflavin, 660 mg; d-calcium pantothenate, 2640 mg; nicotinic acid, 3520 mg; cyanocobalamin, 3.96 mg; choline chloride, 25344 mg; menadione sodium bisulfite complex, 440 mg. Mineral concentrations in % (and source): zinc, 1.496 (zinc oxide); iron, 1.188 (ferrous sulfate); manganese, 0.0748 (manganous oxide); copper, 0.198 (copper oxide); iodine, 0.01 (ethylene diamine dihydroiodide).

b) Concentration in %: chlortetracycline, 4.4; sulfamethazine, 4.4; penicillin, 2.2.

c) Concentration in %: dl-alpha-tocopheryl acetate, 50.

blood samples were then drawn in a similar manner at 1,2,3,4,5,7,10,24,38 and 52 days post-injection. Samples were drawn into a pre-heparinized syringe and transferred to heparinized centrifuge tubes, thoroughly mixed and centrifuged at 1450 x g for 15 min. Plasma was harvested and stored in plastic tubes under nitrogen at -20 C until analyses could be performed. Urine and feces were collected daily for 10 days post-injection. Twenty ml of 50% HCl was added to each urine collection container to prevent microbial degradation. Total urine volumes were recorded. The urine was then brought to a constant volume with distilled deionized water, mixed and a 20 ml aliquot taken which was stored at -20 C for Se analysis. Feces were placed in plastic bags and also stored at -20 C for future processing.

Following the collection period, the pigs were moved to a pen in the grower-finisher barn. The same diet was offered ad-libitum until 60 days post-injection when the pigs were taken off experiment.

Analyses

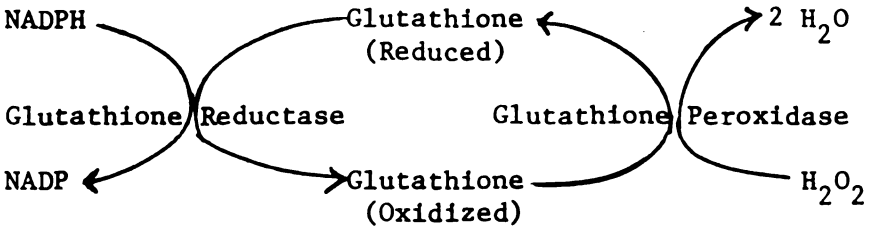
Selenium was analyzed spectrofluorometrically by the method of Whetter and Ullrey (1978). Duplicate samples (0.25 to 1.0 ml depending on Se content) and duplicate standards (0, 0.025, 0.05, 0.1, 0.2, 0.3 ug of Se/ml) were pipetted into acid washed 50 ml erlenmeyer flasks and digested with 2 ml HNO_3 and 2 ml $HClO_4$. Minerals were

dissolved by the addition of 2 ml 1.2 N HCl. Chelation by addition of 8 ml of EDTA solution (14.2 g/l) and neutralization by addition of approximately 1 ml of NH_4OH followed. One drop of cresol red solution (0.01 g in 1.0 ml H_2O, 1 drop 1:1 NH_4OH and H_2O to 50 ml) was added and HCl (1.2N) or NH_4OH (concentrated) were used to adjust the solution to an orange-pink color.

Five ml of 2,3-diaminonaphthalene (1.0 mg per ml .12 N HCl) was added which complexed with the Se to form diazoselenol which is light sensitive and fluorescent. This was extracted with cyclohexane, transferred to test tubes and fluorescence determined in an Aminco-Bowman spectrophotofluorometer SPF-125 (American Instrument Co., Urbana, IL 61801; excitation at 376 nm and emission at 510 nm). Plasma Se concentrations (ug/ml) were calculated using a curvilinear regression line determined from similarly processed standards and corrected for sample volume. Statistical examination of the inherent error in the procedure has been previously reported (Whetter and Ullrey, 1978). Mean recovery (+/- standard deviation) of added sodium selenite in 84 determinations was 98.7 ± 7.1%. The mean difference (+/- standard deviation) between values determined by the proposed method and other AOAC methods was -0.03 ± 0.60 %.

Plasma glutathione peroxidase activity was determined by the coupled assay procedure of Paglia and Valentine (1967) as modified by Lawrence et al. (1974). It is based

on the reaction:



The reaction was monitored using a Beckman DU-Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH 44074) at 340 nm with a Varian Model 9176 chart recorder. The plasma sample (0.025 ml) was added to a 1 ml cuvette containing a phosphate buffer solution, pH=7.0, (0.915 ml) to which NADPH (0.12 mg/ml) and glutathione reductase (1.0 EU/ml) had been added. Reduced glutathione solution (0.050 ml; 12.3 mg/ml) was added and the solutions mixed. An H_2O_2 solution (0.010 ml; 0.124 ml 30% H_2O_2 and 99.876 ml H_2O) was then added to initiate the reaction, and the absorbance was monitored for several minutes. To account for any spontaneous degradation of NADPH, a blank was run simultaneously with distilled deionized water replacing the sample. Concentrations of the solutions were such that any changes in the rate of the reaction were due only to the change in the activity of the sample. Hydrogen peroxide was used as the substrate for the reaction to ensure evaluation of GSH-Px independent of GSH-S-transferases which show activity when organic hydroperoxide substrates are used (Prohaska and Ganther 1977). Corrected absorbance changes per minute were converted to EU per ml of sample by multiplication by a

conversion factor determined by the sample size, the molar extinction coefficient of 6.22×10^3 for NADPH and the stoichiometry of the reaction of 2 moles GSH formed per mole NADPH oxidized.

Due to the results of the studies by Zhang et al. (1986), Koller et al. (1984a), and Langlands et al. (1981) discussed earlier, care was taken to analyze samples at a constant time after collection and to make sure each sample was treated the same. All samples in this experiment were analyzed for GSH-Px activity approximately 4 months after they were collected. All samples from the same collection period were analyzed at the same time.

Plasma proteins were determined by the modified Lowrey method (Hartree, 1972) for standardization of GSH-Px units. Diluted plasma samples were added to test tubes containing a potassium-sodium tartrate:sodium bicarbonate:sodium hydroxide solution. After incubation at 50 C, a sodium tartrate:copper sulfate solution was added followed by diluted Folin-Ciocalteu reagent and a second incubation at 50 C. Absorbance was measured using a Gilford Stasar II spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH 44074) at 650 nm. A curvilinear regression line determined from similarly processed standards was used to calculate plasma protein levels.

Urine samples were analyzed for Se using the fluorometric procedure described above. Aliquots varied from 0.25 to 5.0 ml, depending on Se concentration.

Feces were weighed, lyophilized and finely ground

using a Wiley mill. Selenium content was determined using 0.1 to 0.5 g aliquots, depending on Se concentration.

Data were analyzed by SAS general linear model procedures for repeated measures using least square means (SAS Institute Inc. Cary, NC 27511).

Results and Discussion

Figure 1 illustrates the effect of the different treatments on plasma GSH-Px activity. The vehicles alone had no effect as shown by the constant decline in activity to a low level. This indicates that the diet used in this experiment was definitely deficient in Se. One pig which had been injected with the peanut oil vehicle died 41 days post-injection of a subsequently diagnosed Se deficiency. The plasma GSH-Px activity of pigs receiving sodium selenite and barium selenate rose to similar levels initially. By day 10, pigs receiving selenium in either form had higher ($P<0.01$) GSH-Px activities than those injected with the vehicles alone. The difference between the forms of Se was apparent by 24 days post-injection when barium selenate-injected pigs had higher ($P<0.05$) activities than those of sodium selenite-injected pigs, and these plasma activities were sustained longer with barium selenate (52 vs 24 days, respectively). This is shorter than the efficacy period demonstrated by MacPherson and Chalmers (1984) who administered barium selenate subcutaneously to cattle at 3.55 mg (1 mg Se)/kg bwt.

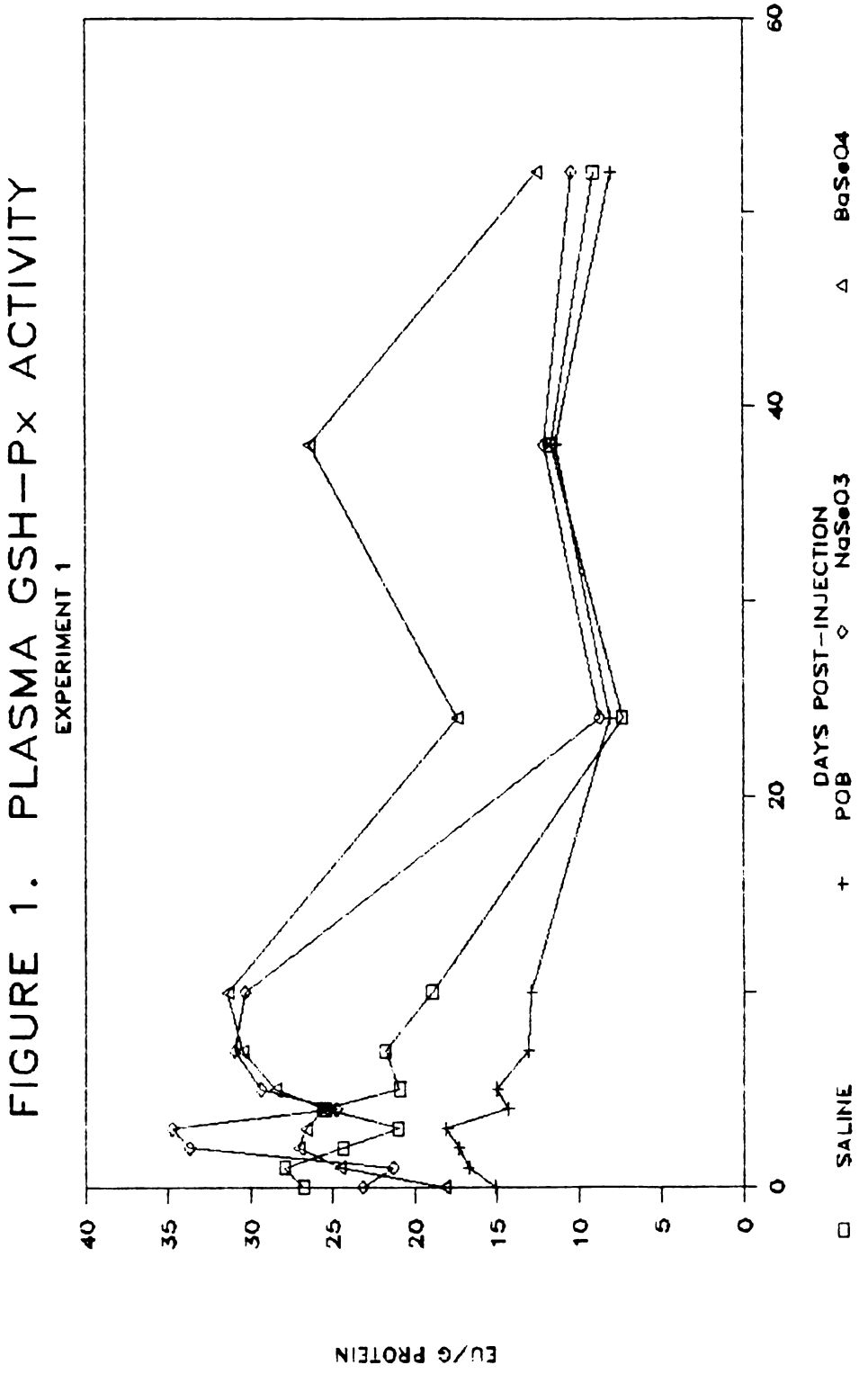


FIGURE 1. PLASMA GSH-Px ACTIVITY
EXPERIMENT 1
EU/G PROTEIN
0
5
10
15
20
25
30
35
40
0
20
40
60
DAYS POST-INJECTION
□ SALINE
+ POB
◇ NaSeO3
△ BaSeO4

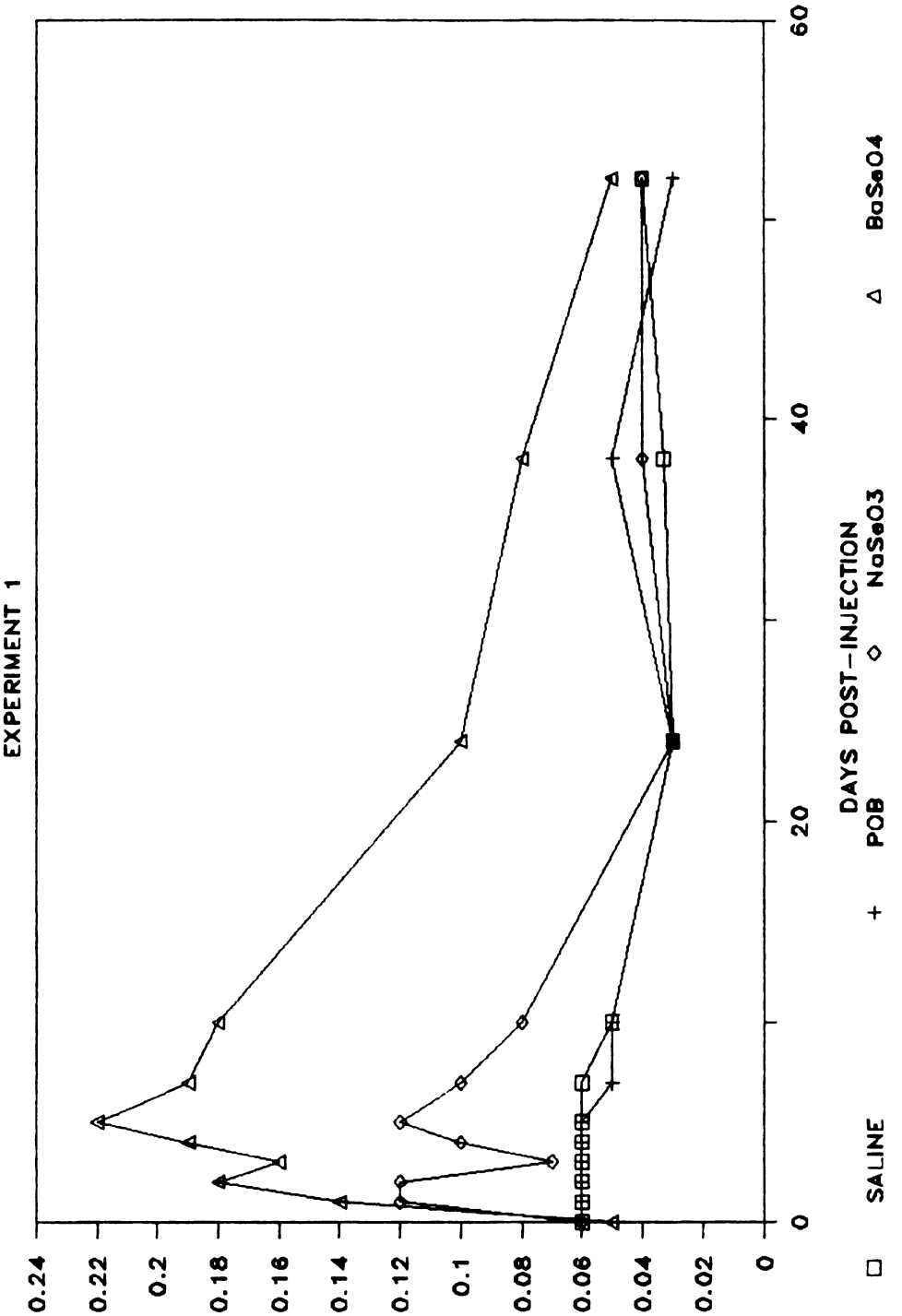
Enzyme activity in whole blood remained elevated for 140 days.

