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Comparative Mutagenicity of Urinary Metabolites of Nitropolycylic Aromatic Hydrocarbons 1-Nitropyrene, 2-Nitrofluoranthene, and 3-Nitrofluoranthene

by

Michael N. Boucher

A technical report submitted to the Faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in Public Health in the Department of Environmental Sciences and Engineering.

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ABSTRACT

MICHAEL N. BOUCHER. Comparative Mutagenicity of Urinary Metabolites of Nitropolycylic Aromatic Hydrocarbons 1-Nitropyrene, 2-Nitrofluoranthene, and 3-Nitrofluoranthene. (Under the Direction of Dr. LOUISE M. BALL)

This study compared the mutagenicity of urinary metabolites of prevalent air pollutants 1-nitropyrene (1-NP), 2-nitrofluoranthene (2-NFA), and 3-nitrofluoranthene (3-NFA). Rats were injected intraperitoneally with specific doses and their urine was analyzed for mutagenicity by an Ames plate incorporation assay using Salmonella Typhimurium. Resulting data yielded revertant excretion rate plots used for comparative analysis. To achieve maximum mutagenicity, 1-NP's urinary metabolites required exogenous activation by S9, 2-NFA's did not vary significantly with S9 activation, while 3-NFA's was decreased by S9 metabolism. 2-NFA's mutagenic metabolites' revertant excretion rate was a quarter of the rates of 3-NFA's and 1-NP's mutagenic metabolites. The strongest mutagenicity of urinary metabolites during the 48 hrs after injection was from 1-NP which was double the amounts from 3-NFA and 2-NFA. However, the estimated total urinary mutagenicity showed 1-NP and 2-NFA potentially created equal amounts while 3-NFA 's amount was only half 1-NP's amount. All three compounds' urinary mutagen excretion rates are formation rate limited.

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I. INTRODUCTION

1-Nitropyrene (1-NP) and nitrofluoranthenes (NFA) are prevalent air pollutants contributing significantly to mutagenic activity of ambient organic particles in both cities and rural areas (Aray, *et al* 88; Ramdahl, *et al* 86; Tokiwa, *et al* 87). The everyday operation of combustion engines exhausting into the atmosphere built these pollutants' concentrations high enough to initiate scientific concern for human health. Assessment of 1-NP's mutagenicity is fairly complete implicating 1-NP as a potent mutagen and furthering concern about the carcinogenicity of Nitro-PAHs. The task continues to correlate current results to human risk and better categorize other nitroarenes and air pollutants according to their potential mechanisms of action.

This study provides a preliminary investigation into the mutagenicity of actual *in vivo* metabolites of 2-NFA and 3-NFA. Characterization of 1-NP's *in vivo* fate and *in vitro* mutagenicity is established while only *in vitro* studies for 3-NFA and 2-NFA are available. This study uses the Ames bacterial assay to examine the mutagenicity of urinary metabolites of 2-NFA and 3-NFA. Sprague Dawley rats are injected intra-peritoneally with varying doses of 1-NP, 2-NFA,

and 3-NFA dissolved in acetone. Developed Excretion Rate Plots illustrate each compounds' mutagenicity and kinetics. Considering the past in vitro research results of the NFAs and using this study's *in vivo* results for all three compounds, preliminary characterizations of the urinary mutagenic metabolites of 2-NFA and 3-NFA are established using 1-NP as the reference point.

II. LITERATURE REVIEW

II. A. Background

II. A. 1. Formation of Nitro-PAHs

1-NP and 3-NFA are immediately formed by-products of combustion due to the interactions of nitrogen oxides (NOx), nitric acid, and fluoranthene molecules produced by incomplete combustion of fossil fuels (Pitts, *et al* 85; Ramdahl, *et al* 86). 1-NP, 3-NFA, and 2-NFA can be formed any time after combustion during atmospheric chemical interactions between the by products fluoranthene and dinitrogen pentoxide (N_2O_5) catalyzed by a OH radical with NOx (Pitts, *et al* 85; Ramdahl, *et al* 86; Zielinska, *et al* 87). Atmospheric conditions of radiant energy, constituents' concentration and degradation rates, organic particle interactions, and other forms of natural conditions determine the rate of nitro-PAH formation. 2-NFA has been found in the most abundance of the nitro-PAHs on ambient organic particles, although not found directly in diesel emissions as are 1-NP and 3-NFA (Aray, *et al* 87; Ramdahl, *et al* 86). Nitro-PAH formation even occurs in the charring process of some foods (Kinouchi, *et al* 86). All three compounds are prevalent air pollutants due to their organic particle host sheltering them from external degradation forces.

II. A. 2. Biological Reactivity of Nitro-PAEs

Nitro-PAHs can be absorbed via inhalation and/or ingestion during normal everyday exposure. (King *et al*, 83; Bond & Sun 86; Bond, *et al* 86). Research has shown Nitro-PAHs require biological activation and many are considered potent mutagens and subsequently, potential carcinogens (Ball, *et al* 84; Ball and King 85; Ball and Lewtas 86; Consolo, *et al* 89; Hirayama, *et al* 88). Indeed, Howard (*et al* 83) and Dietrich (*et al* 88) showed 1-NP and 3-NFA metabolites can form a DNA adduct. DNA adducts are suggestive of a chemical's carcinogenic potential because they are proof of the chemical's ability to directly interact with DNA.

In vivo metabolism of Nitro-PAHs involves many factors due to the enterohepatic circulation. The compound may undergo oxidation, acetylation, and conjugation in the liver where the conjugated metabolite may then be transported to the intestines. Intestinal flora may reduce the metabolite while B-Glucuronidase- and Sulfatase-containing bacteria may deconjugate the metabolite allowing absorption into the enterohepatic circulation. Ball (*et al* 84) demonstrated the importance of gut flora in the metabolism reduction of nitro-PAHs followed by N-acetylation to the mutagenic acetylated metabolites. Oxidation of Nitro-PAHs via the liver's mixed function oxidase results in ring epoxidation and ring hydroxylation (King, *et al* 87; Howard, *et al* 85; Ball, *et al* 84; King *et al* 84). Oxidative and reductive pathways are both believed to produce DNA adducts (Djuric, *et al* 86).

Additionally, Ball (*et al* 84) found ring oxidation is a major route for nitro-PAH metabolism *in vivo*. Time dependence of specific metabolite production signified the activity of the enterohepatic circulation in nitro-PAH metabolism (Ball & King, 85). Several bacterial strains are capable of these pathways, but unfortunately multiple strains can not easily be used simultaneously. The use of urinary metabolites provides a non-invasive look at *in vivo* metabolism. Bacterial strains recreate a pseudo metabolism model to view the resulting body's exposure to reabsorbed potentially mutagenic metabolites via the enterohepatic circulation.

Characterizing nitro-PAHs via animal metabolism is limited in scope since human P-450 metabolism of nitro-arenes may differ greatly from rodents', questioning the rodent model's reliability as predictors of human metabolism (Howard, *et al* 90). Consequently, no direct human health risks can be inferred, but we can develop a preliminary appraisal of nitro-PAHs' *in vivo* metabolism, mutagenicity, and potential carcinogenicity.

II. A. 3. Analytical Techniques

II. A. 3. (a). *In Vivo* Metabolism Study

Six week old, male, Sprague Dawley rats were selected for this *in vivo* metabolism study. These rats are fairly competent nitroarene metabolizers and cytochrome P-450 participates in nitro-reduction by

Sprague Dawley rat liver microsomes (Belisario, *et al* 90). Although the extent of P-450 epoxidation of nitroarenes differ between species (Howard, *et al* 88), a majority of previous research has accepted the Sprague Dawley rat as the standard model (Ball & King, 85; Belisario, *et al* 90; Howard, *et al* 85; Howard, *et al* 88). To minimize confounding between rat samples, all rats were treated identically. Acclimatization time, food, water, cage type, injection and collection times, age, and gender were consistent for all rats.

In vitro studies have shown similar metabolism rates for both 1-NP and NFAs so *in vivo* rates could be proportionally similar. 1-NP's past metabolism research shows a majority of the dose is excreted in the first 24 hrs, approximately 15% in urine and 40% in feces (Ball, *et al* 84; Ball & King, 85; Kinouchi, *et al* 90). Considering Stocking's (89) findings of 2-NFA's slow *in vitro* metabolism, an extended collection period of 48 hrs ensured the majority of sample was collected. Only urinary metabolites were collected as the fecal metabolites should not significantly elevate mutagenicity levels above background in Salmonella Typhimurium strains as evidenced by 1-NP (Ball, *et al* 84).

Initial excretion of 1-NP metabolites before 8 hrs primarily consisted of the oxidative products, diols and hydroxy metabolites, which are not potent mutagens. After 8 hrs, the enterohepatic circulation enabled ring oxidation, nitroreduction, and N-acetylation metabolism to occur creating the potent mutagen 6-hydroxy-NAAP

with peak mutagenicity levels attained around 12 hrs (Ball & King, 85; Howard, *et al* 85; Kinouchi, *et al* 86). Shorter collection periods were required to better characterize the initial portion of excretion. DMSO proved to be an inefficient transport for nitro-PAHs into the systemic system (Ball, *et al* 85) while acetone provided an increased delivery efficiency. Less than 2% of metabolites excreted degrade by bacteria or enzymes between collection and analysis (Ball, *et al* 84). Immediately freezing samples upon collection and storage in a dark freezer ensured samples authenticity until use in the Ames assay.

II. A. 3. (b). **Ames Salmonella Assay**

The Ames Salmonella Typhimurium assay is widely accepted for chemical and urinary mutagenicity research and specifically, the TA98 strain is frequently used for, and most responsive to nitro-PAH (Ball *et al* 84; Ball *et al* 85; Howard, *et al* 87; Zeiger, *et al* 87; Zielinska, *et al* 87). Salmonella Typhimurium strain TA98 is modified with a -1 frameshift disabling its histidine production capability. Lacking a repair mechanism, a frameshift mutation is required to reset the gene frame for proper function. Nitroreductase is a key metabolic activity of TA98 as nonreductase deficient strains decrease mutation rate of 1-NP, a known nitroreductase dependent mutagen (Consolo, *et al* 89). The bacteria is grown in a histidine deficient medium with the chemical of interest. Reversion back to the wild state capable of producing

histidine is evidence of a frameshift mutation of the genome. The assumption is that any chemical able to effect a mutation of the genome is capable of other unidentifiable mutations of the genome presenting it as a potential carcinogen. Many mutagens require exogenous activation by S9, a mammalian system which provides a mixed function oxidase dependent on a NADPH, glucose-6-phosphate electron generating system. S9 is from Aroclor 1254 induced animals which provides a wide range of P-450 mixed function oxidases (Maron & Ames, 83). The Ames bacterial assay has an overall positive predictive value of only 62% (Tennant, *et al* 87), however, is over 90% efficient in correlating mutagenic nitroarenes as carcinogenic in rodents (Zeiger, 87).