Plasma Se levels followed the same general trend as GSH-Px activity (Figure 2). There was a marked increase in plasma Se levels one day post-injection for those pigs receiving either form of selenium. For sodium selenite-injected pigs this corresponded with the peak levels seen. Barium selenate-injected pigs had peak values between 2 and 5 days post-injection. Both sodium selenite- and barium selenate-treated pigs had increases ($P<0.005$) in plasma Se over control pigs at day 10. By 38 days post-injection, pigs receiving sodium selenite had plasma selenium values similar to the control pigs. Those receiving barium selenate still had elevated ($P<0.005$) plasma selenium levels which remained elevated through 52 days post-injection. Plasma Se levels did not exceed 0.25 ug/ml at any sampling point. This is below the limit of 0.50 ug/ml which Mahan and Moxon (1979) determined as the point where selenosis signs appear.

There was a shift in the correlation between plasma Se and GSH-Px activity during the first 10 days post injection for those pigs receiving Se, indicating that not all of the plasma Se was associated with GSH-Px. Whanger et al. (1978a) demonstrated the same phenomenon in sheep by injecting sodium selenite subcutaneously and shifting the usually strong positive correlation of blood Se to GSH-Px activity to a very weak correlation.

Injection site reactions were minimal. Those pigs

FIGURE 2. PLASMA SELENIUM LEVELS



injected in the thigh had no impairment of movement. Those receiving injections in the flank had no visible swelling or inflammation, but a palpable lump was present for 10 days post-injection.

There was a marked effect of treatment on the excretion of the Se dose given (Figure 3, Figure 4). Total daily fecal Se was constant in those animals acting as controls. An increase in total daily fecal Se was seen within 3 days post-injection for those pigs injected with Se in either form. The pattern of secretion varied between sodium selenite and barium selenate. Fecal excretion of Se remained elevated for those pigs receiving barium selenate. The elevation in fecal Se is not in agreement with the findings of Burk et al. (1972). The fecal levels of Se from rats fed a basal diet containing 0.004 ppm Se did not vary following injections of up to 200 ug Se as sodium selenite. Daily total urinary Se for Se-treated pigs had the greatest increase post-injection, as expected (Figure 4). Sodium selenite injection provided the highest daily excretion which occurred at 24 hr post-injection for urine and at 48 hr for feces. As a percent of dose, Se from barium selenate was retained longer ($P<0.05$). By 10 days post-injection, combined urinary and fecal Se accounted for 29.0% of the Se from sodium selenite and 12.3% of the Se from barium selenate (Table 2).

The Se status of the animal prior to injection with a Se supplement has a marked effect on the retention of the

FIGURE 3. FECES SELENIUM

EXPERIMENT 1

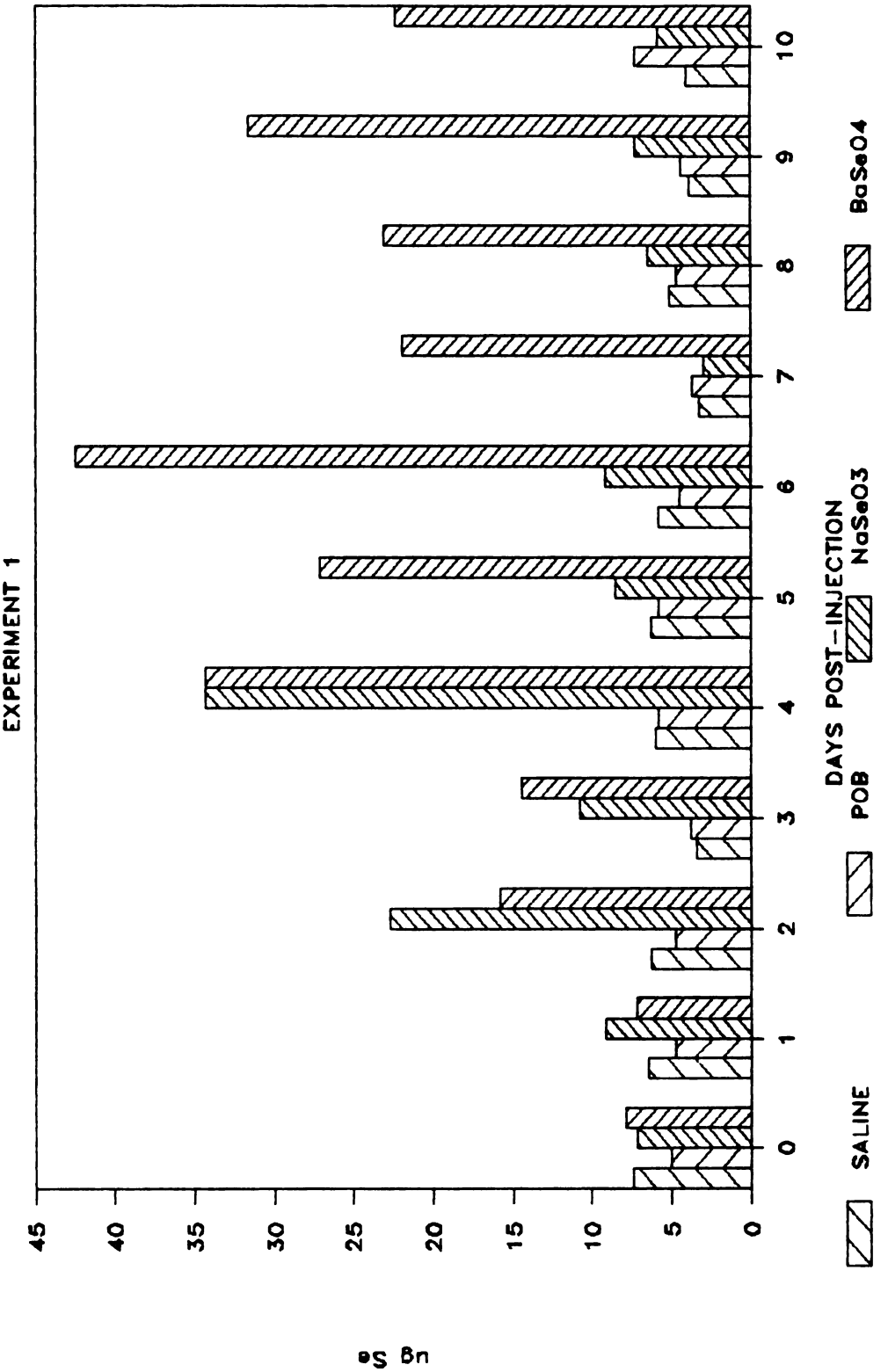


FIGURE 4. URINE SELENIUM

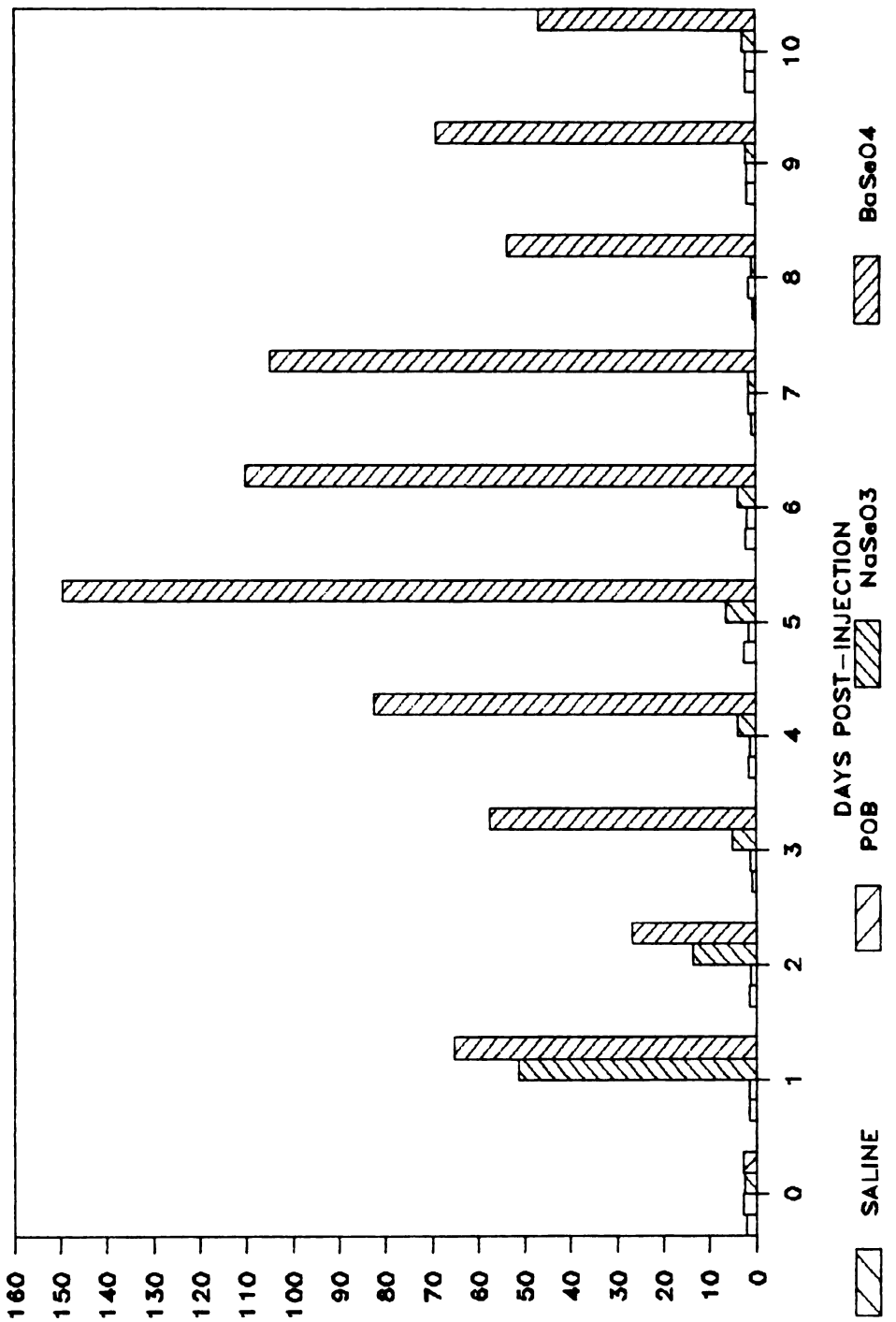


Table 2. Experiment 1 Ten Day Urine and Fecal Se Excretion as Percent of Dose Given

	Sodium selenite	Barium selenate	SEM
Urine (a)	18.27	9.81	3.30
Feces (a)	10.75 (b)	2.52 (c)	1.46
Total	29.02 (b)	12.33 (c)	3.12

a) Average of three pigs.
b,c) Means with different letters within a row are significantly different ($P<0.05$).

dose. Deficient or marginally adequate animals show an increased total retention and retention time. Burk et al. (1972) measured retention and excretion patterns in rats injected with $^{75}SeO_3^-$. The lower the initial Se status of the rat, the greater the retention of the injected dose. The initial Se status of the rat had no effect on total fecal Se excretion, but urinary excretion of the dose increased as Se status increased.

This confirms work done by Muth et al. (1967). Ewes were used to evaluate retention time of a 5 mg dose of ^{75}Se as sodium selenite. The retention of the Se in deficient ewes was greater than that in adequate ewes. Two vehicles were tested; saline and a 5% peanut oil:beeswax mixture. The peanut oil mixture improved the retention in deficient ewes over that of the saline. The vehicle made little or no difference in retention when Se-adequate ewes were used.

Calves born with adequate serum Se levels due to prepartum supplementation of the dam showed no physiologic response to injections of Se and vitamin E given at birth and 14 days of age (Weiss, 1983). Calves of unsupplemented dams responded to both injections. Mahan et al. (1979) found that in pigs fed a diet supplemented with 0.1 ppm Se, an intramuscular injection of sodium selenite elevated plasma Se levels for less than 2 weeks. The pigs on this study can be considered marginally adequate at the beginning. This allowed determination of the experimentally effective periods for parenteral administration of sodium selenite and barium selenate in the vehicles used to be 21

and 52 days, respectively.

From this preliminary study it was concluded that barium selenate was biologically effective as a Se source and was useful as a long term supplement. The next objective was to improve the duration of the effective period.

Experiment 2

Methods to slow the release of Se from the injection site involve the use of various carriers. The Romansky formulation works adequately for both barium selenate and sodium selenite but is impractical for use on a commercial basis due to the variability in composition of beeswax. Thus, the development of a new vehicle was required.

Buckwalter and Dickson (1948) introduced a long-lasting formulation of procaine penicillin G in oil which used aluminum monostearate as the thickening agent. Aluminum monostearate has thixotropic properties (Parrott 1970). When allowed to sit undisturbed, a thixotropic mixture will thicken and gel. When it is agitated at the same temperature, the mixture will become thin and will flow. The thixotropic property is desirable in a long acting parenteral Se supplement. Gelling of the product would prevent sedimentation of the Se as the product sat on the shelf. It would also retain the dose in a compact bolus at the injection site, reducing surface area and slowing absorption. Compounds which have thixotropic properties are long-chain or linear polymers such as sodium salts of carboxymethylcellulose or polyvinylpyrrolidone in water and aluminum monostearate in oil. When placed under pressure, such as being forced through a hypodermic syringe, the chains orient parallel to each other and flow easily. When allowed to rest, the chains intertwine and gel.

To form an even suspension of a powder in a vehicle a surface-active or wetting agent is often used. The choice of the agent employed depends on whether the vehicle is water or oil. The surface-active agent serves to balance the polar and non-polar groups of the insoluble compound with those of the vehicle, allowing more efficient mixing of the two phases. The hydrophil-lipophil balance (HLB) system is used to classify surface-active agents (Parrott 1970). It is a subjective scale. Agents with a low HLB value are oil soluble and those with a high value are water soluble.

Various thixotropic compounds and appropriate wetting agents were used in this study in an attempt to prolong the efficacy of barium selenate given parenterally.

Methods

A. Assembly of the parenteral preparations

Four formulations were chosen for this experiment.

The first was a modification of the peanut oil-beeswax preparation used in experiment one. Barium selenate was measured into sterilized vials under UV light. A small weighed amount of sorbitan monooleate (HLB=4.3; No. S-6760, Sigma Chemical Co., St. Louis, MO 63178) as the wetting agent was added along with two sterilized mixing beads, and the vials were sealed. Peanut oil with 2% beeswax was treated as in experiment one and assembly was performed as previously described.

Assembly of the second preparation paralleled that of

the first. The vehicle used was a mixture of peanut oil and aluminum monostearate (5% w:w), comparable to that of Buckwalter and Dickson (1948), which had been sterilized by dry heat at 160 C for 2 hr before addition to the barium selenate. Sorbitan monooleate was again added as the wetting agent. Aluminum monostearate was prepared using the method recommended in Remington's Pharmaceutical Sciences (1970). A 2% aqueous solution of KOH was added to a 6% solution of stearic acid in ethanol. This solution was then added to a 2.5% aqueous solution of potassium alum. A white precipitate formed which was then washed with ethyl ether and acetone. The resulting powder was boiled in methanol, filtered and washed with acetone to remove free stearic acid and aluminum distearate.

Preparations three and four were water-based. Distilled deionized water was used. Barium selenate and polyoxyethylene sorbitan monooleate (HLB=15.0; No. P-1754, Sigma Chemical Co., St. Louis, MO 63178) were again weighed into vials. Prior to addition to the barium selenate-polyoxyethylene sorbitan monooleate mixture, the vehicles were autoclaved 15 min at 121 C and 12 kg/cm^2 Formulation three utilized a low viscosity carboxymethylcellulose sodium salt (8% w:v; No. C-8758, Sigma Chemical Co., St. Louis, MO 63178) as the thickening agent. Formulation four involved a mixture of polyvinylpyrrolidone (20% w:v; No. K 26-28, General Aniline and Film Corp., New York, NY 10014) and the low viscosity

carboxymethylcellulose sodium salt (4% w:v).

All four preparations had equivalent concentrations of 17.5 mg $BaSeO_4$ (4.83 mg Se)/ml designed to be administered subcutaneously at 1 ml/5 kg bwt.

B. Pigs

Sixteen crossbred pigs averaging 5.8 kg were used in this experiment. The protocol closely paralleled the first experiment except the collection period was 21 days. The diet was the same formulation as that used in experiment one and contained 0.05 ppm Se by assay. Twenty-four hr urine and feces collections were made for the first 10 days and additional 24 hr collections were made at 12, 15, 18, and 21 days post-injection. Blood samples, drawn as previously described, were taken initially and at 1, 2, 3, 4, 7, 10, 14, 21, 28, 35, 42, 49, 63, 77, 91, and 120 days post-injection. An aliquot of whole blood was retained before processing. Hematocrit (McGovern et al., 1955) and whole blood hemoglobin concentrations (Crosby et al., 1954) were determined. Erythrocytes were washed twice with heparinized saline following harvesting of the plasma. All samples were stored in 5 ml plastic tubes under nitrogen at -20 C until analyzed.

Following the balance study the pigs were moved to the grower-finisher barn and fed ad libitum the same diet. Forty days post-injection the diet was changed to match the pigs requirements for the finishing phase (Table 3). The new diet was found to contain 0.06 ppm Se.

Between 127 and 152 days post-injection, the pigs were

Table 3. Experiment 2 Grower Diet

Ingredient	% Composition
Corn	72.84
Soybean meal (44 % crude protein)	23.50
Calcium carbonate	1.10
Mono-di-calcium phosphate	1.50
Salt	.50
MSU vitamin-trace mineral premix (a)	.50
Aureomycin-50 (b)	.05
Vitamin E premix (c)	.01
	100.00

a)Vitamin concentrations (per kg): retinyl acetate, 660,00 USP units; cholecalciferol, 132,000 USP units; riboflavin, 660 mg; d-calcium pantothenate, 2640 mg; nicotinic acid, 3520 mg; cyanocobalamin, 3.96 mg; choline chloride, 25344 mg; menadione sodium bisulfite complex, 440 mg. Mineral concentrations in % (and source): zinc, 1.496 (zinc oxide); iron, 1.188 (ferrous sulfate); manganese, 0.0748 (manganous oxide); copper, 0.198 (copper oxide); iodine, 0.01 (ethylene diamine dihydroiodide).

b)Concentration in %: chlortetracycline, 11.

c)Concentration in %: dl-alpha-tocopheryl acetate, 50.

killed by electric shock and exsanguination. Samples of liver, kidney and sternomandibular muscle were taken for Se analysis. The injection site was located and half the tissue was fixed in buffered formalin for histopathologic evaluation. The remaining tissue was retained for Se analysis.

Analyses

Plasma, urine and feces were analyzed for Se as previously described.

Plasma, whole blood and washed erythrocytes were evaluated for GSH-Px activity approximately 4 months after they were collected. Whole blood and washed erythrocytes were diluted with distilled deionized water 21 fold and a 0.05 ml aliquot assayed.

The hemoglobin content of the washed erythrocyte suspension was also determined.

Plasma proteins were determined by the modified Lowrey procedure.

Tissue selenium values were determined using the procedure described above. The samples (5 g liver and kidney, 10 g muscle) were predigested in HNO_3. Volumes of the predigests were then brought up to 50 ml and an aliquot (5 ml muscle, 3 ml liver, 1 ml kidney) used in the assay.

Results and Discussion

There were no significant biological differences between the treatments. Plasma, erythrocyte and whole blood GSH-Px activities all exhibited similar responses (Figures 5,6,7). A sharp initial rise in plasma enzyme activity peaked 7 days post-injection (Figure 5). Activity then declined slowly. A second peak of a similar magnitude occurred 90 days post-injection and was followed by a rapid decline. The decrease in activity seen at 60 days post-injection may be attributed to daily variation in the GSH-Px analysis.

Erythrocyte activity followed the same general pattern (Figure 6). The first rise peaked between 14 and 21 days post-injection. The second was seen between 60 and 70 days post-injection and was much smaller than the first. The first peak may be due to erythrocyte turnover. The lifespan of a pig erythrocyte is 62-71 days (Schalm, 1965). New erythrocytes being released into the circulation would have incorporated adequate Se from the injection. Whole blood GSH-Px activities showed the same two peaks, but peak heights were transposed (Figure 7). An early peak at 10-14 days was followed by a much higher peak between 63 and 90 days post-injection.

The simultaneous drop in plasma, erythrocyte and whole blood GSH-Px activity can be a normal occurence in pigs of that age. Because all samples from a collection period were analyzed on the same day, there may be confounding of the

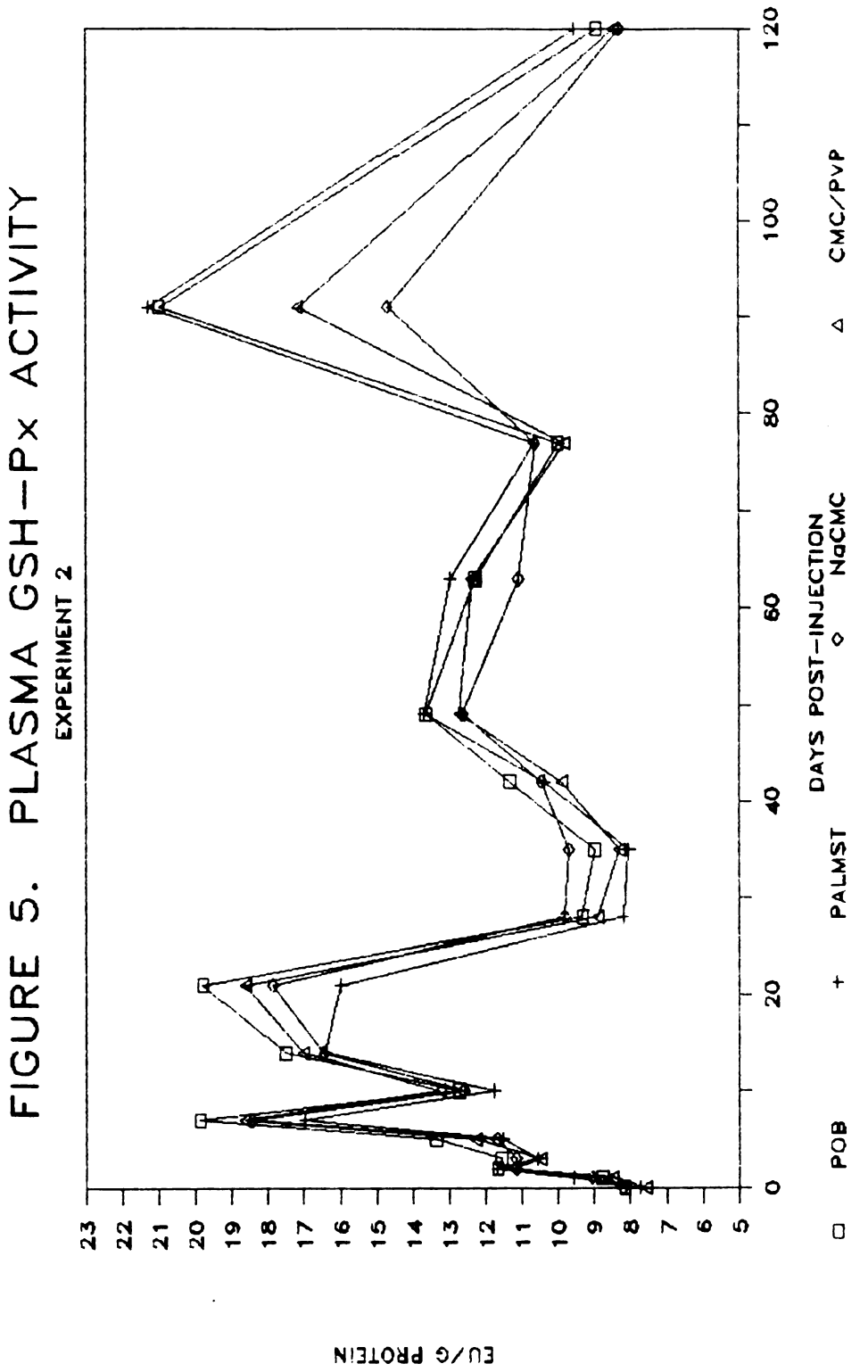


FIGURE 5. PLASMA GSH-Px ACTIVITY
EXPERIMENT 2
EU/G PROTEIN
DAYS POST-INJECTION
□ POB
+ PALMST
◇ NaCMC
△ CMC/PVP

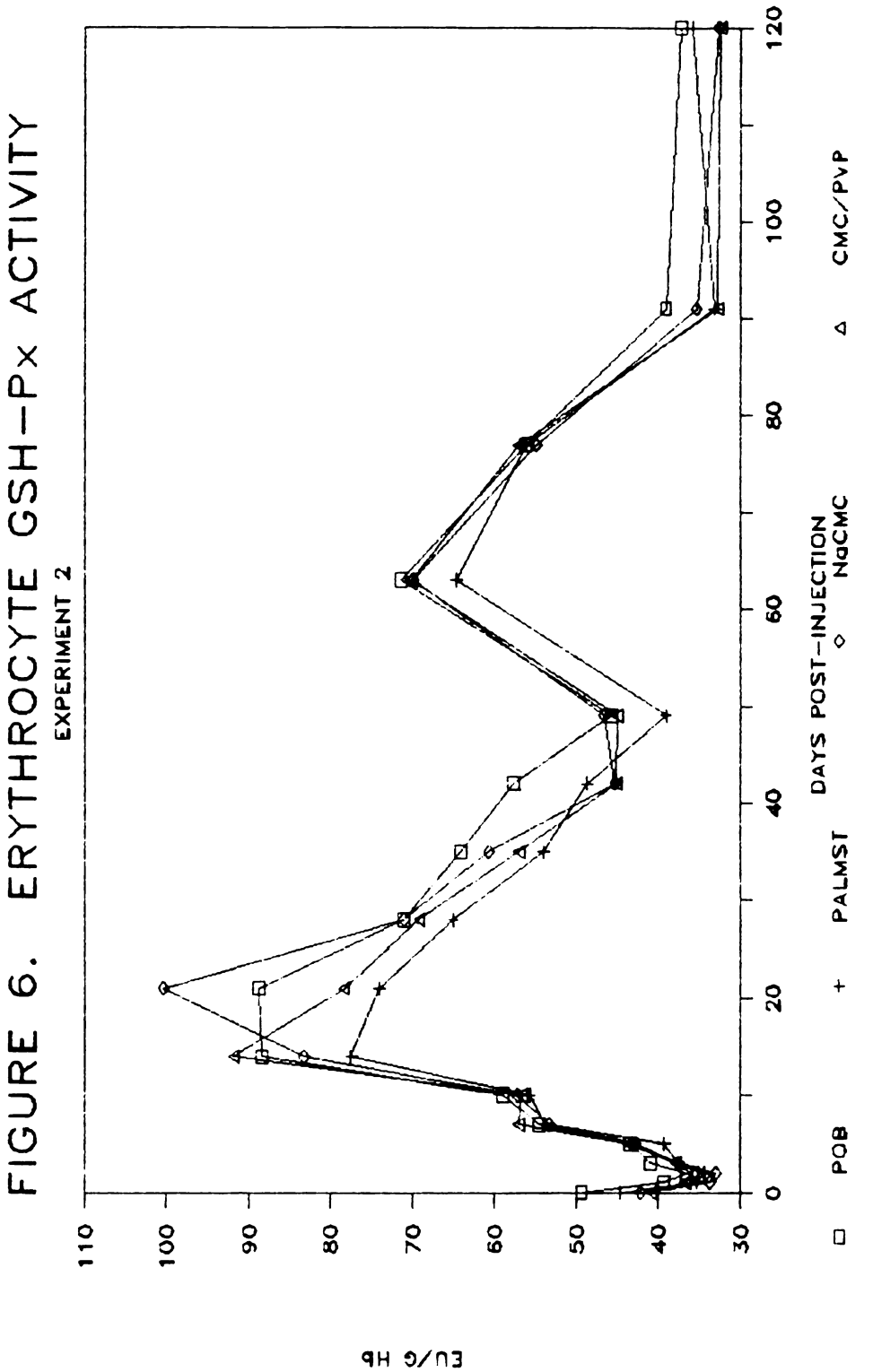


FIGURE 6. ERYTHROCYTE GSH-Px ACTIVITY
EXPERIMENT 2
EU/G Hb
110
100
90
80
70
60
50
40
30
0
20
40
60
80
100
120
DAYS POST-INJECTION
□ POB
+ PALMST
◇ NaCMC
△ CMC/PVP

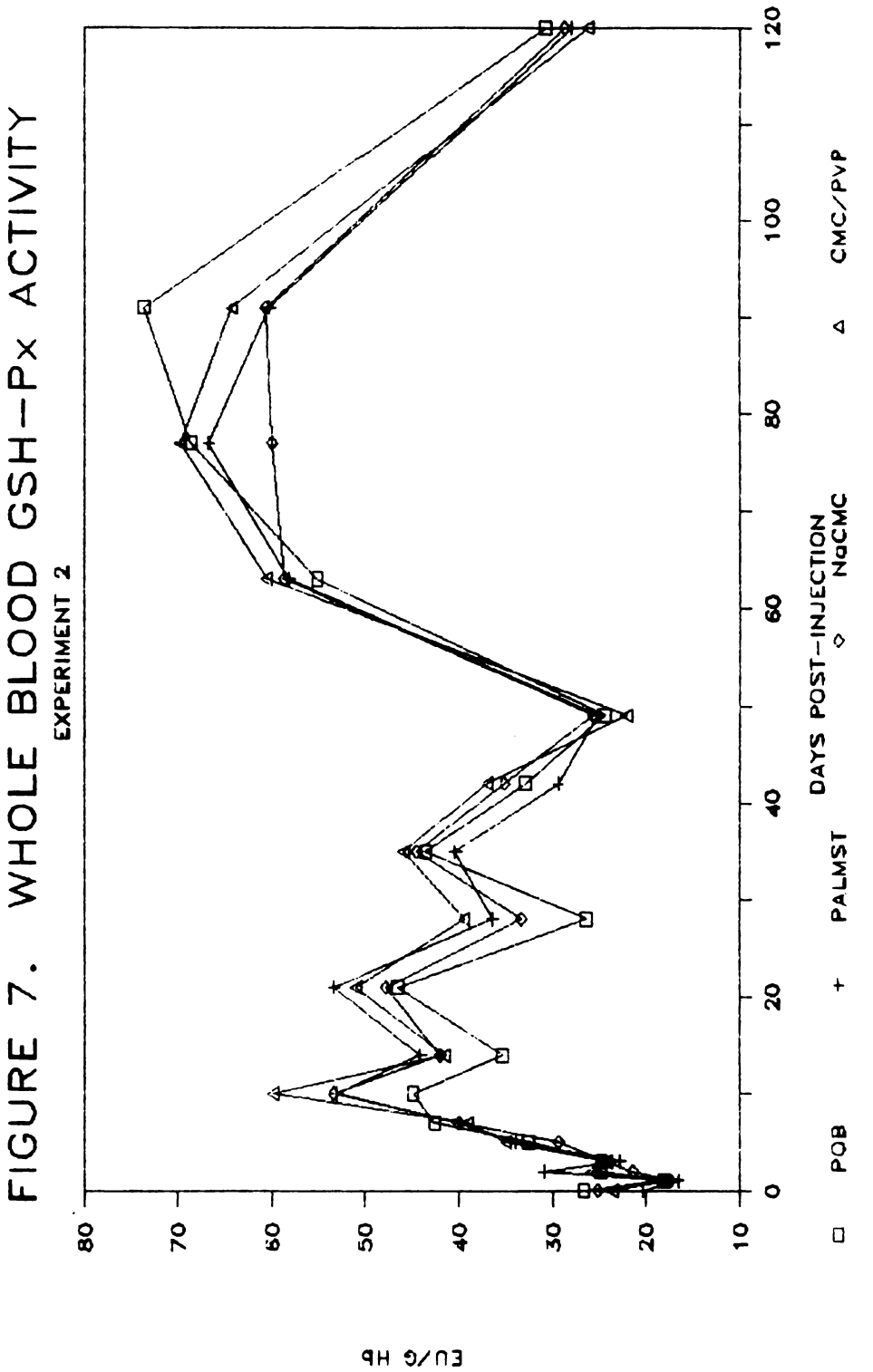


FIGURE 7. WHOLE BLOOD GSH-Px ACTIVITY
EXPERIMENT 2
EU/G Hb
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
DAYS POST-INJECTION
POB
PALMST
NaCMC
CMC/PVP

results by daily variation in the GSH-Px assay despite care taken to prevent it.