II. A. 3. (c). Toxicokinetic Analysis

Toxicokinetics has progressed rapidly over the past two decades becoming an extremely useful tool for invasive and non-invasive *in vivo* studies. Particularly of interest to this study is the analysis of urinary excretion data. The urinary excretion rate of a compound is identical to the plasma concentration level over time (Shargel & Yu, 85). Excretion rate data provide a profile of internal workings of the animal. Excretion rate models consider the whole animal as one compartment, however, the flux in and out of the animal may vary with time providing more than one rate constant outlined by Figure 1.

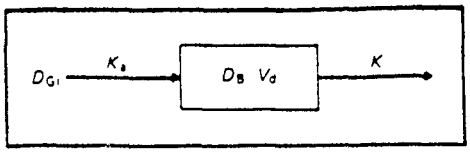

Figure 1 One-compartment pharmacokinetic model for first-order drug absorption and first-order elimination

(Reprinted from Shargel and Yu, 85)

The rate of uptake and excretion of a substance is a function of the rate constants, Ka, and Ke, which can both be estimated from excretion rate data. The standardized method using an Excretion Rate Plot is shown in Figure 2 (Shargel & Yu, 85). The slope of the terminal end of the profile estimates the excretion rate constant, Ke, while Ka, is the residue slope created by excretion line's data points subtracted from the initial positive slope's data points. If Ka >>Ke, then the Y intercept is the total cumulative amount of substance excreted which also equals the total area under the curve (AUC). Also if Ka>>Ke then the substance's excretion rate is formation rate limited (Shargel and Yu, 85). The AUC can also be determined by using the linear excretion rate to estimate the X intercept and calculating the AUC via the trapezoidal rule. Once the AUC is known, a Sigma Minus Plot, Figure 3 (Shargel and Yu, 85), can plot the amount left to be excreted over time. The Sigma Minus Plot's slope better estimates the excretion rate constant Ke since all excretion rate data are used rather than just the last few terminal values.

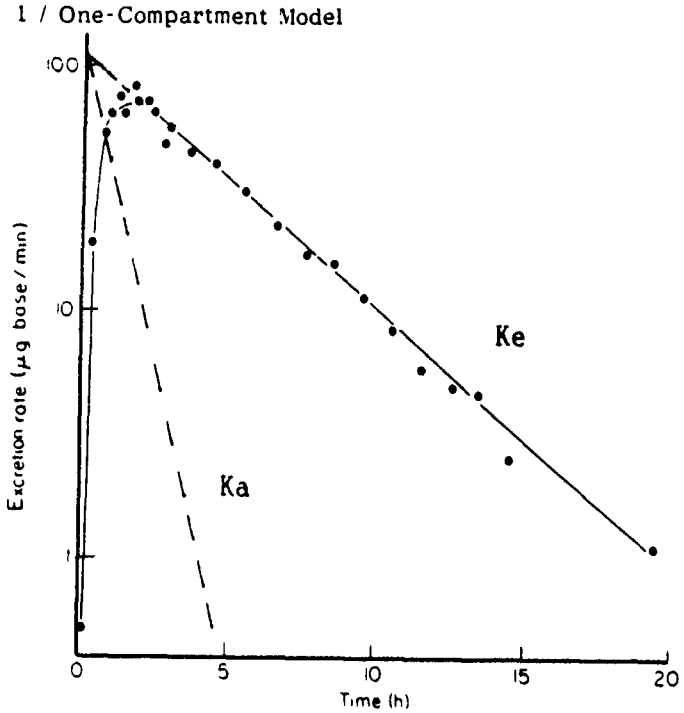

Fig. 2 Linear Axis Excretion Rate Plot to determine Ke and Ka (Reprinted from Gibaldi and Perrier, 82)

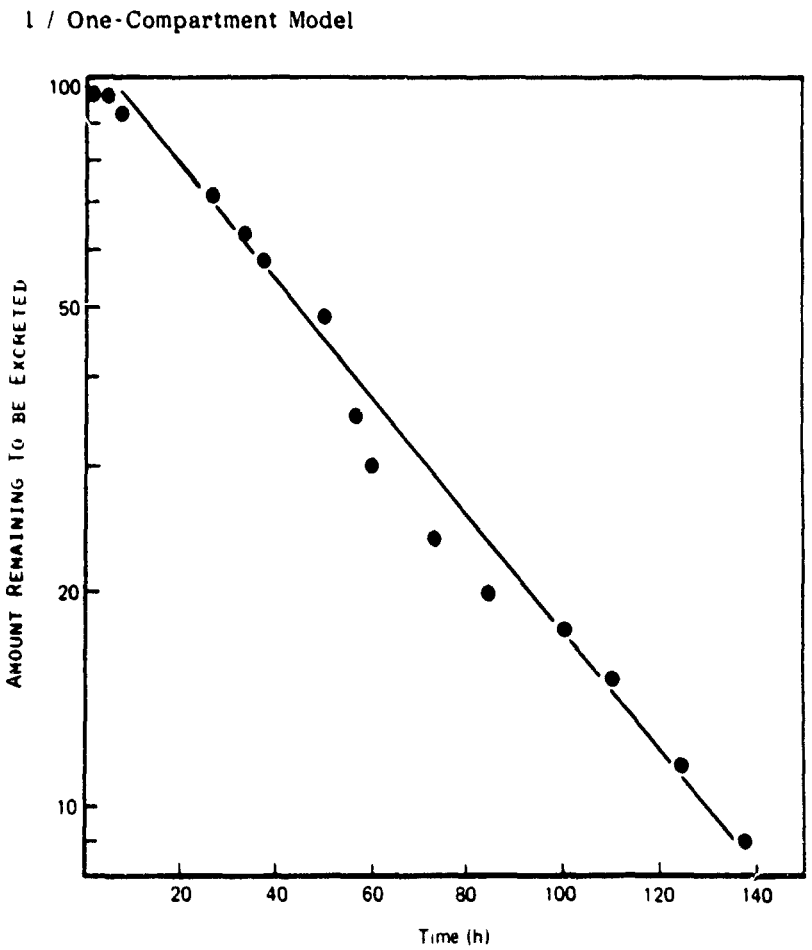

Fig. 3 Sigma Minus Plot to determine Ke (Shargel & Yu, 85)

II. B. 1-Nitropyrene Mutagenicity Characterization

In vitro experiments initially charted each of 1-NP's metabolites which were eventually found via *in vivo* research. The mutagenicity of 1-NP *in vitro* metabolites is decreased by S9 while curiously, its urinary metabolites are activated by S9 (Ball, *et al* 84). 1-NP's *in vivo* pathway significantly differed from the *in vitro* route due to the *in vivo* metabolism process, however, both paths essentially achieved similar active mutagenic metabolites.

The majority of the dose after injection of rats was excreted in bile as glutathione, cysteinylglycine, and cysteine conjugates (Bond, *et al* 85; Howard, *et al* 85; Kinouchi, *et al* 90). Intestinal flora in the intestines play significant roles in reducing 1-NP and metabolizing its conjugates to reactivated metabolites enabling reabsorption via the enterohepatic circulation (EHC) (Ball & King, 85; Howard, *et al* 85; Kinouchi, *et al* 86; Kinouchi, *et al* 90). These reabsorbed metabolites could be hydrolyzed in intestine and reduced by bacterial nitroreductase to reactive n-hydroxyl arylamine (NAA) derivatives (Ball & King, 85; Howard, *et al* 85; Kinouchi, *et al* 86; Kinouchi, *et al* 90). This transformation to NAA may be common for all nitroarenes, but to what extent is unknown (Ball & King, 85; Kinouchi, *et al* 90). The metabolite 6-hydroxy-NAAP accounted for the highest portion of urine mutagenicity excreted at 12 hours after dosing and required S9 for maximum bacterial mutagenicity (Ball, *et al* 84; Ball & King, 85). S9 may further

metabolize the 6-OH-NAAP metabolite by esterification eventually generating a nitrenium ion. Similarly, the parent, 1-NP undergoes *in vitro* nitroreduction to a hydroxylamine or nitrenium ion (Ball, *et al* 84). Howard (*et al* 83) showed the nitrenium ion formed by 1-NP can form a dG:C8 adduct N-(deoxyguanosin-8-yl)-1-amino-fluoranthene. However, 1-NP metabolites reduce easier to DNA adducts than the parent 1-NP (Djuric, *et al* 86). The excretion pathways of 1-NP appear to be independent of exposure concentration where fecal remains the major route accounting for twice as much as the urine route (Ball & King 85; Bond, *et al* 85). The fecal does not contain significant mutagenic metabolites while very little parent 1-NP remained unmetabolized in the urine (Bond, *et al* 85; Howard, *et al* 85; Ball and King, 85).

II. C. Characterizations of Mutagenicity of 2- & 3-Nitrofluoranthene

3-NFA is strongly mutagenic in bacterial strains containing nitro-reductase (Ball, *et al* 86), however 2-NFA is considered a weaker mutagen more closely related to 1-NP's levels. Nitroreduction and O-esterification have both been identified as slightly preferential metabolic activators for 2-NFA and 3-NFA respectively (Consolo, *et al* 89). 2-NFA (Zielinska, *et al* 87; Belisario, *et al* 90) and 3-NFA (Consolo, *et al* 89; Belisario, *et al* 90; Howard, *et al* 88; Stocking, 89) like, 1-NP (Ball & King, 85) were shown to be dependent on nitroreduction *in vitro* where as their metabolites are more dependent on

esterification (McCoy, *et al* 83; Consolo, *et al* 89). Both NFAs are less dependent than 1-NP on nitroreductase *in vitro*, however, a reductase lacking bacterial strain still decreased 3-NFA activity by 49-69% (Consolo, *et al* 89). Moreover, 3-NFA *in vitro* metabolism pathway is through epoxide intermediates (Howard, *et al* 88) and the resulting phenolic metabolites' mutagenicity remain unexplained (Consolo, *et al* 89). 2-NFA is less active and more dependent on nitroreductase than 3-NFA, but less affected by S9's mixed function oxidase (Zielinska, *et al* 88). Like 1-NP, 2-NFA's mutagenicity was decreased slightly by S9 as was its *in vitro* metabolites 9-hydroxy-2NFA and 8-hydroxy-2NFA (Zielinska, *et al* 87). 3-NFA's *in vitro* mutagenicity like 1-NP's, is decreased by S9 in the bacterial strain TA98 but unlike 1-NP's *in vivo* metabolites, 3-NFA does not produce *in vitro* metabolites more dependent on S9 (Consolo, *et al* 89). Specifically, the 3-NFA-8-ol metabolite was as mutagenic as its parent 3-NFA, but other 3-NFA metabolites were 10 fold less mutagenic (Consolo, *et al* 89). This implies 3-NFA metabolites further metabolism may result in detoxification indicating not all nitro-PAHs' metabolism mechanisms are alike (Ball & Williams, 86). Stocking (89) demonstrated *in vitro* reduction of 3-NFA efficiently produced only the 3-AFA metabolite while reduction of 2-NFA produced 2-AFA which itself underwent acetylation to form 2-NAAFA, but at a considerably slower rate. Indeed 1-NP and 2-NFA metabolites have been shown to retain a strong dependence on nitroreduction while 3-NFA's

primary *in vitro* metabolites were largely dependent on O-esterification (Consolo, *et al* 89). Unlike the hepatic metabolism of 1-NP shown to involve both ring oxidation and nitroreduction (Ball, *et al* 84; Howard, *et al* 85), NFAs were shown to only undergo ring hydroxylation in aerobic and only reduction in anaerobic conditions (Belisario, *et al* 90). Metabolism of these nitro-PAHs seems to be very dependent on their environment suggesting their metabolism may depend on their *in vivo* kinetics. Metabolism may also vary with the *in vivo* model used as P-450 metabolism of 3-NFA and 1-NP vary greatly between species (Howard, *et al* 88).