Plasma Se levels peaked 2 days post-injection (Fig. 8). Levels then gradually decreased to plateau at above initial levels for 120 days post-injection giving a strong indication that Se was being released slowly. The lack of a drop in plasma Se indicates that the drop in blood GSH-Px activity may be due to laboratory variation or to variation in GSH-Px activity due to the age of the pig.

A localized edema at the injection site occurred by 10 hr post-injection. The reaction, in order of increasing magnitude, was least with peanut oil-beeswax followed by peanut oil-aluminum monostearate, carboxymethylcellulose-polyvinylpyrrolidone, and carboxymethylcellulose. The visible edema disappeared by 7 days post-injection for all treatments but a palpable lump remained in those injected with the water-based preparations at 21 days post-injection.

Whole blood hemoglobin values were well within the expected normal range of 10 to 16 g/dl blood (Schalm, 1965) for all treatments (Table 4). Packed cell volumes were also within the 32 to 50% range accepted as normal.

Balance trial results paralleled those found for the peanut oil-beeswax treatment group in experiment one (Table 5). At 10 days post-injection, combined urine and fecal losses accounted for 12.6%, 16.8%, 17.0%, and 16.1% of the Se given for the respective treatments. Fecal excretion peaked at 3-4 days post-injection (Figure 9). The urine Se

FIGURE 8. PLASMA SELENIUM LEVELS

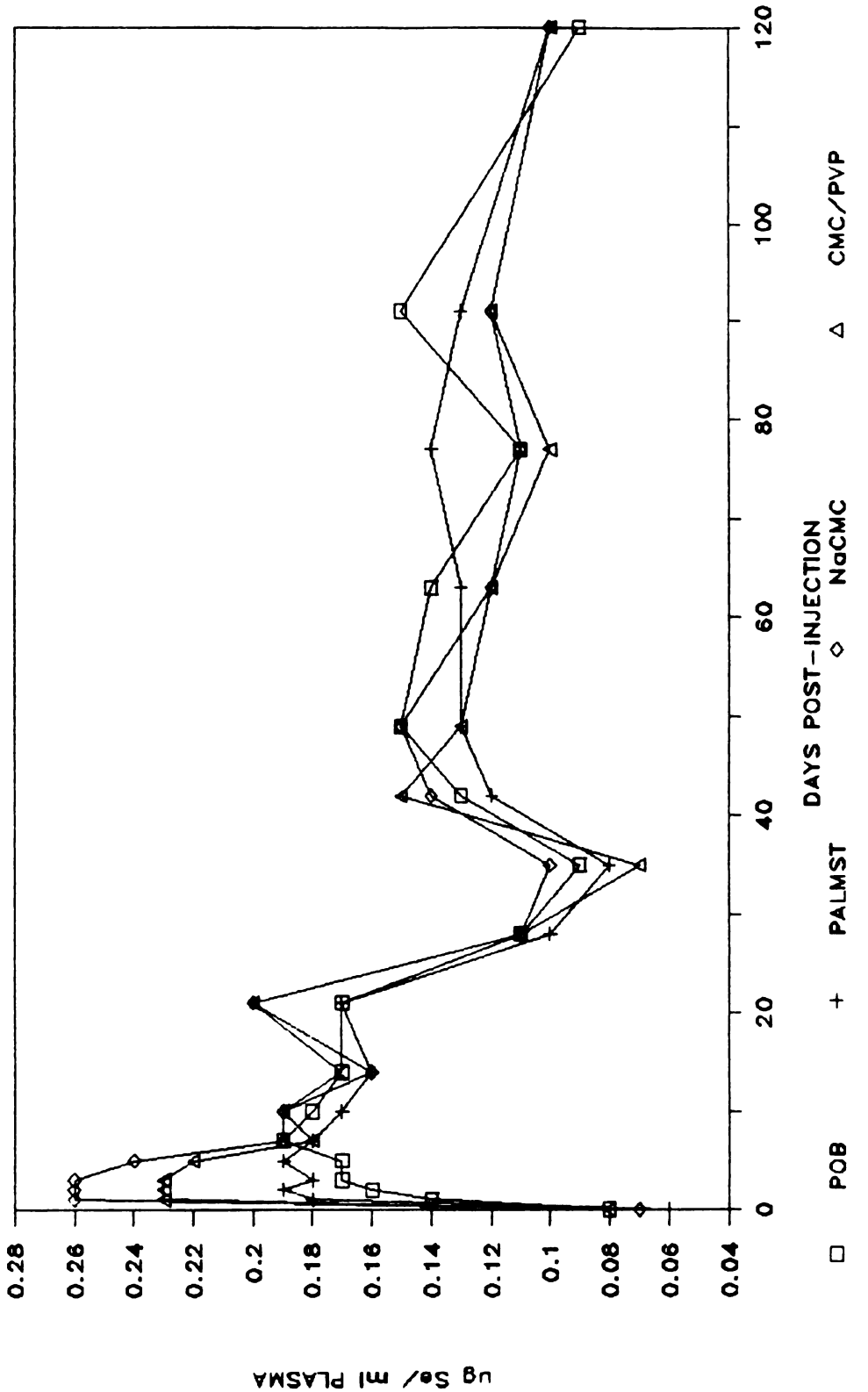


Table 4. Experiment 2 Hematology

Treatment	Days Post-Injection																
	0	1	2	3	5	7	10	14	21	28	35	42	49	63	77	91	120
	Hemoglobin g/dl Whole Blood																
POB (a)	15.4	13.7	12.3	12.0	12.0	11.9	12.9	12.4	13.5	15.4	11.1	11.2	12.4	12.3	14.6	13.2	14.4
PALMST (b)	14.6	13.6	12.6	12.2	11.9	11.8	12.6	12.6	13.4	14.6	11.2	11.6	12.2	12.2	13.6	13.4	14.8
CMC (c)	15.4	14.1	12.9	12.8	12.2	12.2	12.8	12.6	13.0	15.4	11.2	11.1	12.0	12.4	15.5	12.2	15.1
CMC/PVP (d)	14.9	13.6	12.2	12.1	11.5	11.6	12.8	12.6	13.5	14.6	10.6	11.2	12.0	12.7	14.6	12.0	15.1
SEM	.34	.53	.52	.45	.39	.36	.35	.28	.33	.33	.33	.42	.48	.30	.88	.33	.48
	Hematocrit % Packed Cell Volume																
POB	45.8	42.0	38.5	37.7	38.0	38.5	39.5	42.5	42.4	35.9	34.0	34.9	37.5	39.3	41.2	41.8	44.2
PALMST	43.4	41.8	39.0	38.8	36.6	37.7	38.8	42.0	41.6	35,2	35.2	35.2	37.6	39.4	43.8	42.0	44.0
CMC	45.7	43.2	41.1	40.9	38.8	39.3	39.8	40.3	40.7	37.0	34.6	34.6	37.7	39.0	42.4	42.3	45.6
CMC/PVP	44.2	41.6	38.2	38.2	36.5	37.4	39.8	41.9	42.2	37.3	32.5	34.9	36.4	38.7	42.7	42.0	45.5
SEM	1.01	1.47	1.84	1.37	1.07	1.17	1.03	.97	1.02	.75	.76	1.06	1.60	1.05	.95	.93	.86

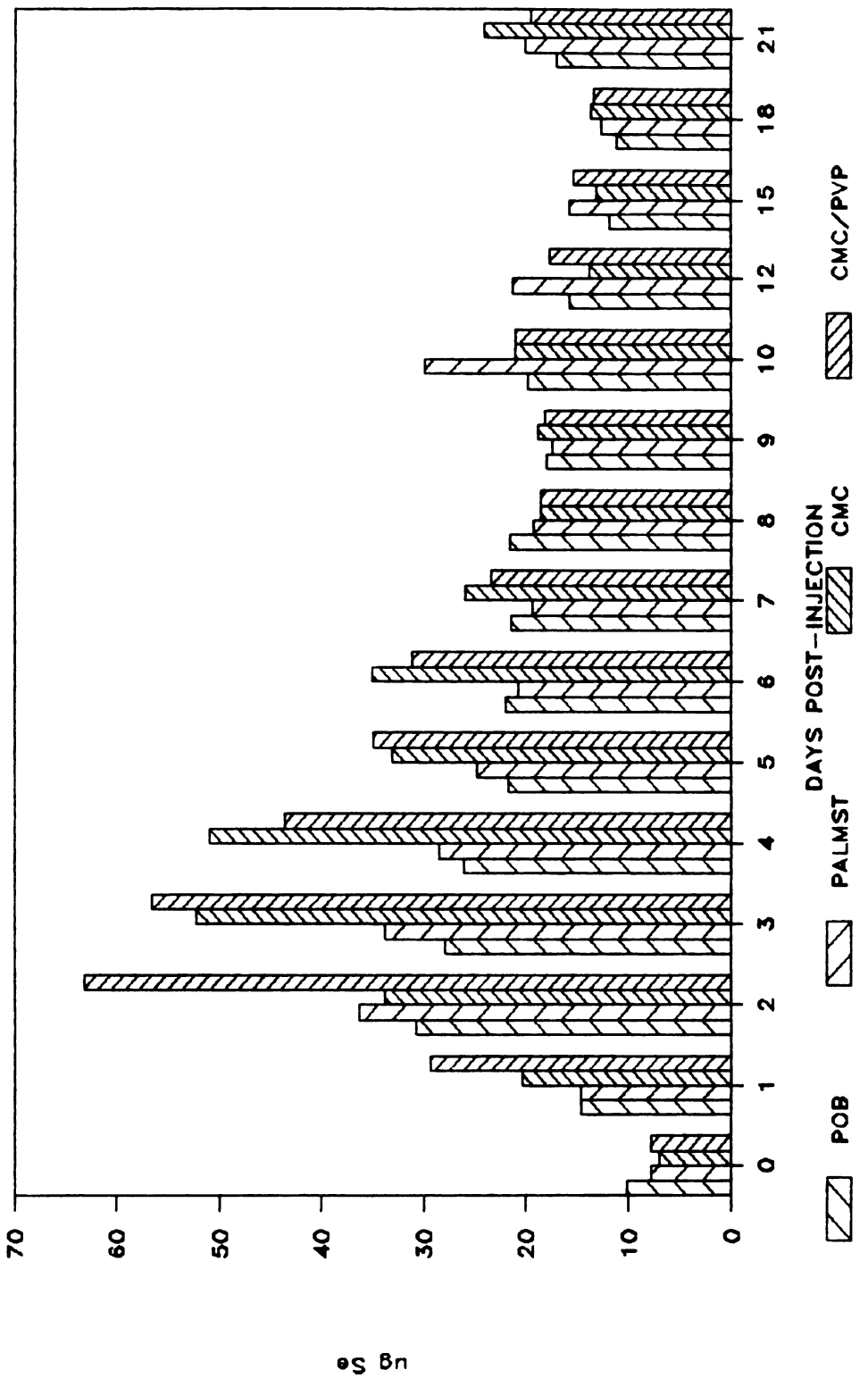
a) Peanut oil-beeswax vehicle with barium selenate.
b) Peanut oil-aluminum monostearate vehicle with barium selenate.
c) Carboxymethylcellulose vehicle with barium selenate.
d) Carboxymethylcellulose-polyvinylpyrrolidone vehicle with barium selenate.

Table 5. Experiment 2 Ten Day Urine and Fecal Se Excretion as Percent of Dose

	POB (a)	PALMST (b)	CMC (c)	CMC/PVP (d)	SEM
Urine (e)	9.40 (f)	13.20 (g)	12.08	10.88	1.15
Feces (e)	3.16 (h)	3.43 (h)	4.88 (i)	5.23 (i)	0.53
Total	12.56 (f)	16.63 (g)	16.96	16.11	1.31

a) Peanut oil-beeswax vehicle with barium selenate.
b) Peanut oil-aluminum monostearate vehicle with barium selenate.
c) Carboxymethylcellulose vehicle with barium selenate.
d) Carboxymethylcellulose-polyvinylpyrrolidone vehicle with barium selenate.
e) Average of four pigs.
f,g) Means with different letters within a row are significantly different ($P<0.05$).
h,i) Means with different letters within a row are significantly different ($P<0.01$).

FIGURE 9. FECES SELENIUM



peak varied from 1 to 3 days post-injection, and collections taken at 21 days still showed slightly elevated Se levels (Figure 10). Fecal excretion of Se was higher ($P<0.01$) when the water-based vehicles were used. Those pigs receiving the peanut oil-beeswax treatment showed lower ($P<0.05$) urinary and total Se excretion than did the pigs receiving the other treatments.