II. D. Metabolism Kinetics of 1-NP, 2-NFA, and 3-NFA

1-NP metabolism *in vivo* has been well characterized. Initial metabolites excreted are phenols and dihydrodiols which rapidly give way in the 8-12 hr period to the acetylated 6-OH-NAAP constituting the majority of the 1-NP dose excreted. Since very little actual parent compound remains unmetabolized, the terminal clearance rate is truly the metabolites' excretion rate. Expanding the comparative picture to estimate NFAs' response, metabolism kinetics provides key evidence as to metabolism opportunities, although NFAs' metabolism rate remains highly varied. 3-NFA in bacterial strains undergoes 90% metabolism to 2-AFA in 6 hrs (Stocking, 89). 2-NFA metabolism is slower with 10% of the parent metabolized in 6 hrs while in 24 hrs, 80% of the parent is metabolized

to 2-AFA and 15% of the parent is metabolized to 2-NAAFA. 2-NFA's nonlinear metabolism rate currently is attributed to a secondary nitroreductase or an oxygen sensitive reductase which is activated (Stocking, 89). 2-NFA and two metabolites, 8 and 9-hydroxy 2-NFA depend on nitroreduction and O-acetylation *in vitro* (Zielinska, *et al* 87), no *in vivo* metabolic pathways have yet been described. Comparing exposure dose to urinary metabolite mutagenicity over time, 1-NP metabolites become more mutagenic as the enterohepatic circulation provides opportunity for acetylation for mutagenic metabolite production (Ball, *et al* 84; Ball & King, 85). The combination of a fast metabolism rate predicted by Stocking (89) and the detoxifying action of metabolism implies most 3-NFA urinary metabolites may be direct acting mutagens (independent of S9 activation) while 2-NFA's metabolites can only be estimated to undergo more acetylation than metabolites of 3-NFA.

III. MATERIALS AND METHODS

III. A. Materials

III. A. 1. *In Vivo* Metabolism Study Materials

Six week old Sprague Dawely rats whose weights ranged from 200-230 grams were purchased from Charles River while the plastic metabolism cages (Nalgene) and animal isolation booth were provided by Department of Laboratory Animal Medicine (DLM), University of North Carolina, Chapel Hill. Granular rat chow was supplied by DLM and ground to powder form. Nitro-PAHs injected: 1-nitropyrene (99.7% purity) was purchased from Midwest Research Institute (Kansas City WO); 3-nitrofluoranthene purchased from Chemsyn Inc. (Lenexa, Ks); and 2-nitrofluoranthene was synthesized and purified by Dr Louise Ball (Dept. of Environmental Science and Engineering, UNC) according to procedures outlined by Kloetzel (*et al* 1955). HPLC grade acetone was supplied by Fisher Scientific and sterile single use syringes were supplied by Bector Dickerson & Co. (Rutherford, N.J.). Sterile 0.22 and 0.40 micron filters were purchased from MSI (Westboro, MA).

III. A. 2. *In Vitro* Ames Assay Materials

Agar-Agar was purchased from U.S. Biochemical Corp (Cleveland, OH). Sodium Azide, 2-nitrofluoranthene, ampicillin trihydrate, and crystal violet were purchased from Aldrich (Milwaukee, WI). B-Glucuronidase,

NADP, and Glucose-6-Phosphate were supplied by Boehringer Mannheim (West Germany). Dextrose was purchased from EM Science (Cherry Hill, NJ). L-histidine, biotin, magnesium chloride, potassium chloride for the Vogel-Brunner medium and S9 salt solution, along with molecular biology grade Dimethyl Sulfoxide were acquired from Fisher Scientific (Fairlawn, NJ). 2-Anthramine was purchased from Sigma Chemical Co (St Louis, Mo). Oxide broth was supplied by Oxoid Ltd (Basingstoke, Hants, England). S9 from Aroclor 1254 treated male rats purchased from Maltox Molecular Toxicology, Inc. (College Park, MD). Phosphate Buffer, PH=7.5, was obtained from the Linberger Cancer Research Center's Tissue Culture Facility (UNC,Chapel Hill). Salmonella Typhimurium strain TA98 was obtained from Dr. Bruce Ames, University of California at Berkeley.

III. B. Methods

III. B. 1. *In Vivo* Metabolism Study

After a one week acclimatization period, Sprague Dawley rats were injected intra-peritoneally through a 25g needle with varying doses of 1-NP, 2-NFA, and 3-NFA of 2 mg, 1 mg, and 0.5 mg in 0.2 ml and 0.1 ml acetone solutions. Three rats were used for each chemical and one control animal per dose received a pure acetone injection. Immediately after the injection, rats were placed in plastic metabolism cages in a controlled, pathogen free environment located in the DLM facility, Beryhill Hall, UNC. A sealed insulated dry ice box located underneath

the cage collected and immediately froze urine samples during the 48 hr collection period. A picture of this apparatus is shown in Fig. 18, Appendix A. Urine was collected at intervals 0-4 hrs, 4-8 hrs, 8-24 hrs, 24-36 hrs, and 36-48 hrs and sterilized by filtration with a 0.4 micron pore size filter and stored at -80 C in darkness until mutagenicity analysis. Rats had continuous access to drinking water and powdered rat chow.

After a brief visual inspection of the injection site for unabsorbed chemical and general appearance of intra-peritoneal cavity, the carcass was incinerated by DLM, UNC. Metabolism cages were cleaned with 95% ethanol solution, then washed with a soap solution and rinsed after each animal's use. All contaminated syringes and materials were doubled bagged, labeled, and disposed of as trace carcinogenic waste through the UNC's hazardous material collection department. Vinyl lab gloves, surgical face mask, and lab coat were worn during animal handling, urine collection, and cage work.

III. B. 2. *In Vitro* Ames Assay

Mutagenicity analysis was via a Salmonella Typhimurium plate incorporation test using TA98 strain with and with out S-9 fraction as outlined by Ames' 82 publication. Quality assurance of the strain was performed on the master plates made from frozen permanents stored at -80° C. Sample colonies for each master plate were verified for proper

characteristics of histidine dependency, rfa, crystal violet, and UV light sensitivity, and ampicillin resistance. A colony was picked off the master plate and incubated in 25 mls of nutrient broth in a dry, dark, shaker at 37° C for 16 hrs to develope an approximate concentration of 1.5 x 10^9 cell/ml. Acceptable urine samples (see Results' paragraph IV. A.) were resterilized through a 0.22 micron filter and divided into three undiluted volumes ranging from 75 uL to 250 uL for 2 mg dosed rat samples and two undiluted volumes ranging from 100 uL to 250 uL for 1 mg dosed rat samples. Two control 0 mg dosed rats' samples were evaluated for background urine mutagenicity for each assay. B-glucuronidase, 100 units/plate, was included in the top overlay to account for glucuronide conjugate metabolite formation. One half ml of 100% activity Aroclor induced S9 (40 ug/100 uL) was added to half the plates for each sample. The top agar mixture was vortexed at low speed for 3 - 4 seconds and poured evenly on to the agar plate. Plates were allowed to solidify on a level surface for approximately 30 minutes then placed inverted in a 100% humidity, 37° C dark incubator for 24 hours. Samples with insufficient amounts of urine to complete an assay were combined with other samples from the same time period and chemical if necessary. The 2 mg dose of 1-NP's 0-4 hr sample and the 4-8 hr sample were combined by time period. All 1 mg doses required combination of the 0-4 and 4-8 hr samples for each rat maintaining separation between individual rats' urine. Duplicate positive controls

using 2-nitrofluorene (50ug) evaluated the responsiveness of the strain while duplicate control plates with 2-anthramine (23 ug) evaluated S9's background activity. Duplicate spontaneous reversion plates verified TA98's background activity and provided a zero dose revertant value for future analysis. Only plates producing twice the spontaneous reversion count were considered evidence of mutation. Since a limited number of samples can be run at any one time, the Ames assay is capable of only a finite number of samples per assay. Samples were stratified by collection time and dose for the assays. This stratification compensated for individual assay variances allowing each compound to receive equal assay bias reducing error in their future comparisons.

The mutagenic activity of each sample was determined by least squares linear regression analysis using the spontaneous revertant rate as the zero dose point. Assay results were recorded as revertant/dose and converted to a revertant/hr rate for each individual sample. When more than one Revert./Dose linear regression line was able to be determined, an average value was used in calculating the revertant rate and the standard deviation identified as error. The background reversion rates of the control rats were subtracted from the PAH dosed animals' rates to produce linear scale and logarithmic scale Revertant Excretion Rate Plots. These plots were used to calculate revertant excretion rates which in turn were used to estimate the total cumulative revertants excreted.

III. B. 3. Quality Assurance of Samples

Before, during, and after the *in vivo* metabolism study, the rats' health, physical condition, eating and drinking habits, and urination volumes were monitored and recorded. Persistence of an unusual observation when confirmed by the visual autopsy caused exclusion of the rat. The brief autopsy examined the injection site and intestines to confirm the compound was properly delivered to the rat.

III. B. 4. Toxicokinetic Analysis

Revertants are an indirect measure of the mutagenic species being excreted. No quantitative results concerning the physical number of mutagens can be inferred from the information provided since the number of revertants produced per mutagenic metabolite is unknown. However, this information can provide an overall view of the mutagen excretion for comparative analysis.

The Logarithmic Revertant Excretion Rate Plot's profile for each compound indicated the animal system appeared to be a one compartment model with first order kinetics concerning mutagen excretion. Applying product excretion kinetic analysis to the revertant data, the combined pseudo absorption and formation rate constant, Ka, and the revertant excretion rate constant, Ke, were estimated. Identifying the highest and lowest possible slopes for each rate constant accounted for the error in the plotted points and for the assumption of a single

compartment model. The lack of data points immediately after injection required the area under the curve, (AUC), be calculated to estimate the resulting range of total cumulative revertants. A Sigma Minus plot using all five values of the Excretion Rate Plot was developed using the AUC values to provide a more accurate estimation of the excretion rate constant. Lastly, the combined absorption and formation rate constant, Ka, was estimated accounting for error using the total cumulative revertant value, AUC, as the Y intercept for the Logarithmic Revertant Excretion Rate Plot.

IV. RESULTS

IV. A. Quality Assurance of Urine Samples

Eighteen of the forty eight rats used provided acceptable samples for use in this study. Table 4 outlines the reasons for exclusion of any rat or selective sample. Notably, at least two rats were used for analysis of each injected compound. Both the 1 mg and 2 mg doses were analyzed whereas the 0.5 mg dose provided an insufficient revertant rate beyond the 8th hour of collection.

Analysis of Ames assay results revealed an 18 September assay of the 8 - 24 hr period samples was unusable for compounds 2-NFA and 3-NFA due to an unusually high revertant rate for the control urine shown in Fig. 37 in the appendix. Fortunately, two other acceptable assays were completed for this sample period.

IV. B. Mutagenicity of Urine Samples

Least squares linear regression analysis of each Ames Assays' Revertants vs Dose plots are presented in Figures 19 through 36 in Appendix A and their numerical slope data listed in Table 5. All future results were based on these linear regression lines whose regression coefficients, Table 6, averaged 0.91 $\pm$ 0.07, indicating the strong revertant-dose relationship of the metabolites. Revertant/Dose values were converted to Revertant/Hour rates, Table 7, allowing comparisons

between samples. Linear axis revertant excretion rate plots for the 2 mg doses Figures 4, 5, and 6 compare rats' mutagenicity excretion rates. These figures also illustrate the significant difference in urination rates between rats during the first 8 hour period of the *in vivo* study. The extremely low revertant rates of the control rats confirm the treated rats' revertant rates are direct results of the injected nitro-PAHs.

IV. B. 1. 1-NP's Urinary Mutagenicity

Maximum mutagenicity required exogenous activation by S9 and was excreted between 7 - 14 hours after injection of the 2 mg dose and 7 - 20 hrs for the 1 mg dose extrapolated from excretion rate plot, Fig. 7. The direct acting mutagenicity behaved similarly but only provided 30% $\pm$ 10% of the activity, shown in Excretion Rate Plots, Figures 8 and 9.

The 1 mg dose provided approximately 33% $\pm$ 13% of the total activity achieved by the 2 mg dose for S9 activated mutagenicity. Moreover, the direct acting mutagenicity in the 2 mg dose was 15% $\pm$ 5% higher than the 1 mg dose.

IV. B. 2. 2-NFA's Urinary Mutagenicity

2-NFA's maximum mutagenicity occurred between 7-24 hrs period for both S9 activated and direct acting mutagens for both 1 mg and 2 mg doses, as shown in Figures 8 and 9. However, S9 dependent mutagenicity

equaled the direct acting mutagenicity for the 1 mg dose. Doubling of the dose resulted in S9 activated mutagenicity increasing 40% ± 30% while direct acting mutagenicity rose 27% ± 3%.

IV. B. 3. 3-NFA's Urinary Mutagenicity

Maximum mutagenicity was direct acting and excreted between 4-10 hrs for the 2 mg and similarly was excreted between 0-15 hrs for the 1 mg dose shown in Fig. 7. Exogenous activation by S9 decreased this mutagenicity by 45% ± 30% for the 2 mg dose, Fig. 9, and 70% ± 25% for the 1 mg dose, Fig. 8. Doubling the dose raised direct acting mutagenicity 40% ± 27% and indirect acting mutagenicity 20% ± 15%.

IV. C. Toxicokinetic Analysis of Revertant Excretion Data

IV. C. 1. 1-NP's Toxicokinetic Analysis

Extrapolation of the logarithmic Excretion Rate Plot's, Fig. 10, terminal end yielded a range of 4,850 ± 2,530 Revert/(48 hr to infinity). Extrapolation of this terminal rate estimates the total mutagenicity produced was 41,478 ± 3,700 revertants. The Sigma Minus Plot, Fig. 11, estimated Ke, the Excretion Rate Constant's range was - 0.061 ± 0.015 (1/hr). The combined absorption/formation rate constant Ka was estimated at 1.14 (1/hr) as shown in Fig. 17. The revertant excretion rate constant Ke is 18 times smaller than Ka, thus mutagen excretion is formation rate limited.

IV. C. 2. 2-NFA's Toxicokinetic Analysis

Extrapolation of the Logarithmic Revertant Excretion Rate Plot's, Fig. 12, terminal end showed the upper bound of the revertant excretion rate was actually 0 rev/hr accounting for error. Therefore the highest excretion rate and the "best fit" excretion rate were used. The terminal end's cumulative revertant rate was 11,680 $\pm$ 8,500 Revert/(48 hr to infinity). Extrapolation of the terminal rate indicates the total mutagenicity was 25,448 $\pm$ 14,170 revertants. The Sigma Minus Plot, Fig. 13, estimated the revertant excretion rate constant, Ke, was - 0.016 $\pm$ 0.058 (1/hr). The combined absorption/formation rate constant Ka was estimated at 1.14 (1/hr) as shown in Fig. 17. The revertant excretion rate constant Ke is 40 times smaller than Ka, thus mutagen excretion is formation rate limited.

IV. C. 3. 3-NFA's Toxicokinetic Analysis

Extrapolation of the Logarithmic Revertant Excretion Rate Plot's, Fig. 14, terminal end yielded a revertant rate of 1,944 $\pm$ 1,497 Revrt/hr. This value estimated the total revertants excreted was 23,240 $\pm$ 4,000 revertants. The Sigma Minus Plot, Fig. 15, estimated the revertant excretion rate constant, Ke, was - 0.079 $\pm$ 0.0043 (1/hr). The combined absorption/formation rate constant Ka was estimated at -1.14 (1/hr) as shown in Fig. 17. The revertant excretion rate constant Ke is 15 times smaller than Ka, thus mutagen excretion is formation rate limited.

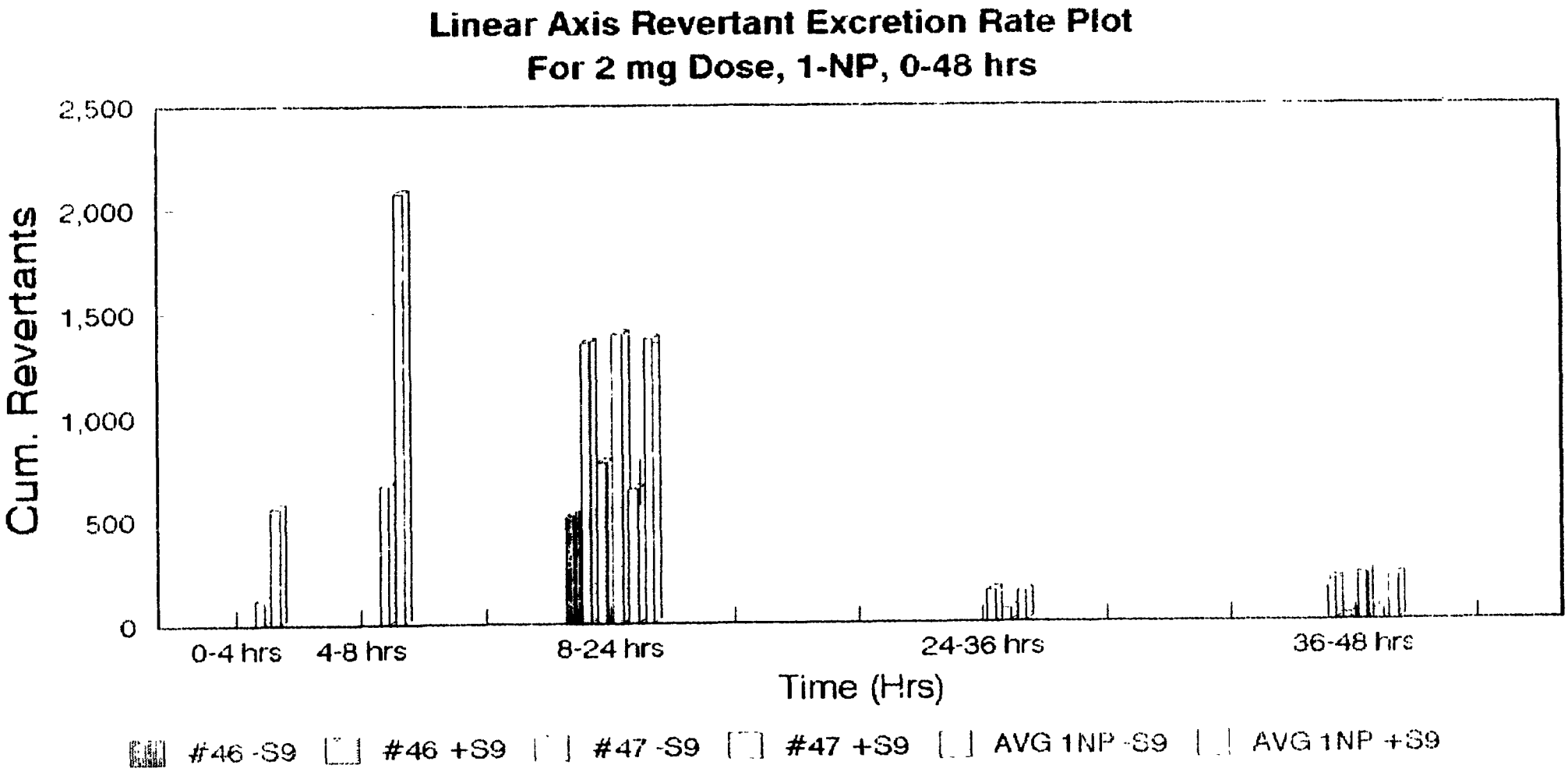
Linear Axis Revertant Excretion Rate Plot
For 2 mg Dose, 1-NP, 0-48 hrs
Cum. Revertants
2,500
2,000
1,500
1,000
500
0
0-4 hrs
4-8 hrs
8-24 hrs
24-36 hrs
36-48 hrs
Time (Hrs)
#46 -S9
#46 +S9
#47 -S9
#47 +S9
AVG 1NP -S9
AVG 1NP +S9