On day 70, one pig from the peanut oil-beeswax treatment group was found dead. Necropsy findings showed no signs of Se/vitamin E deficiency or Se toxicity lesions. Samples of liver, kidney, skeletal muscle and heart muscle were taken at necropsy for Se analysis and contained 0.419, 1.232, 0.058 and 0.135 ppm Se wet basis, respectively. The tissues taken from the other pigs at slaughter showed Se levels at or slightly below these (Table 6). There was no correlation between treatment and tissue Se. Liver values ranged from 0.193 to 0.306 ppm on a wet basis. Kidney contained 1.163 to 1.514 ppm, and sternomandibular muscle contained .038 to .056 ppm, both on a wet basis. These values are below those determined for animals maintained on diets supplemented with Se as sodium selenite. Groce et al. (1971) and Ku et al. (1973) reported skeletal muscle Se concentrations of 0.08, 0.098 and 0.12 ppm wet basis for pigs fed 0.14, 0.54 and 0.44 ppm Se diets, respectively. Ku et al. (1973) also reported liver and kidney Se concentrations of 0.61 and 2.14 ppm wet basis, respectively, for pigs fed the 0.44 ppm Se diet. Because of

FIGURE 10. URINE SELENIUM

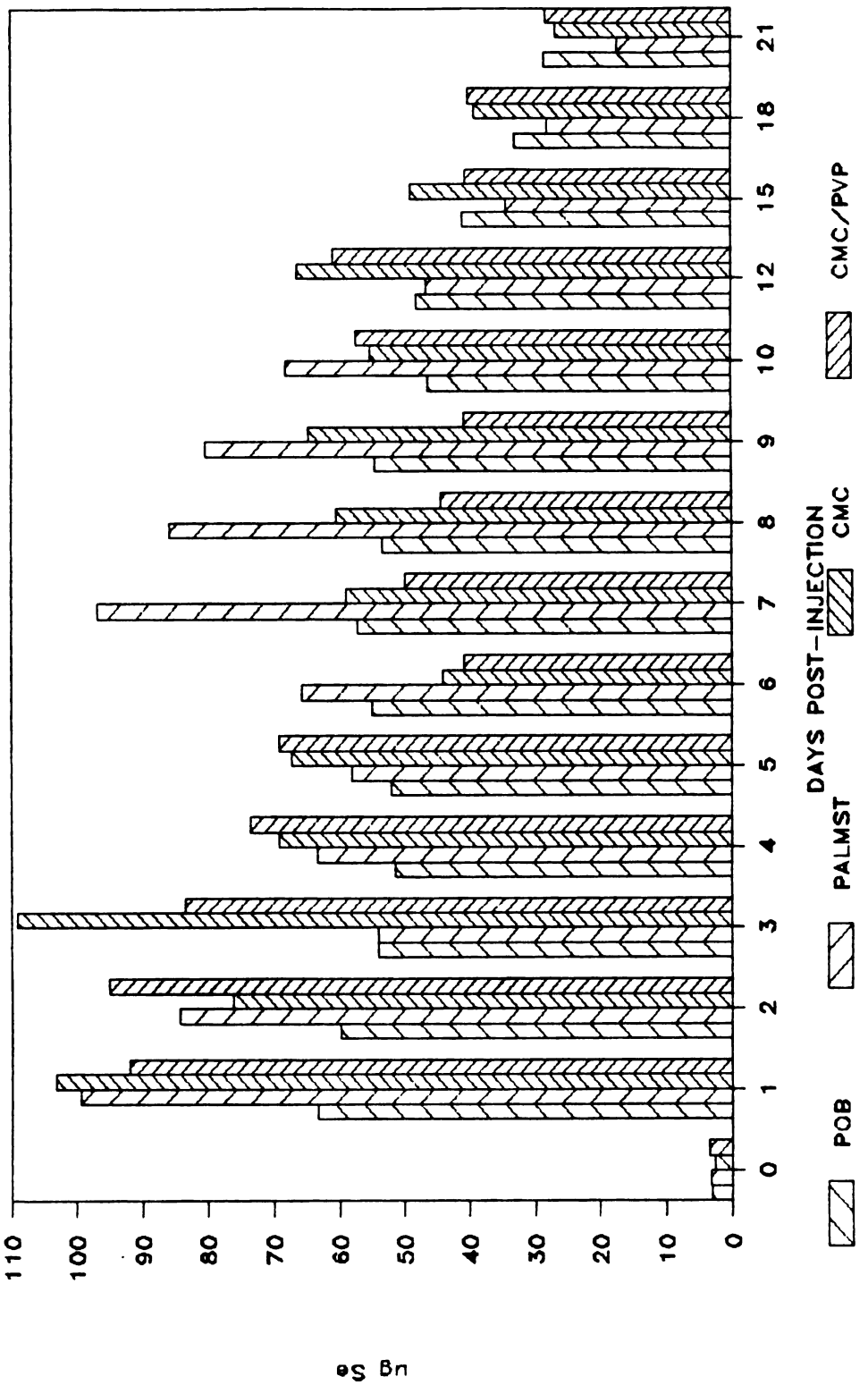


Table 6. Experiment 2 Tissue Se Concentration (a)

Treatment	Pig No.	Liver	Kidney	Skeletal Muscle
POB (b)				
	135-1	.381	1.971	.066
	135-4	.193	1.298	.046
	212-1	.287	1.345	.040
PALMST (c)				
	135-5	.270	1.485	.052
	135-6	.372	1.767	.061
	212-3	.349	1.615	.045
	212-12	.306	1.514	.056
CMC (d)				
	135-2	.264	1.337	.045
	135-8	.374	2.127	.063
	212-5	.286	1.135	.057
	212-10	.266	1.163	.044
CMC/PVP (e)				
	135-7	.343	1.879	.061
	135-13	.236	1.167	.038
	212-2	.210	1.244	.041
	212-4	.283	1.568	.060

a) ug/g tissue, wet basis.
b) Peanut oil-beeswax vehicle with barium selenate.
c) Peanut oil-aluminum monostearate vehicle with barium selenate.
d) Carboxymethylcellulose vehicle with barium selenate.
e) Carboxymethylcellulose-polyvinylpyrrolidone vehicle with barium selenate.

the lack of replication it is difficult to compare the tissue values of the pig that died to those of pigs taken at slaughter.

Tissue concentrations of Se have been evaluated often. Lopez et al. (1968) demonstrated that kidney and liver have the highest Se concentration of any internal organ. Adipose tissue contains negligible Se levels. Van Vleet (1975) showed slightly elevated liver and kidney Se levels when intramuscular injections of sodium selenite were given to calves, lambs and pigs at 0.0825, 0.055 and 0.6 mg Se/kg bwt, respectively. Any increase seen disappeared by 23 days post-injection. Diehl et al. (1975) showed that at lethal sodium selenite dose levels (upwards of 1.65 mg Se/kg bwt) there is an apparent saturation point for kidney and skeletal muscle. Liver was flexible in the amount of Se it could store and appeared to retain excess Se until the kidney could dispose of it.

Selenium analysis of the muscle near the injection site taken at slaughter showed concentrations varying from 0.045 to 0.109 ug Se/g tissue wet basis (Table 7). There was no significant difference between treatments. All treatments showed pigs with tissue Se levels at both extremes. Van Vleet (1975) found there was a slight to substantial initial increase in Se concentration of the muscle surrounding the injection site in calves, lambs and pigs given intramuscular injections previously described. The degree of tissue selenium increase depended on the species injected, and the concentration of Se at the site

Table 7. Injection Site Reaction

Treatment	Pig No.	Lesion	Se ug/g wet basis (a)
POB (b)			
	135-1	--	.066
	135-4	--	.109
	212-1	--	.051
PALMST (c)			
	135-5	+	.061
	135-6	+++	.101
	212-3	--	.045
	212-12	--	.083
CMC (d)			
	135-2	--	.093
	135-8	+	.051
	212-5	--	.052
	212-10	--	.049
CMC/PVP (e)			
	135-7	+/-	.101
	135-13	--	.058
	212-2	--	.051
	212-4	--	.048

a) Se content of muscle tissue isolated from the injection site.
b) Peanut oil-beeswax vehicle with barium selenate.
c) Peanut oil-aluminum monostearate vehicle with barium selenate.
d) Carboxymethylcellulose vehicle with barium selenate.
e) Carboxymethylcellulose-polyvinylpyrrolidone vehicle with barium selenate.

decreased to baseline by 14 days post-injection.

Localized lesions classified as chronic inflammatory responses were seen in three of the 15 pigs slaughtered (Table 7). There was no correlation between treatment group or concentration of selenium remaining at the injection site. The lesions of two pigs were nodular and were located in the subcutaneous fat. Herigstad and Whitehair (1974) demonstrated that intramuscular injections of sodium selenite in water caused localized necrosis at the injection site. The severity of the necrosis depended on the total amount of Se administered.

The sustained plasma Se concentrations and blood GSH-Px activity achieved in this study show all preparations to be equally effective for up to 120 days. The next step was the incorporation of vitamin E into the preparation. Most currently used parenteral vitamin E supplements are aqueous dispersions which allow rapid release of vitamin E. Because the water-based vehicles were equivalent to the oil-based vehicles with regard to the Se portion of the preparation, they were tested for compatability with alpha-tocopherol and alpha-tocopheryl acetate for use in experiment three. The polyvinylpyrrolidone vehicle was immiscible with either form of the vitamin. The carboxymethylcellulose vehicle formed a homogeneous mixture which did not separate rapidly.

Experiment 3

To ensure complete effectiveness of a parenteral for the prevention of NMD it should contain both Se and adequate vitamin E . This study was designed to evaluate the effects of the addition of pharmacologic doses of vitamin E into the Se-containing parenteral already formulated.

Mahan et al. (1973) compared the effects of injections of Se and alpha-tocopherol alone or in combination for the prevention of NMD in pigs. When administered alone, alpha-tocopherol reduced the incidence of NMD slightly. Selenium alone and Se-alpha-tocopherol injections prevented further occurrence of NMD during the experimental period.

Steel et al. (1981) described studies with sheep grazing pastures where NMD had occurred. A single oral drench of 4000 USP alpha-tocopheryl acetate was adequate to prevent the occurrence of NMD. Treatment was considered to be effective for 6 weeks based on periodic monitoring of plasma alpha-tocopherol levels. When animals under similar conditions were injected intramuscularly with 750 USP alpha-tocopheryl acetate, whole blood alpha-tocopherol levels were maintained at 1.25 to 1.5 ug/ ml for 62 days. When the dose was increased to 1650 USP alpha-tocopheryl acetate. the plasma alpha-tocopherol levels exceeded 2.0 ug/ml by 15 days post-injection and were consistently rising after 62 days. The maintenance of elevated blood tocopherol levels indicates that tocopheryl acetate may act

as an absorption delaying factor but the initial slow elevation of blood tocopherol concentration has caused doubt as to the immediate effectiveness of alpha-tocopheryl acetate when it is administered parenterally.

The vehicle has a great affect on parenterally administered alpha-tocopherol. Aqueous vehicles tend to release alpha-tocopherol more rapidly from the injection site, using plasma tocopherol levels as an indicator (Steele et al., 1981; Newmark et al., 1975). Early work by Overman et al. (1954) compared aqueous and oil parenteral formulations of alpha-tocopherol, both administered at 90.9 USP per dose. The aqueous formula allowed a plasma alpha-tocopherol rise of 1.1 ug/ml while the oil formula allowed only an increase of 0.5 ug/ml. Increasing the amount administered in oil to 454.5 USP gave an increase of 0.8 ug/ml, well below that expected.

When alpha-tocopheryl acetate is given orally it is hydrolysed and the free alcohol absorbed (Blomstrand and Forsgren, 1968). When the gut is bypassed by parenteral administration, there is some question whether the acetate is cleaved by other tissues. Newmark et al. (1975) looked at the biopharmaceutical factors in parenteral administration. Dogs were injected intramuscularly with alpha-tocopheryl acetate in an aqueous suspension at 5 USP/kg bwt. There was a rapid rise in plasma alpha-tocopheryl acetate level peaking at 50 ug/ml 1 to 4 hr post-injection, indicating a rapid mobilization of alpha-

tocopheryl acetate from the injection site. Plasma alpha-tocopherol levels increased gradually to 5 ug/ml 24 hr post-injection, indicating slow cleavage of the ester. In a subsequent experiment, canine whole blood was incubated in vitro at 37 C with alpha-tocopheryl acetate. There was no detectable cleavage of the ester after 3 hr which was attributed to a lack of the enzymes in whole blood necessary to cleave the ester.

Jensen et al. (1983) demonstrated that an injection of 200 USP alpha-tocopheryl acetate in an aqueous dispersion would cause a sudden increase in erythrocyte resistance to lipid peroxidation, a measure of antioxidant status. Blood tocopherol levels did not show a corresponding increase. Pigs fed 200 USP alpha-tocopheryl acetate daily required 2 weeks to show an increased erythrocyte resistance and the resulting resistance was lower than that seen in injected pigs. Dietary treatment produced blood tocopherol levels slightly higher than those of injected pigs.

Studies in humans have shown similar results. Rindi and Perri (1958) injected healthy adult men intramuscularly with 300 USP dl-alpha-tocopheryl acetate in an aqueous emulsion. Blood samples drawn over a 48 hr period indicated a rapid increase during the first 8 hr post-injection of plasma alpha-tocopheryl acetate levels which then slowly decreased. A slow increase in plasma alpha-tocopherol levels from 7 to 12 ug/ml was seen beginning at 8 hr post-injection and continuing through 32 hr post-

injection.

The safety as well as the efficacy of a dose of vitamin E must be determined. Diets containing 220 USP alpha-tocopheryl acetate per kg, fed to chicks for three weeks, produced a depression in iodine uptake by the thyroid (March, 1973). Dietary vitamin E as dl-alpha-tocopheryl acetate fed to chicks at 1000 USP/kg had no effect on growth but chicks fed 2200 USP/kg showed markedly reduced growth rates. These excess levels depressed mitochondrial respiration by 55% over basal diet levels.

The results of repeated parenteral administration of vitamin E vary according to dosage form, route of administration, and number of doses . Lorch et al. (1985) showed that intravenous administration of alpha-tocopheryl acetate in a water-based vehicle to premature human infants at levels ranging from 26.6 to 50.5 USP/kg bwt per day over periods ranging from 23 to 55 days were associated with pulmonary deterioration, thrombocytopenia, liver failure, ascites, and renal failure. Toxicity signs were not seen by Knight and Roberts (1985) who administered, subcutaneously, 90.9 USP alpha-tocopherol or 100 USP tocopheryl acetate per kg bwt per day to pre-colostral rabbit pups over a period of 6 days. Tissue distribution varied with form of the vitamin. Liver tocopherol levels were higher in those pups given the free alcohol. Tocopheryl acetate administration resulted in high tocopheryl acetate levels in the lung. Single parenteral

administrations of alpha-tocopheryl acetate up to 1650 USP, given to weanling sheep, have not produced clinical toxicity signs.

Practical problems associated with parenteral products dictated additions to the formulas previously used. Under normal storage conditions, parenteral products are susceptible to microbial degradation. To prevent bacterial growth, a preservative is added. Thimerosal is the commercial name for sodium ethylmercurithiosalicylate. At concentrations of 1:10,000 it is a non-irritating bacteriostatic agent suitable for parenteral use (Parrot, 1970).

In addition to preventing microbial attack, a product must be protected against oxidative damage. Vitamin E is a potent anti-oxidant itself. Addition of a second agent to minimize loss during storage was indicated. Various compounds are used in foods to maximize the retention of tocopherols. Ascorbyl palmitate is one of a group of vitamin C esters which are used in this capacity. When combined with tocopherols these esters act as synergists (Pongracz, 1973). Ascorbic acid esters and tocopherol are widely used in combination for preserving canned and frozen foods and in meat curing. The optimal ratio for maximizing the anti-oxidant properties is a ratio of ascorbyl palmitate to alpha-tocopheryl acetate of 1:2.5.

Because of the post-injection swelling seen in the pigs, the low viscosity carboxymethylcellulose salt was replaced with a high viscosity salt (No. C-5013, Sigma

Chemical Co., St. Louis, MO 63178) at 2.5% w:w.

Materials and Methods

A. Assembly of the parenteral preparations

Barium selenate and polyoxyethylene sorbitan monooleate were weighed under UV as before. Before sealing, the vials were purged with nitrogen. The tocopherol (No. T-3251, Sigma Chemical Co., St.Louis, MO 63178) or tocopheryl acetate (No. T-3376, Sigma Chemical Co., St. Louis, MM 63178) were weighed into acid-washed vials. A mixture of distilled deionized water, the high viscosity carboxymethylcellulose sodium salt, ascorbyl palmitate (No. 14536, Alfa Products, Danvers, MA 01923) and thimerosal was made. This mixture was added to the tocopherol or tocopheryl acetate to volume. Two acid washed mixing beads were added, the vials were purged with nitrogen, sealed and autoclaved for 15 min at 121 C and 12 kg/cm^2. After cooling, the tocopherol mixture was removed from the vial using a sterilized syringe and introduced into the vial containing the barium selenate. Following thorough mixing, the vials were stored in the dark at 4 C until used.

B. Lambs

Eleven ewes were housed in a covered pen. Two weeks prior to the expected arrival of the first lamb the normal trace mineral salt offered was replaced by one unsupplemented with Se (Table 8). Ewes were maintained on alfalfa hay with water and the salt offered ad-libitum until lambs were weaned. Lambs were born over a 17 day

Table 8. Experiment 3 Trace Mineral Salt

Ingredient (a)	% Composition
Zinc	1.508
Manganese	.805
Iron	.455
Copper	.029
Cobalt	.006
Iodine	.007
Salt	min. 92.55
	max. 94.96

a) Source: zinc oxide, manganous oxide, ferrous sulfate, copper oxide, cobalt carbonate, calcium iodate, sodium chloride.

Table 9. Experiment 3 Creep Ration

Ingredient	% Composition
Corn	53.975
Oats	15.0
Soybean meal (44 % crude protein)	25.0
Molasses	5.0
Limestone	1.0
Protector CTC 10 (a)	.025

a) Vitamin concentrations (per kg): retinyl acetate, 297,000 USP units; cholecalciferol, 148,500 USP units; dl-alpha tocopheryl acetate, 440 USP units; riboflavin, 297 mg;d-calcium pantothenate, 1113 mg; nicotinic acid, 1610 mg; cyanocobalamin, 1.24 mg; menadione dimethylpyrimidinol bisulfite, 248 mg. Antibiotic concentration in %: chlortetracycline, 2.2.

period.

Lambs were randomly assigned to one of four treatment groups. Treatment was initiated 3 days after birth. Lambs were weighed and an initial blood sample was drawn from the jugular vein. The lambs were then immobilized with xylazine and two liver needle biopsies taken. Tissue samples were stored under nitrogen at -20 C until assays could be performed. This procedure was repeated 10 days later. Subsequent blood samples were drawn at 1, 2, 3, 7, 10, 14, 28, 60, and 90 days post-injection. Packed cell volume and hemoglobin concentration were determined for the fresh blood samples. An aliquot of whole blood was retained. Plasma was divided into three aliquots. Erythrocytes were washed twice with heparinized saline, and all blood fractions were stored in plastic tubes under nitrogen at -20 C.

Two weeks following the arrival of the first lamb, a creep feeder was added to the pen. The first lambs began utilizing the feeder soon after. The same creep ration formula (Table 9) was offered during the remainder of the experiment. The initial shipment of creep ration was not enough to last the entire experiment. A second shipment was required and was offered to the lambs 75 days after the creep was initially introduced. The first shipment contained 0.073 mg Se, 3.45 USP alpha-tocopheryl acetate and 1.43 USP alpha-tocopherol/ kg as fed. The second shipment contained 0.063 mg Se, 3.13 USP alpha-tocopheryl acetate and 0.75 USP alpha-tocopherol/kg as fed. Lambs had

free access to the alfalfa offered to the ewes and water ad libitum.

Analyses

Plasma samples were analyzed for Se as previously described.

Glutathione peroxidase activity was determined for all blood fractions. A 0.05 ml aliquot of plasma was used. Whole blood and washed erythrocytes were diluted 81- and 121-fold, respectively and a 0.05 ml aliquot used. All samples were analyzed for GSH-Px activity 24 to 26 days after drawing.

Vitamin E assays were conducted according to the procedure of Bieri et al. (1979). Duplicate plasma samples (0.5 ml) and duplicate standards (0.0. 0.5, 1.0, 2.0, and 4.0 ug each of alpha-tocopherol and alpha-tocopheryl acetate/ml) were aliquoted into tubes containing 0.2 ml saturated ascorbic acid and 0.1 ml apocaretenal (1 ug/ml) in hexane. Apocaretenal was used as an internal standard. Sow plasma was added to the standards as a background organic matrix. Methanol (1.0 ml) was added to denature plasma proteins. The tubes were purged with nitrogen and capped. After vortexing 1 min, hexane with 0.05% betahydroxytoluene (3.0 ml) was added. The tubes were again purged with nitrogen, capped and again vortexed 1 min. Centrifugation at 1450 x g for 10 min followed. The hexane layer was then transferred to a 25 ml erlenmeyer

flask and the hexane evaporated under vaccuum until dry. The remaining residue was dissolved in methanol (0.5 ml), filtered into 0.5 dram vials and stored under nitrogen at -20 C until analyzed by high performance liquid chromatography (HPLC). Samples were analyzed less than 24 hr after extraction by passing 100 ul of the methanol solution through HPLC instrumentation (Waters and Associates Inc., Milford, Mass.) consisting of a model 45-M solvent delivery system, a model U6K universal liquid chromatograph injector and a model 440 absorbance detector. The detector was connected to a Servogor 120 recorder. A Bondapak C18 reverse phase, 3.9 mm x 15 cm column was used. The apocaretenal peak was detected by monitoring the eluent at 436 nm. Tocopherol and tocopheryl acetate were detected by monitoring at 280 nm. The peak heights of the standards, corrected by the internal standard, were used to calculate a curvilinear regression line from which sample concentrations were determined.

The liver biopsy samples were analysed in a manner similar to the plasma samples. The tissue was thawed using 0.3 ml saturated ascorbic acid solution (3.0 g/ml HPLC water) and transferred to a test tube. Tocopherol, equivalent to the lowest standard was added to each tube. Tocol was added as an internal standard (0.1 ml of 10 ug/ml methanol). Following addition of 2.0 ml methanol, tubes were purged with nitrogen and homogenized using a Polytron model PT 10-35 (Brinkmann Instruments, Inc., Westbury, NY 11590) for 10 seconds. Tubes were then vortexed and the

procedure continued as described above. Tissue tocopherol concentrations were determined using a curvilinear regression line calculated from corrected peak heights. The values obtained, less the background level added, were divided by sample weight to determine the concentration in the biopsy samples.

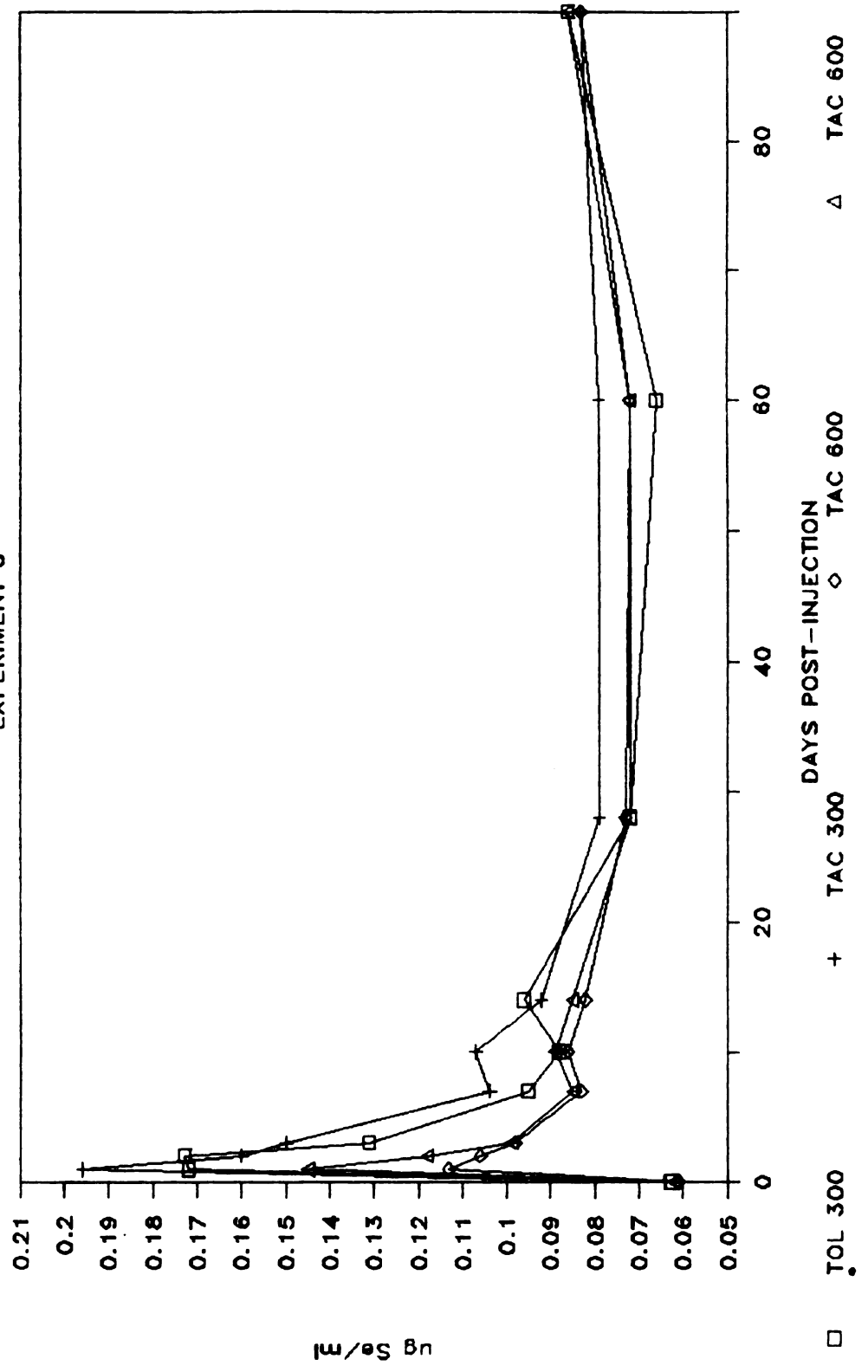
Between 91 and 103 days post-injection the lambs were killed by electric shock and exsanguination. Samples of liver, kidney, sternomandibular muscle and the injection site were taken for Se analysis.

Results and Discussion

The response of the animals to the Se portion of the product closely paralleled experiment two. Plasma Se values reached maximum levels by 24 hr post-injection and then declined to plateau at above-adequate levels which were maintained throughout the experimental period (Figure 11). Animals receiving 300 USP of vitamin E showed significantly higher ($P<0.05$) plasma Se levels 24 hr post-injection than those receiving 600 USP. This difference disappeared by 7 days post-injection. The form of vitamin E did not influence plasma Se levels.

Whole blood, erythrocyte and plasma GSH-Px values also followed the same general trend as those in experiment two (Figures 12, 13, 14). Due to the staggered initiation of treatment, all samples for the same period post-injection were not analyzed on the same day. Daily variations in the

FIGURE 11. PLASMA SELENIUM LEVELS



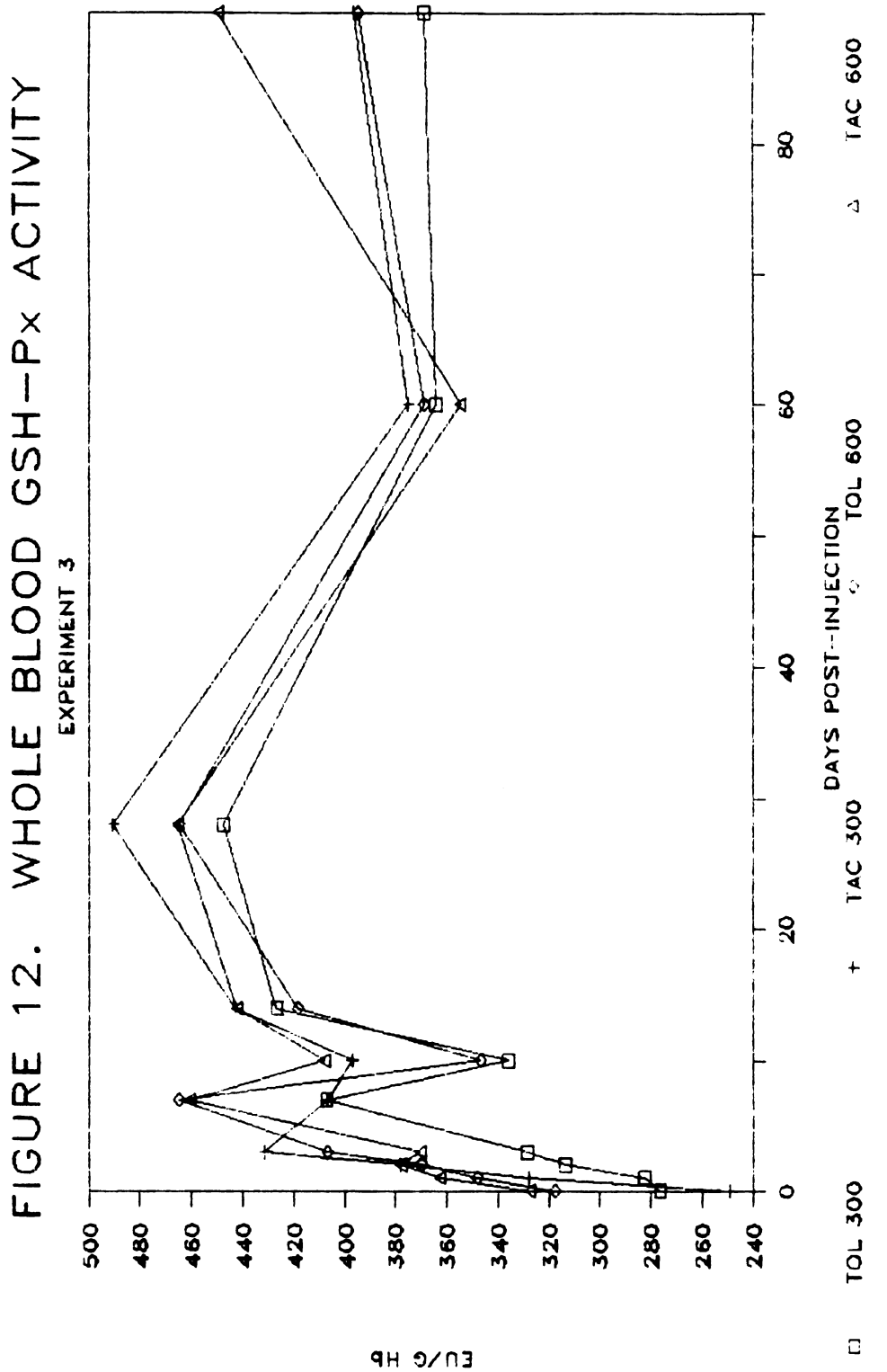


FIGURE 12. WHOLE BLOOD GSH-Px ACTIVITY
EXPERIMENT 3
EU/G Hb
500
480
460
440
420
400
380
360
340
320
300
280
260
240
0
20
40
60
80
DAYS POST-INJECTION
□ TOL 300
+ TAC 300
◇ TOL 600
△ TAC 600