Figure 4

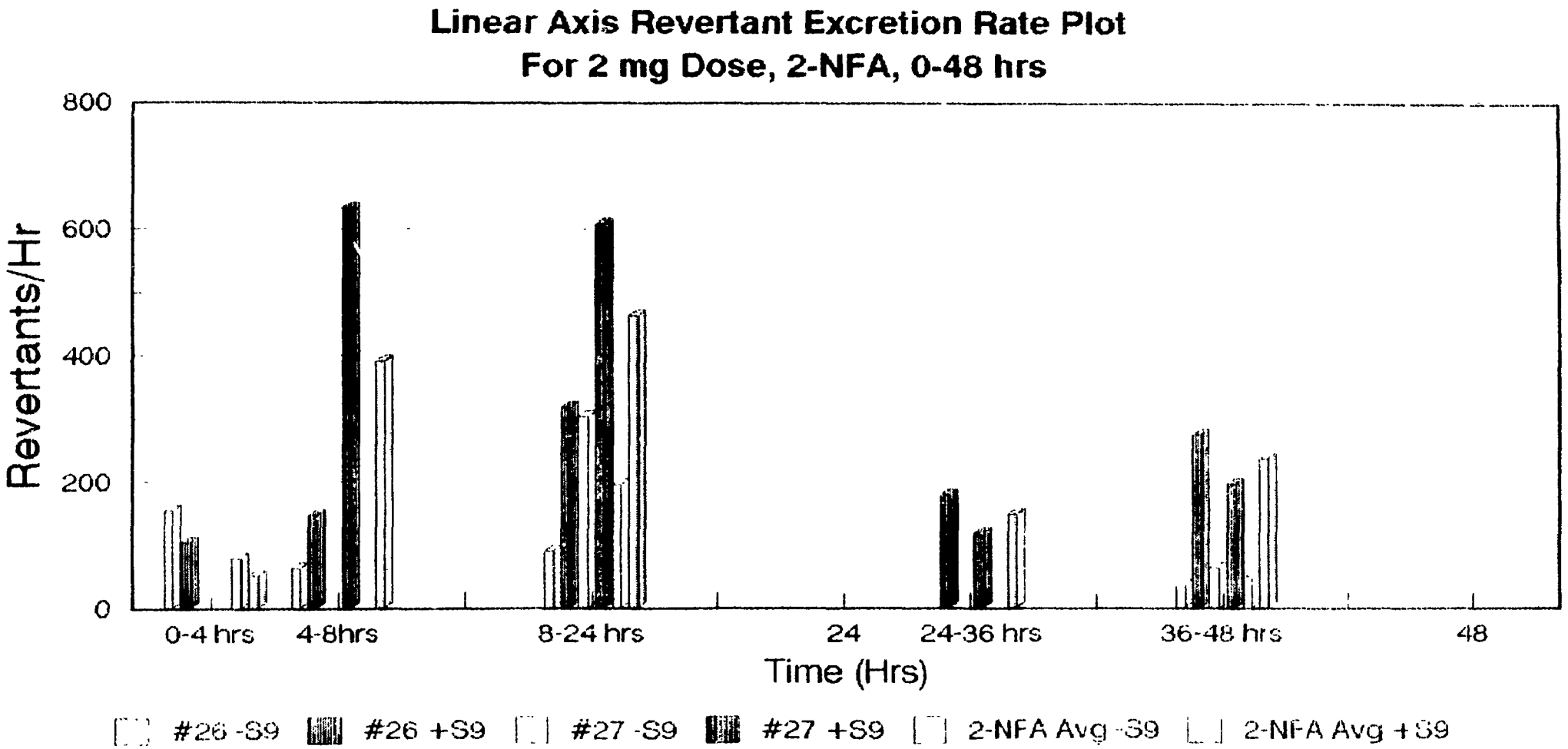
Linear Axis Revertant Excretion Rate Plot
For 2 mg Dose, 2-NFA, 0-48 hrs
800
600
400
200
0
Revertants/Hr
0-4 hrs
4-8hrs
8-24 hrs
24
24-36 hrs
36-48 hrs
48
Time (Hrs)
#26 -S9
#26 +S9
#27 -S9
#27 +S9
2-NFA Avg -S9
2-NFA Avg +S9

Figure 5

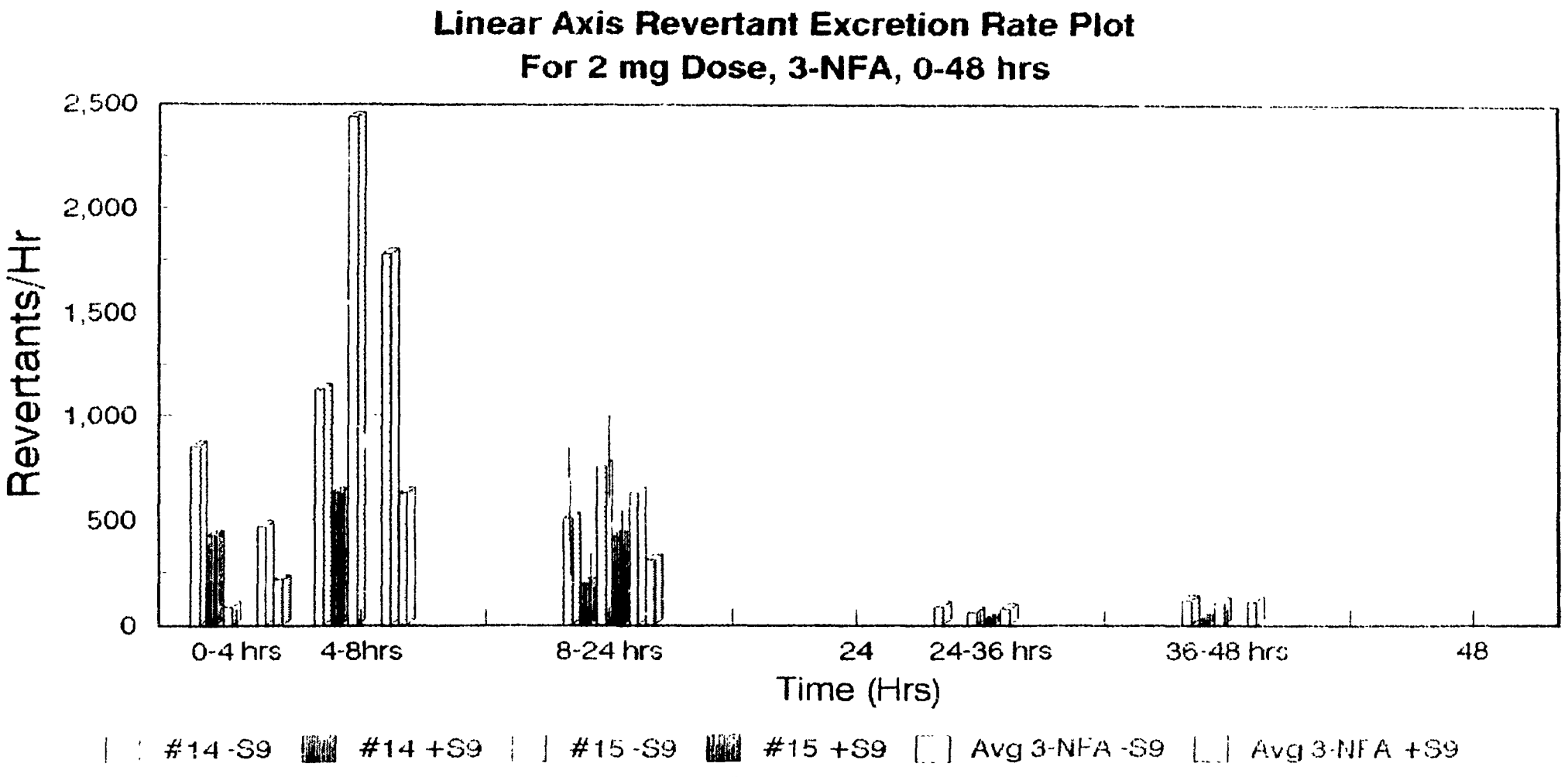
Linear Axis Revertant Excretion Rate Plot
For 2 mg Dose, 3-NFA, 0-48 hrs
Revertants/Hr
2,500
2,000
1,500
1,000
500
0
0-4 hrs
4-8hrs
8-24 hrs
24
24-36 hrs
36-48 hrs
48
Time (Hrs)
#14 -S9
#14 +S9
#15 -S9
#15 +S9
Avg 3-NFA -S9
Avg 3-NFA +S9

Figure 6

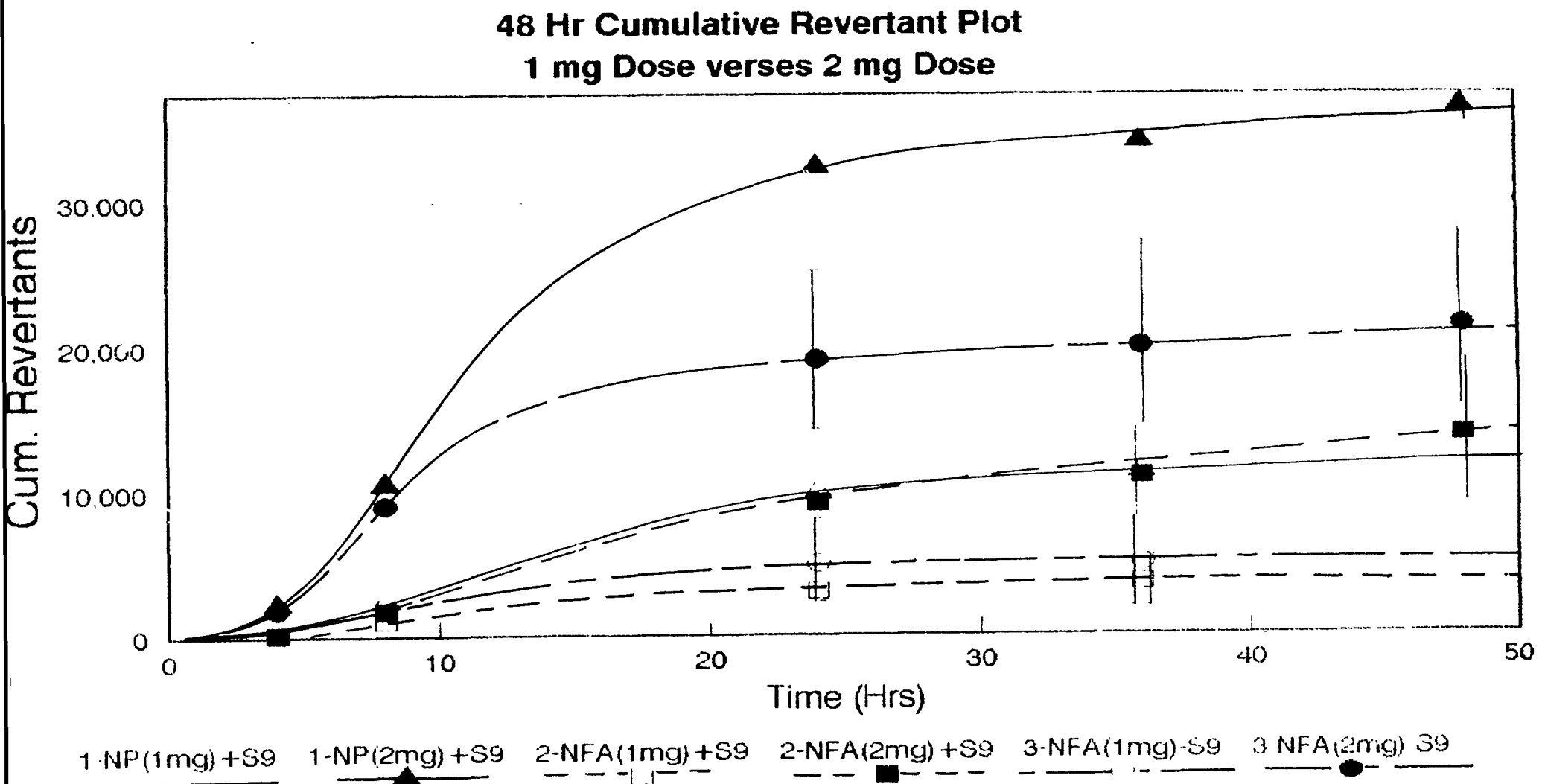
48 Hr Cumulative Revertant Plot
1 mg Dose verses 2 mg Dose
Cum. Revertants
30,000
20,000
10,000
0
0
10
20
30
40
50
Time (Hrs)
1-NP(1mg)+S9
1-NP(2mg)+S9
2-NFA(1mg)+S9
2-NFA(2mg)+S9
3-NFA(1mg)-S9
3-NFA(2mg) S9