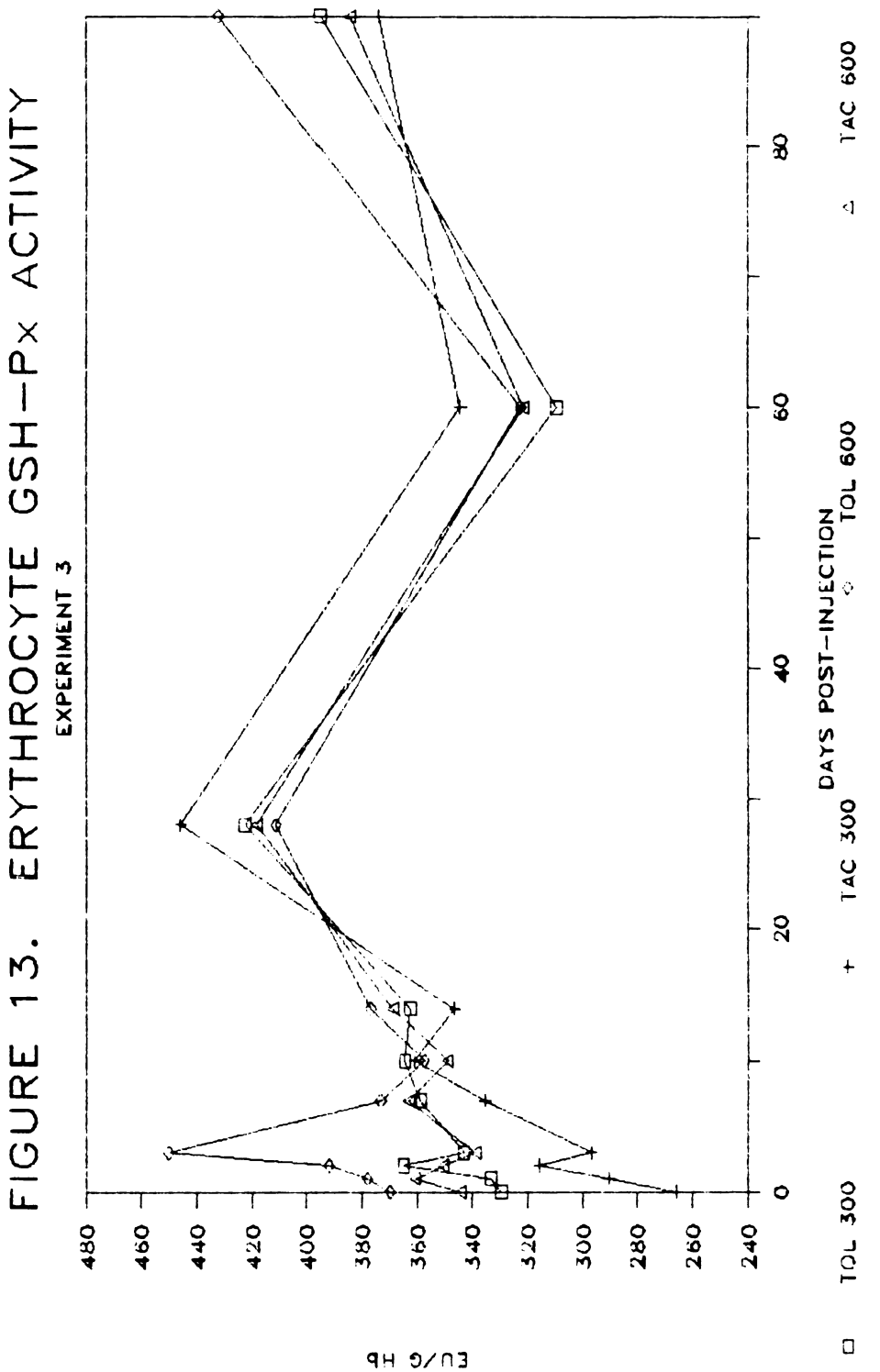
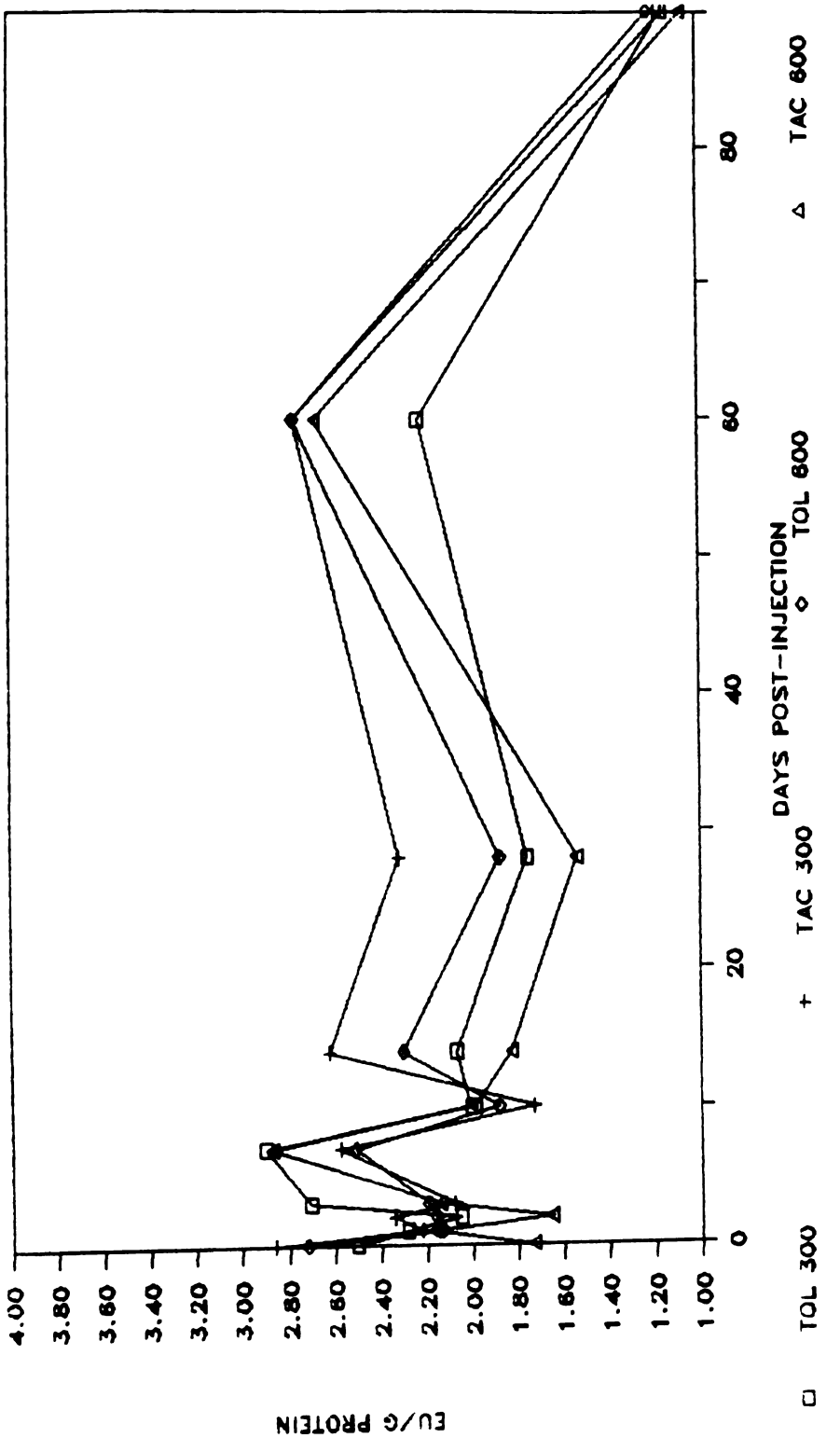


FIGURE 13. ERYTHROCYTE GSH-Px ACTIVITY
EXPERIMENT 3
EU/G Hb
480
460
440
420
400
380
360
340
320
300
280
260
240
0
20
40
60
80
DAYS POST-INJECTION
□ TOL 300
+ TAC 300
◇ TOL 600
△ TAC 600

FIGURE 14. PLASMA GSH-Px ACTIVITY

EXPERIMENT 3



GSH-Px analysis were effectively eliminated. In sheep, plasma GSH-Px is a very small fraction of the total blood GSH-Px activity. Because of this, whole blood and erythrocyte activities, which account for the major portion of the activity, are very similar. An early jump in GSH-Px activity was followed by a gradual increase in activity to 28 days post-injection. At 3 days post-injection, there was a significant increase ($P<0.05$) in the erythrocyte GSH-Px activity for those lambs receiving 600 USP of tocopherol. A similar increase in whole blood activity was not seen, so this difference may be due to experimental error. GSH-Px activities then decreased slightly at 60 days post-injection followed by a second rise at 90 days post-injection.

Hemoglobin and hematocrit values determined on the fresh blood samples did not vary outside the normal range for lambs (Table 10). Accepted values are 25 to 50% for hematocrit and 9 to 16 g hemoglobin per deciliter whole blood (Anonymous, 1986).

Analysis of the plasma samples for vitamin E showed little difference between treatments (Table 11). Initial plasma alpha-tocopherol values ranged from 0.4 to 2.2 ug/ml. Samples taken at 24 hr showed an elevation ($P<0.05$) in tocopherol which continued through 48 hr. By 10 days post-injection, the plasma tocopherol levels had decreased to below initial levels. There was no detectable rise in plasma alpha-tocopheryl acetate in any of the samples taken. Newmark et al. (1975) demonstrated that peak

Table 10. Experiment 3 Hematology

Treatment	Days Post-Injection									
	0	1	2	3	7	10	14	28	60	90
	Hemoglobin, g/dl Whole Blood									
T. 300 (a)	10.8	10.3	9.9	10.2	9.9	9.7	10.1	11.2	12.2	12.4
Ta. 300 (b)	10.9	10.4	10.2	9.9	8.7	8.5	8.7	10.8	12.6	12.7
T. 600 (c)	10.9	10.8	10.5	10.4	9.8	9.8	9.7	11.2	12.3	12.7
Ta. 600 (d)	10.5	10.4	9.6	9.8	8.9	9.5	10.2	11.4	11.8	12.4
SEM	.98	.96	.86	.74	.82	.82	.81	.55	.62	.42
	Hematocrit, % Packed Cell Volume									
T. 300	31.9	30.7	29.6	30.0	30.0	30.0	31.3	34.0	32.2	34.8
Ta. 300	30.8	29.7	29.9	28.9	26.0	25.1	26.8	34.1	35.4	35.5
T. 600	32.4	32.6	31.2	31.4	31.0	30.2	30.5	35.4	36.7	37.4
Ta. 600	31.9	30.7	29.4	29.3	28.4	30.4	31.4	36.9	35.7	37 3
SEM	2.76	2.89	2.52	2.32	2.14	2.26	2.15	1.57	1.56	.86

a) Tocopherol 300 treatment average of 6 lambs.
b) Tocopheryl acetate 300 treatment average of 5 lambs.
c) Tocopherol 600 treatment average of 5 lambs.
d) Tocopheryl acetate 600 treatment average of 5 lambs.

Table 11. Experiment 3 Effect of Injection Treatments on Plasma Alpha-tocopherol

Treatment	Tocopherol 300 (a)	Tocopheryl acetate 300 (b)	Tocopherol 600 (b)	Tocopheryl acetate 600 (b)
Days Post-Injection				
0 (c)	.84	1.46	1.12	.95
1 (d)	1.36	1.58	1.50	1.80
2 (d)	1.84	1.88	2.10	2.20
3	1.36	1.38	1.60	1.40
7	.90	1.04	.88	.72
10	.96	.84	.85	.68

a) Average of 6 lambs
b) Average of 5 lambs
c,d) Rows with different letters differ significantly ($P<0.05$).

tocopherol and tocopheryl acetate levels occur well before 24 hr post-injection when injections of tocopherol or tocopheryl acetate are given intramuscularly. Because injections were given subcutaneously in this experiment, slowed release was expected and the first sampling time was set at 24 hr post-injection. The magnitude of the plasma tocopherol values seen in this experiment were far below those expected with doses this large. Knight and Roberts (1986) demonstrated serum levels of 43 ug/ml in rabbit pups injected with alpha-tocopherol at 100 USP/kg bwt by 24 hr post-injection.

The results of the liver biopsy samples were inconclusive due to the small amount of tissue obtained (Table 12). Thirteen of twenty lambs had elevated liver tocopherol in at least one biopsy taken at 10 days over that taken prior to injection. The distribution according to treatment is shown.

Tissue samples taken at slaughter had Se levels comparable to those found in the pigs (Table 13). Liver Se concentrations ranged from 0.234 to 0.447 ppm wet basis. Kidney contained 1.001 to 1.658 ppm and sternomandibular muscle contained 0.050 to 0.103 ppm, both on a wet basis. Again, there was no effect of the vitamin E dose or form on the Se values obtained.

The injection site in 17 of the 21 animals was readily identified with no difference between treatments. A cyst had formed in the subcutaneous fascia. There was no

Table 12. Experiment 3 Liver Biopsy Weights and Effect of Treatment

Treatment	Avg. Biopsy Weight mg, wet	Increase/ Decrease (a)
Tocopherol 300	23.9	3/3
Tocopheryl acetate 300	24.1	3/1
Tocopherol 600	21.4	2/5
Tocopheryl acetate 600	24.5	5/0

a) Number of lambs per treatment showing increase/decrease.

Table 13. Experiment 3 Tissue Se Concentration (a)

Treatment	Liver	Kidney	Skeletal Muscle
Tocopherol 300 (b)	.306	1.296	.071
Tocopheryl acetate 300 (c)	.302	1.333	.073
Tocopherol 600 (c)	.299	1.259	.069
Tocopheryl acetate 600 (c)	.318	1.387	.063

a) ug/g tissue, wet basis.
b) Average of 6 lambs.
c) Average of 5 lambs.

infiltration into the underlying muscle, and it was easily removed. The cysts varied from 0.5 to 1.5 cm in diameter and contained a granular material. Analysis for Se and Ba indicated that a substantial amount of the salt remained at the injection site in those lambs with cysts. Due to the shape and consistency of the cyst, quantitative information was not possible. Best estimates indicate from 0% to 40% of the dose remained at the injection site. The material analysed showed wide variation in the ratio of Ba to Se indicating dissociation of the salt was occurring.

Concern over repeated usage of the parenteral designed for a single administration necessitated the next experiment.

Experiment 4

Repeated dosing with currently available Se-vitamin E products should be carefully monitored. While a wide margin of safety exists, large doses of sodium selenite may produce toxicity signs as previously discussed. Van Vleet et al. (1974) injected pigs at 7 days of age and again at weaning with varying doses of sodium selenite and alpha-tocopheryl acetate. A twelve fold increase in the dosage resulted in only a slight growth depression. Tissue Se levels showed no evidence of residual Se at 80 and 125 days post-injection.

Anaphylactic reactions have been reported following administration of currently used Se-vitamin E products. The severity and frequency of these reactions varies with species. Horses are most often affected. Signs include excitement, sweating, trembling, ataxia, respiratory distress and cardiac dysfunction (Package Insert, Burns Bio-tech). No distinction has been made as to the factor (Se, vitamin E, or vehicle) eliciting the reaction. Guinea pigs given intramuscular injections of dl-alpha-tocopheryl acetate showed severe tissue reactions at the injection site (Nockels, 1983).

Experiment four was conducted to evaluate repeated dosing of lambs with the vitamin E-Se product used in experiment 3. The dose level of 300 IU of E was selected because of the greater amount of vehicle present and

because of the form of the vitamin. The alcohol form would be physiologically active without further processing in the liver and, in theory, would have a more marked effect if one were present. The greater amount of vehicle would provide for an increased stimulus of an anaphylactic reaction.

Methods

Twin lambs were housed under the conditions described in experiment 3. Three days after birth the lambs were injected with the tocopherol 300 formulation in the right flank at the dose per kg bwt given in experiment 3. A blood sample was drawn prior to injection. Subsequent blood samples were taken at 1, 3, 7, 10, and 21 days post-injection. On day 21, a second injection at the normal dose per kg bwt was given in the right flank area after the blood sample was drawn. Again further samples were taken at 3, 7, 10, 14, and 21 days post-injection. On day 30 following the second injection, a third injection was given in the left flank at the same dosage level. Subsequent blood samples were taken at 3, 7, 14, and 21 days post-injection. All samples were handled and analyzed as previously discussed.

Eighty days after initiation of treatment, the lambs were killed by electric shock and exsanguination. Samples of liver, kidney, sternomandibular muscle, and the injection site were taken for analysis.