Figure 7

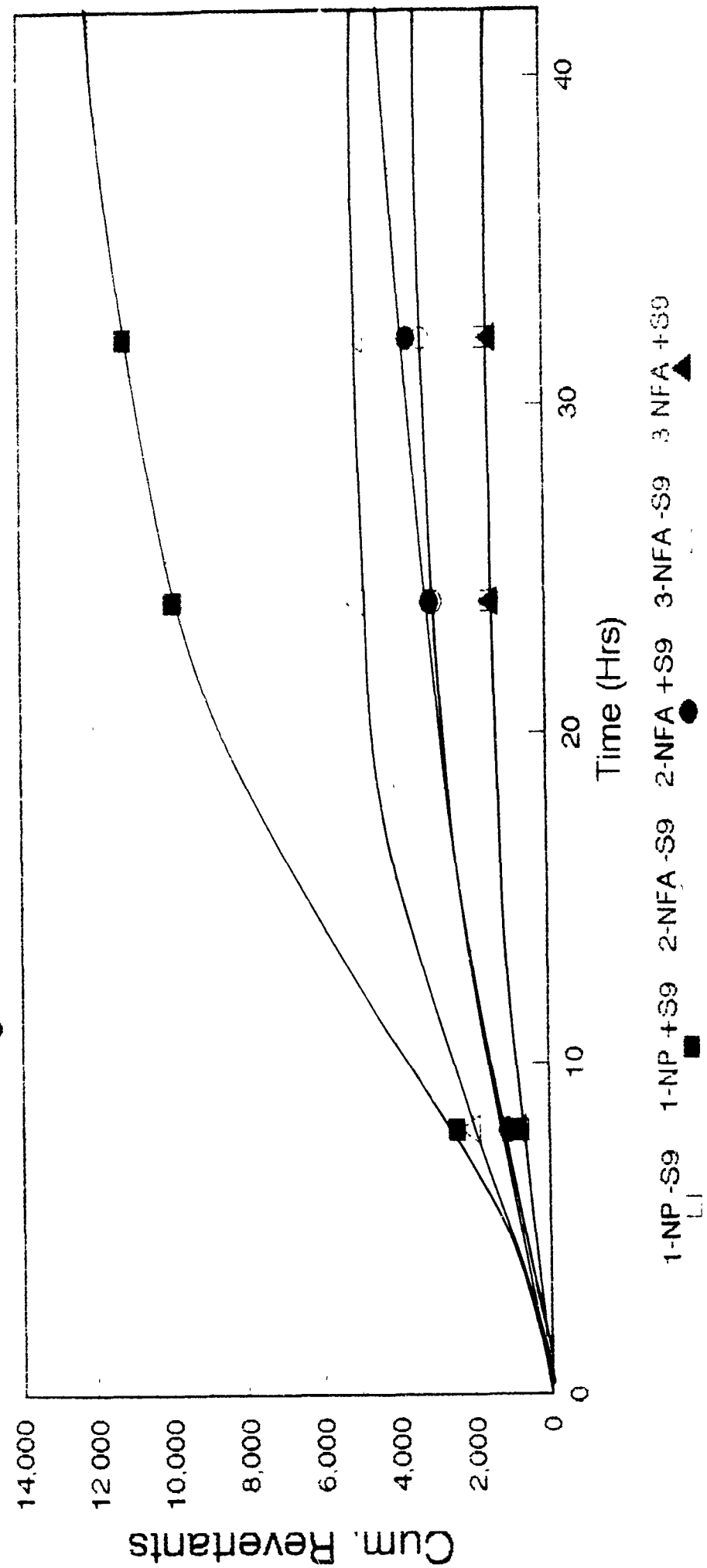
48 Hr Cumulative Revertant Plot
1 mg Doses of 1-NP, 2-NFA, & 3-NFA
Cum. Revertants
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
0
10
20
30
40
Time (Hrs)
1-NP -S9
1-NP +S9
2-NFA -S9
2-NFA +S9
3-NFA -S9
3 NFA +S9

Figure 8

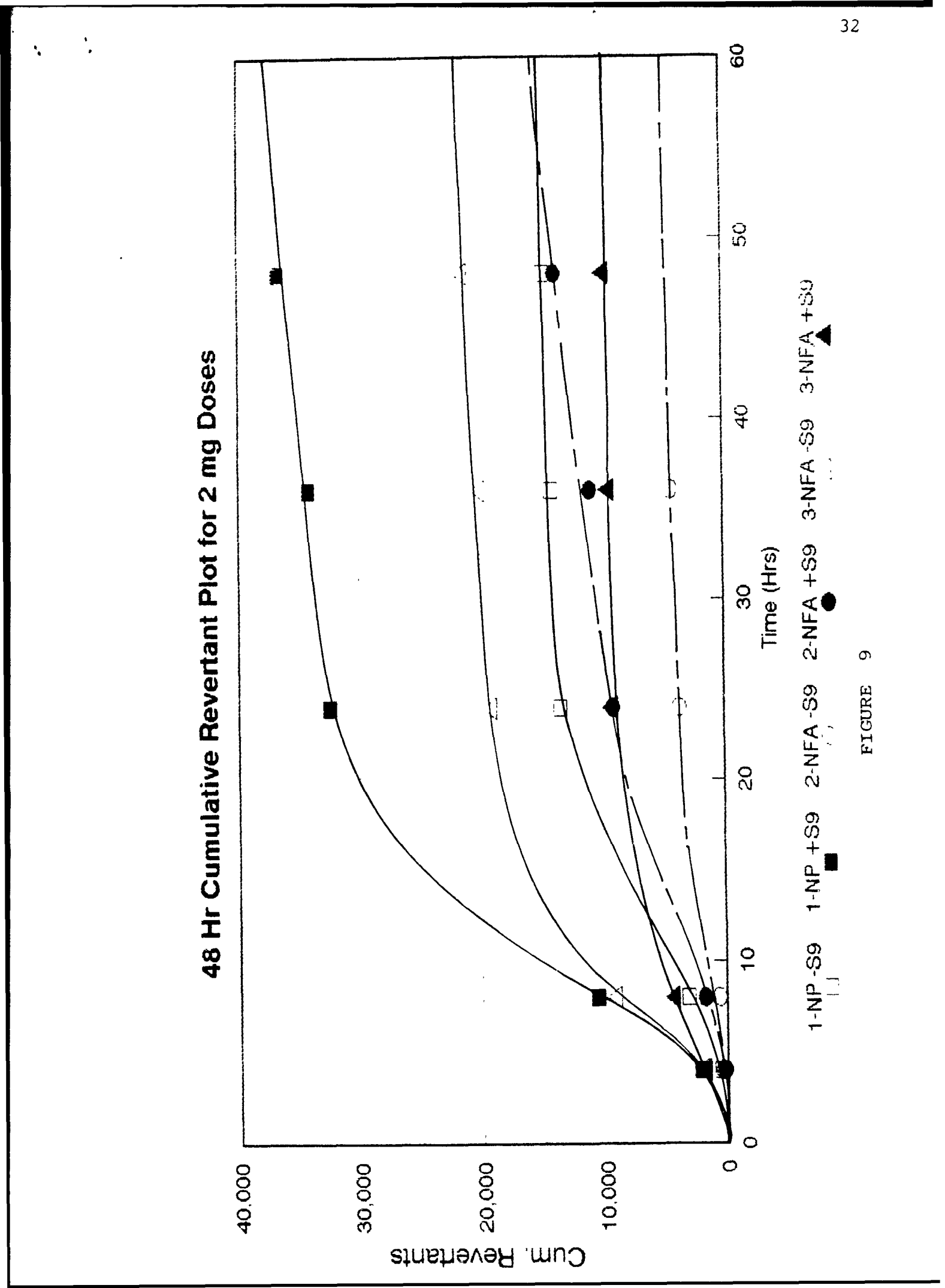
48 Hr Cumulative Revertant Plot for 2 mg Doses
Cum. Revertants
40,000
30,000
20,000
10,000
0
0
10
20
30
40
50
60
Time (Hrs)
1-NP -S9
1-NP +S9
2-NFA -S9
2-NFA +S9
3-NFA -S9
3-NFA +S9

FIGURE 9

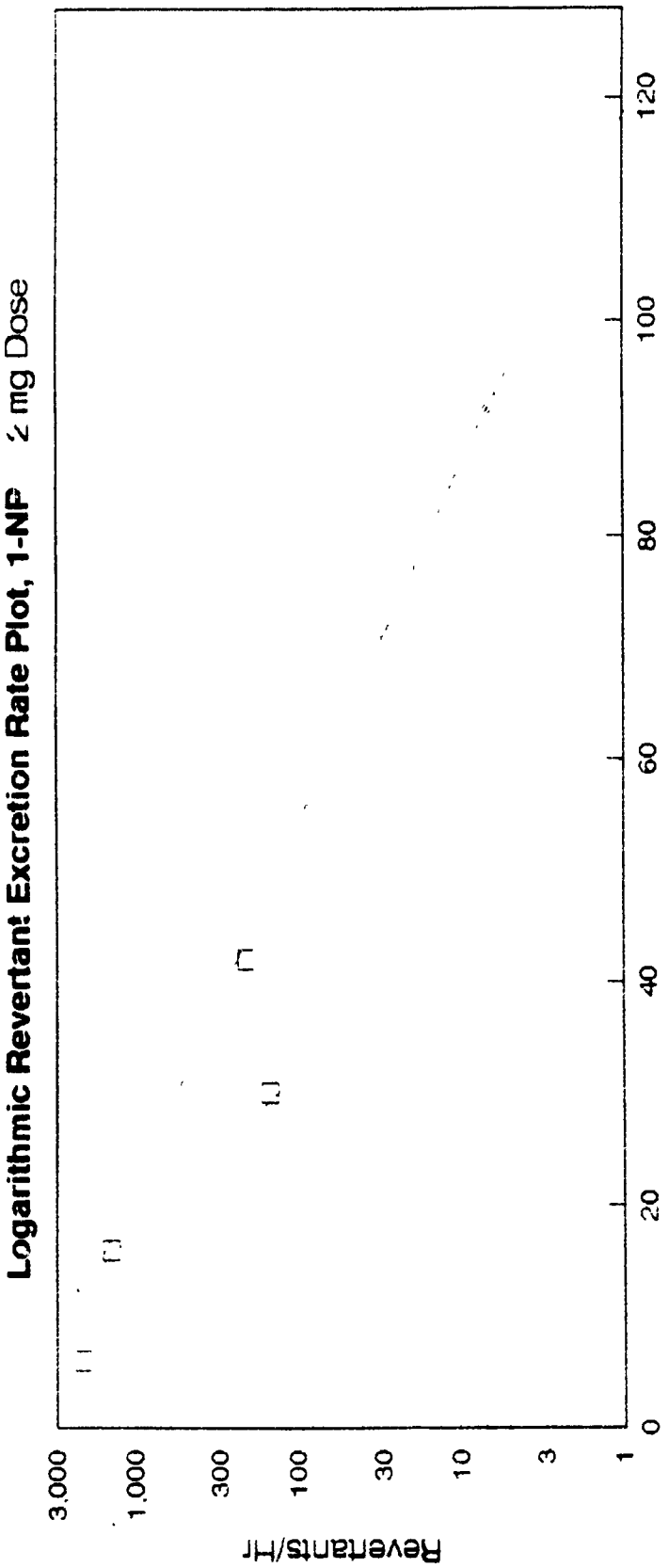
Logarithmic Revertant Excretion Rate Plot, 1-NP 2 mg Dose
Revertants/Hr
3,000
1,000
300
100
30
10
3
1
0
20
40
60
80
100
120
Time (Hrs)
1-NP +S9

Figure 10

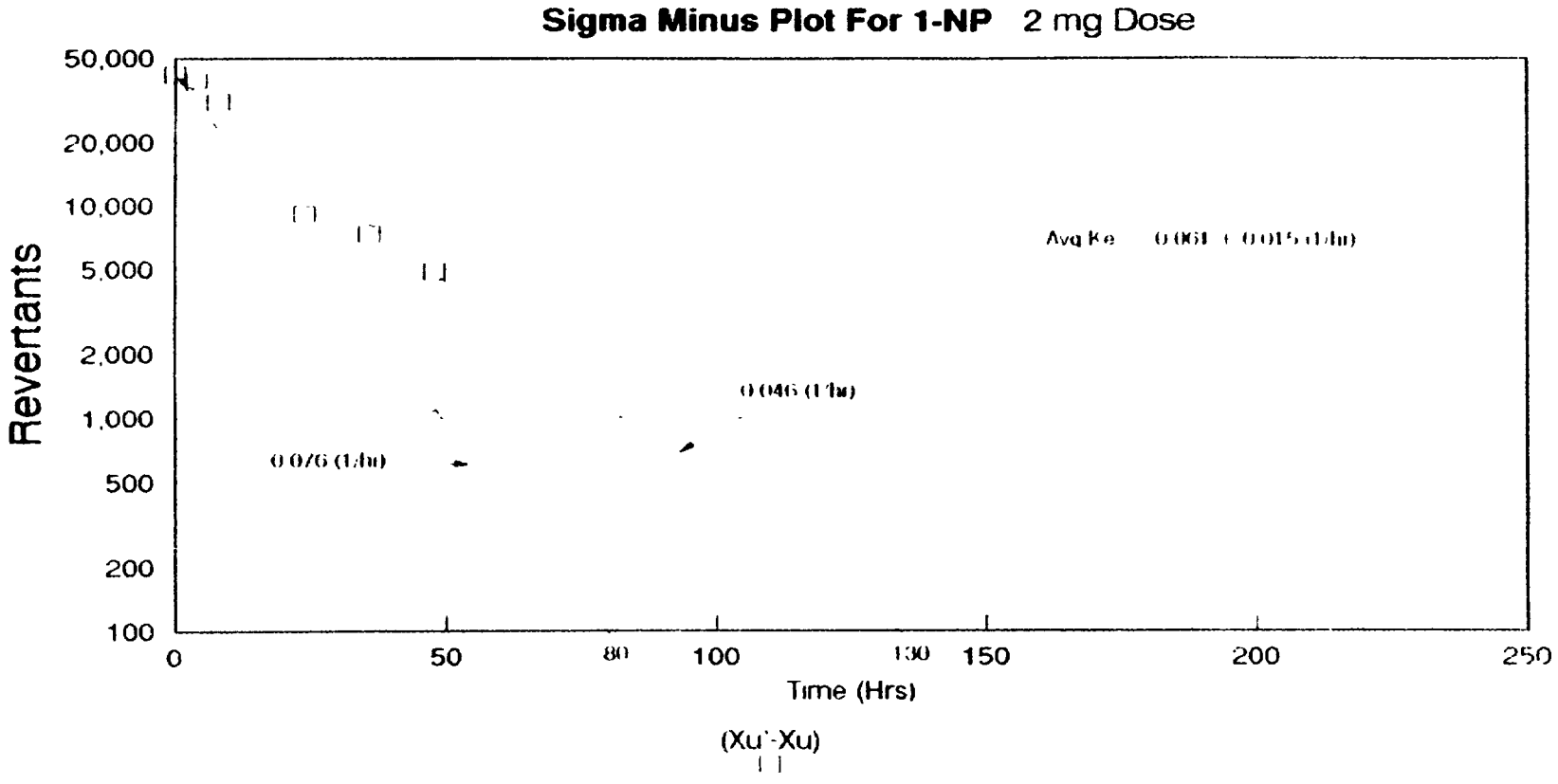
Sigma Minus Plot For 1-NP 2 mg Dose
Revertants
50,000
20,000
10,000
5,000
2,000
1,000
500
200
100
0
50
80
100
130
150
200
250
Time (Hrs)
0.046 (1/hr)
0.076 (1/hr)
(Xu'-Xu)

Figure 11

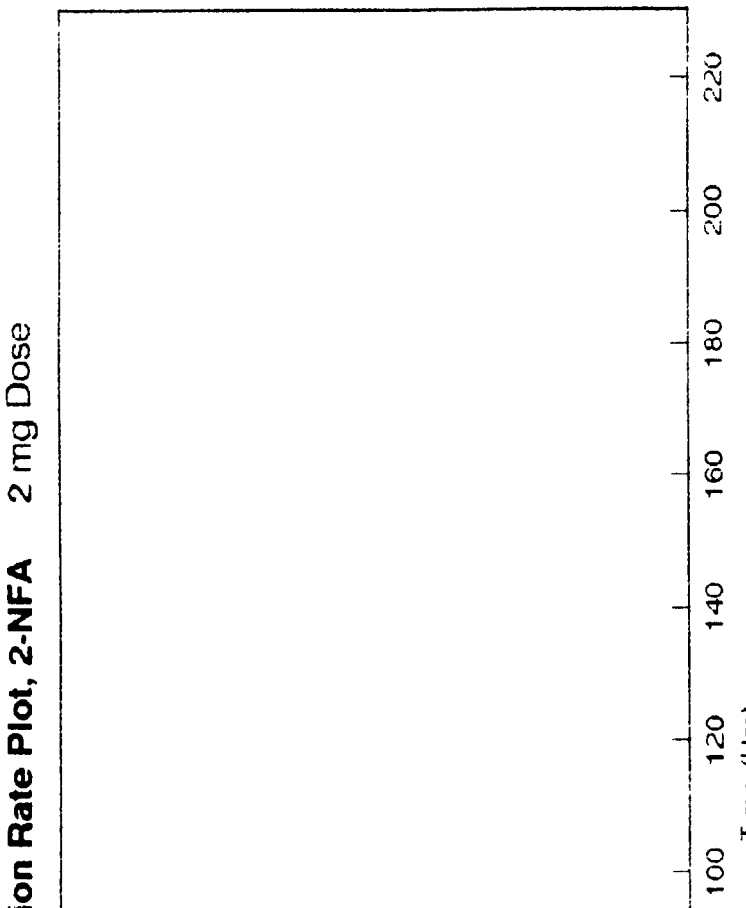
Logarithmic Revertant Excretion Rate Plot, 2-NFA 2 mg Dose
Revertants/Hr
1,000
500
200
100
50
20
10
5
2
1
0
20
40
60
80
100
120
140
160
180
200
220
Time (Hrs)
2NFA +S9