Results and Discussion

Plasma, whole blood and erythrocyte GSH-Px activities are shown in Figures 15 and 16. The response to the first injection was lower than that seen in experiment 3. The second injection elicited a slight increase in whole blood GSH-Px activity. The third injection caused the greatest increase in whole blood and erythrocyte GSH-Px activity.

Hemoglobin and hematocrit values are shown in Table 14. There was no deviation outside the normal ranges for lambs.

Plasma Se increased markedly following the first injection (Figure 17). The second and third injections did not cause additional peaks to occur. Toxic plasma Se levels were not seen throughout the experimental period.

From analysis of the tissues taken at slaughter it was determined that liver and kidney Se concentrations were higher than the values determined for lambs given a single injection. Liver, kidney, and sternomandibularis muscle averaged .718, 1.322, and .090 ug Se/g, wet basis, respectively. These values are comparable to tissue Se levels seen in lambs fed diets which are naturally high in Se. Ullrey et al. (1977) reported Se levels of .618, 1.301, and .167 ug/g wet basis for liver, kidney and sternomandibular muscle, respectively, for lambs fed diets containing .199 mg natural Se per kg.

The site of the first injection was readily identifiable when the second injection was given in the

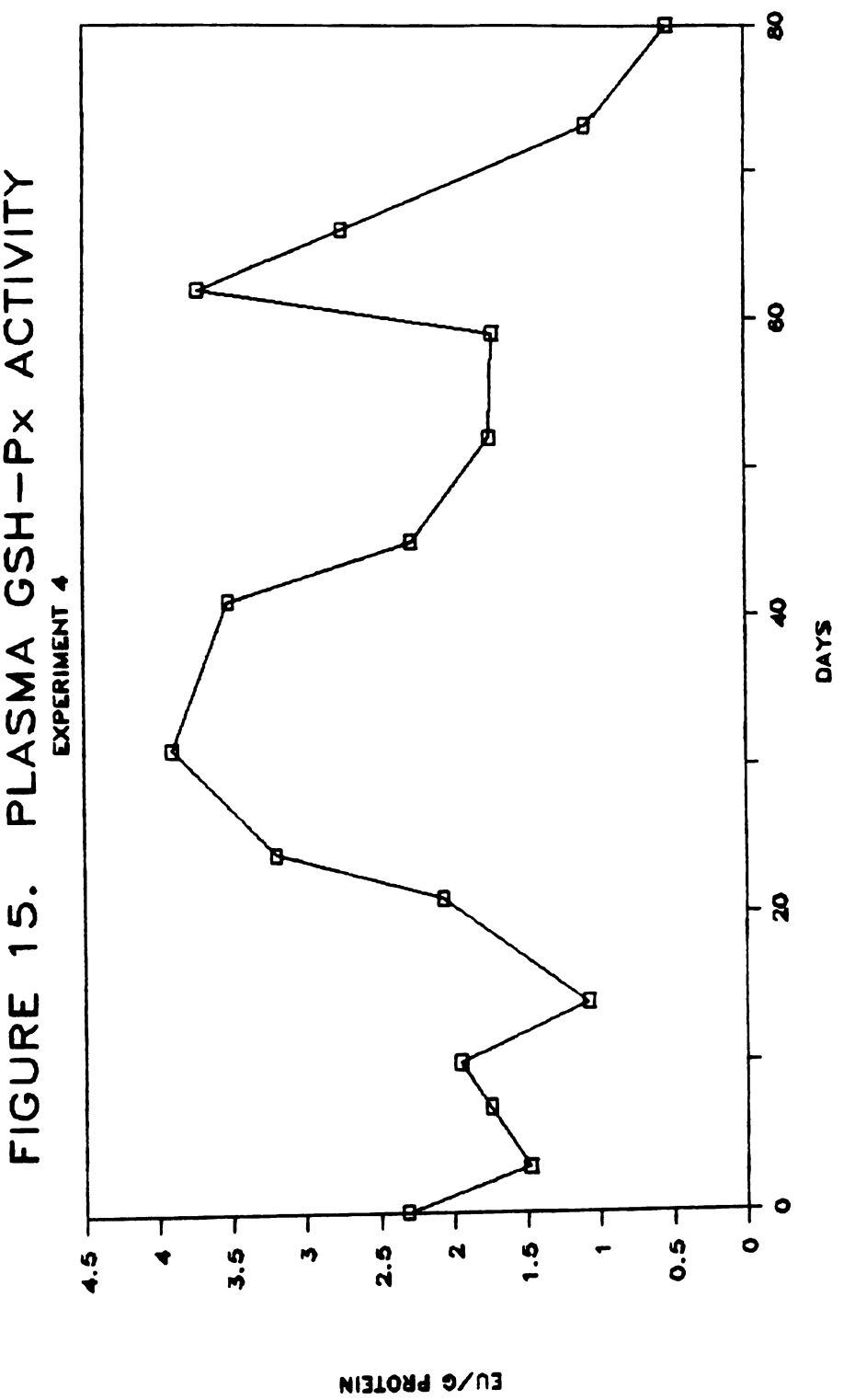


FIGURE 15. PLASMA GSH-Px ACTIVITY
EXPERIMENT 4
EU/G PROTEIN
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
DAYS

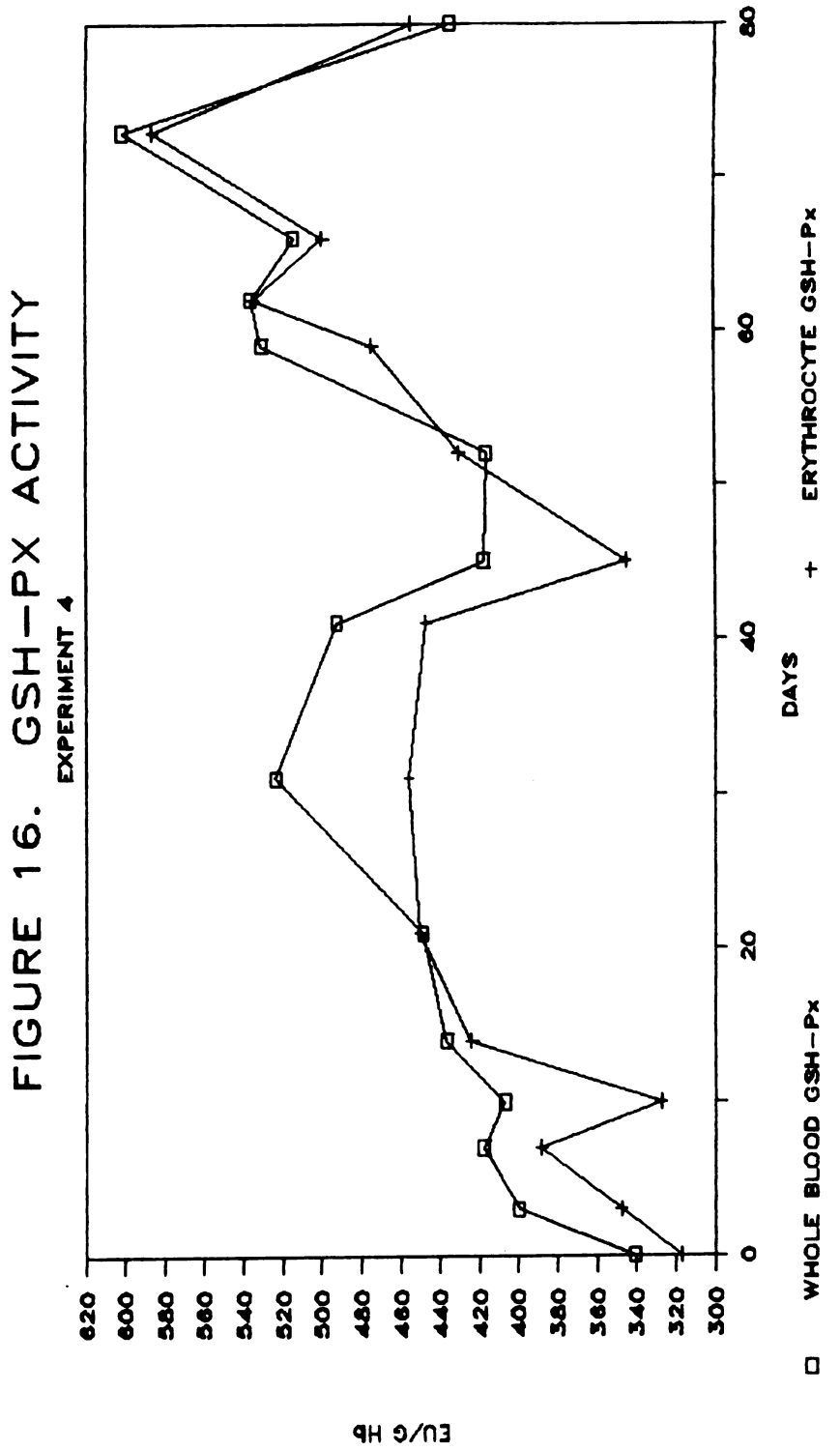


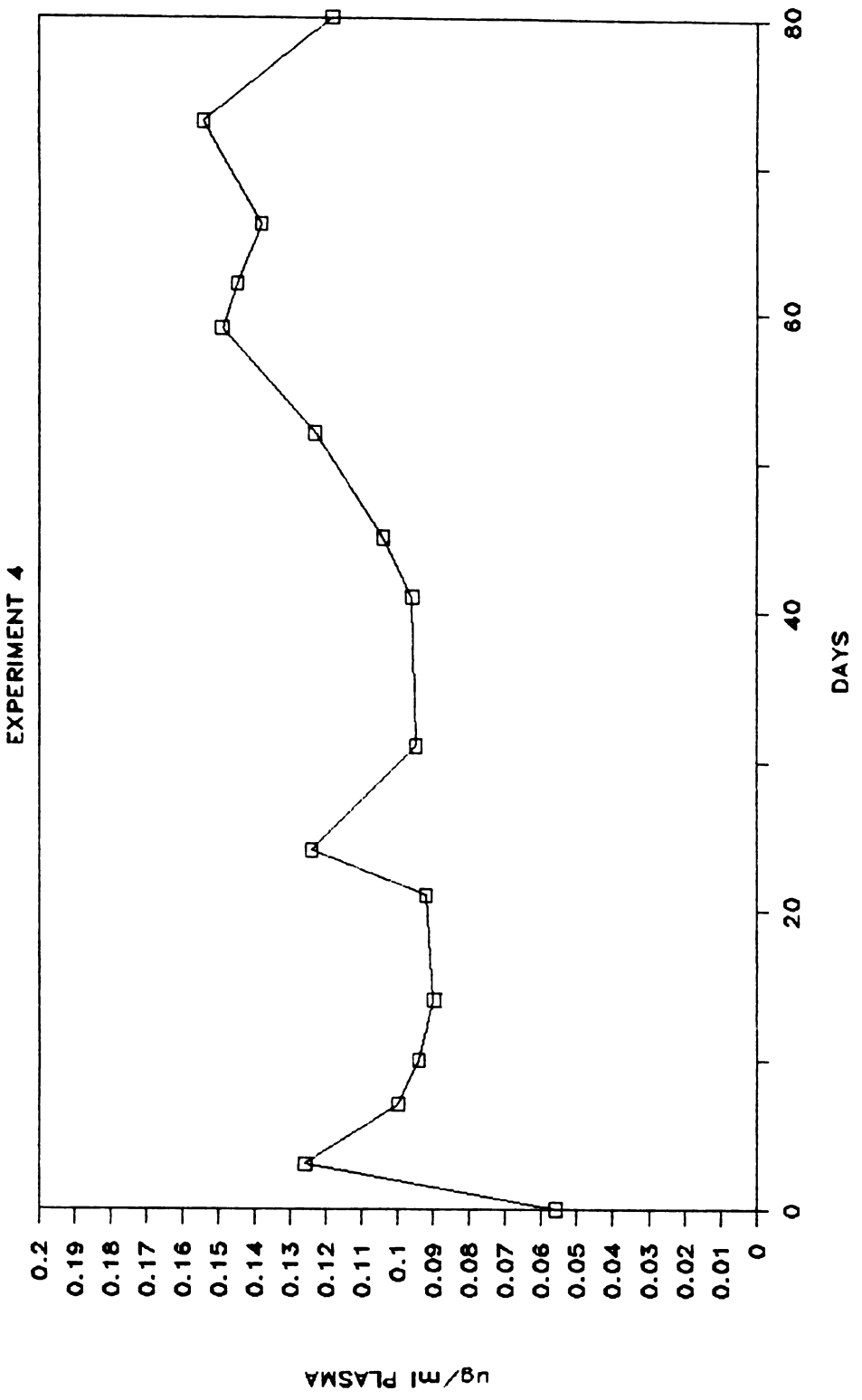
FIGURE 16. GSH-PX ACTIVITY
EXPERIMENT 4
EU/G Hb
620
600
580
560
540
520
500
480
460
440
420
400
380
360
340
320
300
0
20
40
60
80
DAYS
□ WHOLE BLOOD GSH-Px
+ ERYTHROCYTE GSH-Px

Table 14. Experiment 4 Hematology

Days on Experiment	Hb g/dl (a)	Hct % PCV (a)
0 (b)	11.3	35.2
3	11.4	35.2
7	10.6	32.8
10	10.0	31.1
14	10.2	31.8
21 (b)	10.4	31.4
24	9.8	30.4
28	10.0	30.4
31	9.8	29.4
35	10.2	31.4
42	12.7	30.0
51 (b)	10.6	31.5
54	10.9	30.8
58	11.0	30.8
65	11.6	33.2
72	11.4	33.2

a) Average of 2 lambs.
b) Injection given on this day.

FIGURE 17. PLASMA SELENIUM



same area. There was some lameness following the second injection which was attributed to the volume given. The third injection was given in the left flank and, again, lameness was noted. At slaughter, two cysts were isolated, one from the first two injection sites and one from the third injection site, which were similar in appearance to those described in experiment 3. Both were easily removed from the underlying fascia.

There was no anaphylactic reaction to any injection given indicating that the lambs were not sensitized by the product ingredients.

Conclusions

Barium selenate is safe and effective as a parenteral supplement of Se. The parenterals evaluated in this study supported plasma Se levels and plasma, whole blood and erythrocyte GSH-Px activity for up to 120 days following a single injection. This exceeds the effective period for commercially available Se products.

The vehicles tested allowed a greater percent retention of the injected dose of barium selenate over saline with sodium selenite. Fecal excretion was higher than expected and total excretion remained elevated longer when barium selenate was administered than when sodium selenite was used.

Vitamin E, when incorporated into a compatable water-based preparation containing barium selenate, was not as effective as desired. Plasma alpha tocopherol levels were increased, but not to the degree expected. No significant difference was detected between injection of alpha-tocopherol and injection of alpha-tocopheryl acetate. Lambs receiving either form had similar responses and circulating alpha-tocopheryl acetate was not elevated in those animals receiving that form of the vitamin.

The preparations containing barium selenate alone produced a minimal reaction following injection. Recovery of the injection site showed mobilization of the entire amount injected and little or no tissue damage in the area surrounding the injection site. The addition of vitamin E

and a preservative to the carboxymethylcellulose-barium selenate product induced cyst formation at the injection site. There was no biological effect of addition of vitamin E on the action of the barium selenate. Enzyme activities and plasma Se levels were similar to those achieved with the product containing only barium selenate.

A formulation of barium selenate and vitamin E is possible. It is felt that too many changes were made in the formulations used in the third experiment. A preparation of barium selenate and alpha-tocopherol without ascorbyl palmitate may not cause cyst formation. An additional possibility is the simultaneous parenteral administration of a water miscible vitamin E preparation and the barium selenate carboxymethlycellulose formulation seperately. These will be topics of further studies.

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