Figure 12

Sigma Minus Plot For 2-NFA

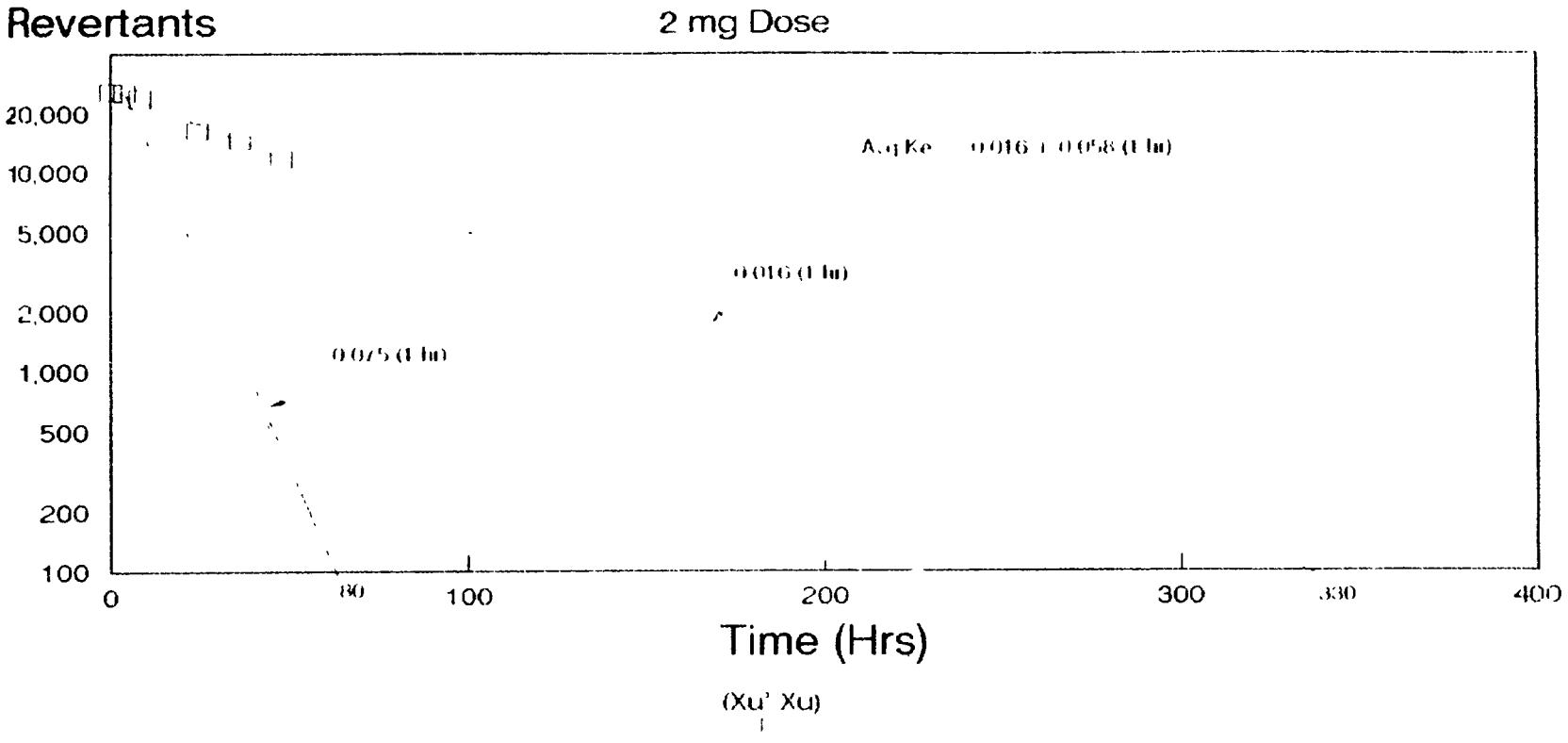

Figure 13

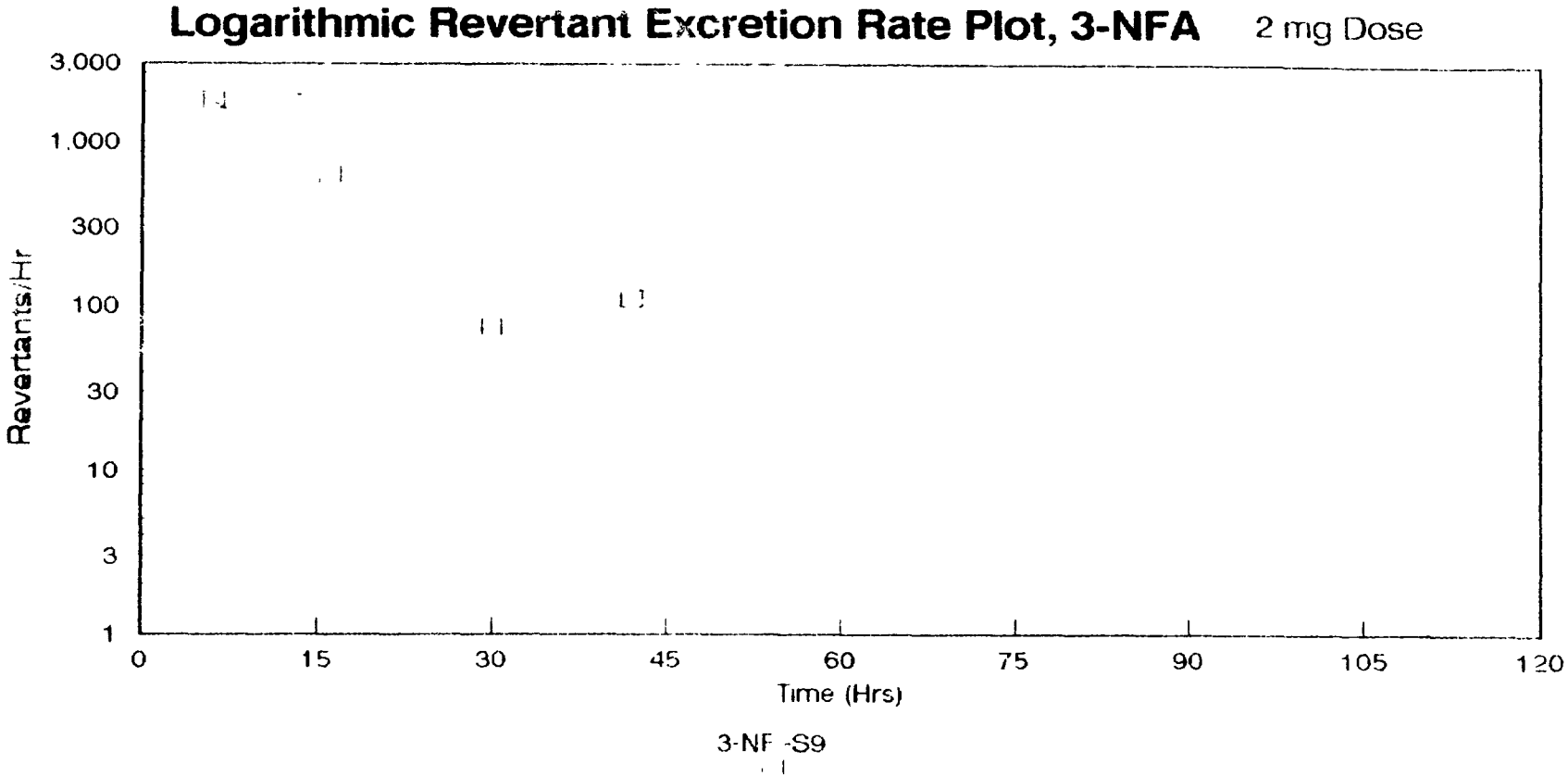
Logarithmic Revertant Excretion Rate Plot, 3-NFA
2 mg Dose
3,000
1,000
300
100
30
10
3
1
Revertants/Hr
0
15
30
45
60
75
90
105
120
Time (Hrs)
3-NF -S9

Figure 14

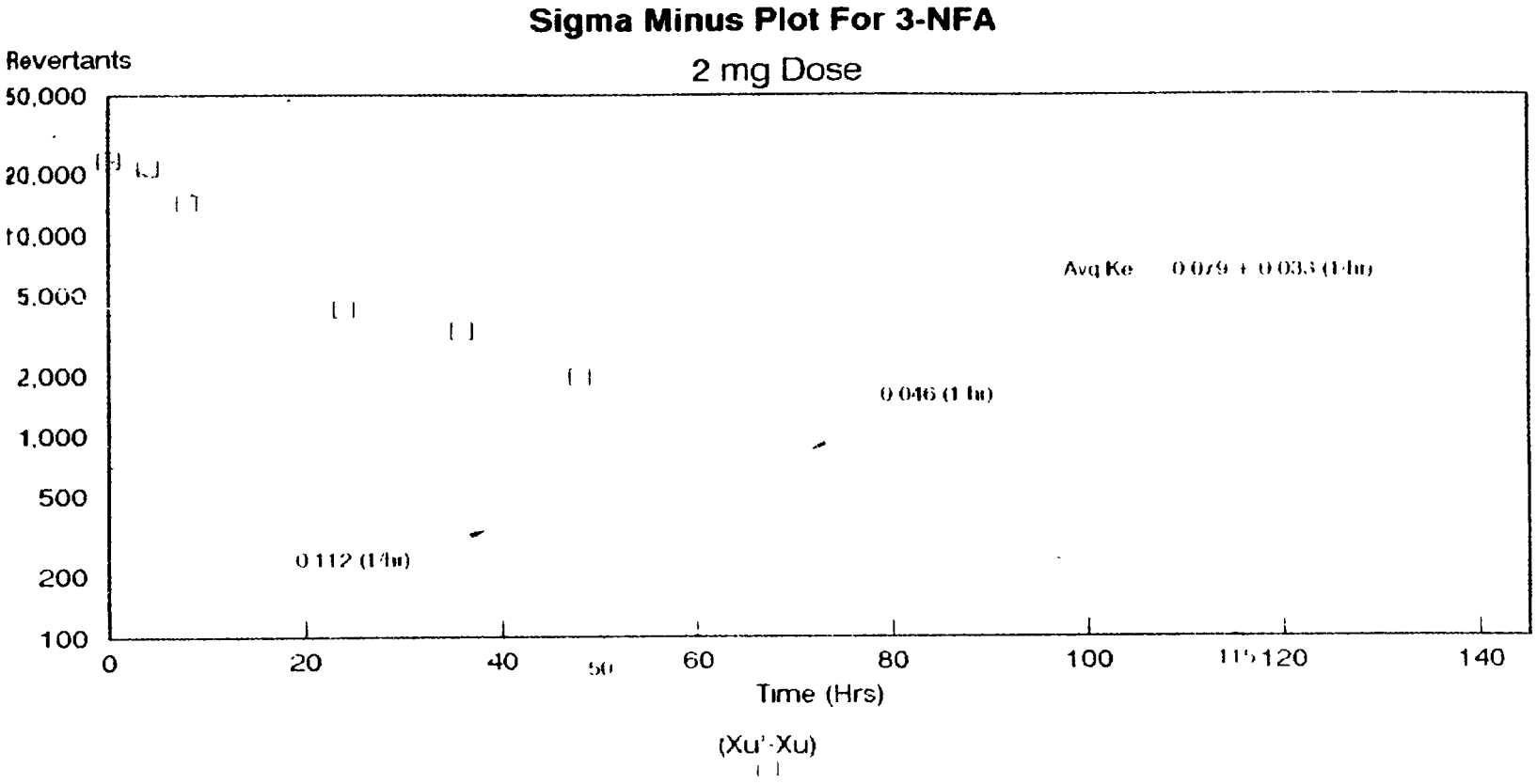
Sigma Minus Plot For 3-NFA
2 mg Dose
Revertants
50,000
20,000
10,000
5,000
2,000
1,000
500
200
100
0
20
40
60
80
100
120
140
Time (Hrs)
Avg Ke
0.046 (1 hr)
0.112 (1/hr)
(Xu'-Xu)

Figure 15

Estimated Total Cumulative Revertants Plot

2 mg Dose, 1-NP, 2-NFA, & 3-NFA

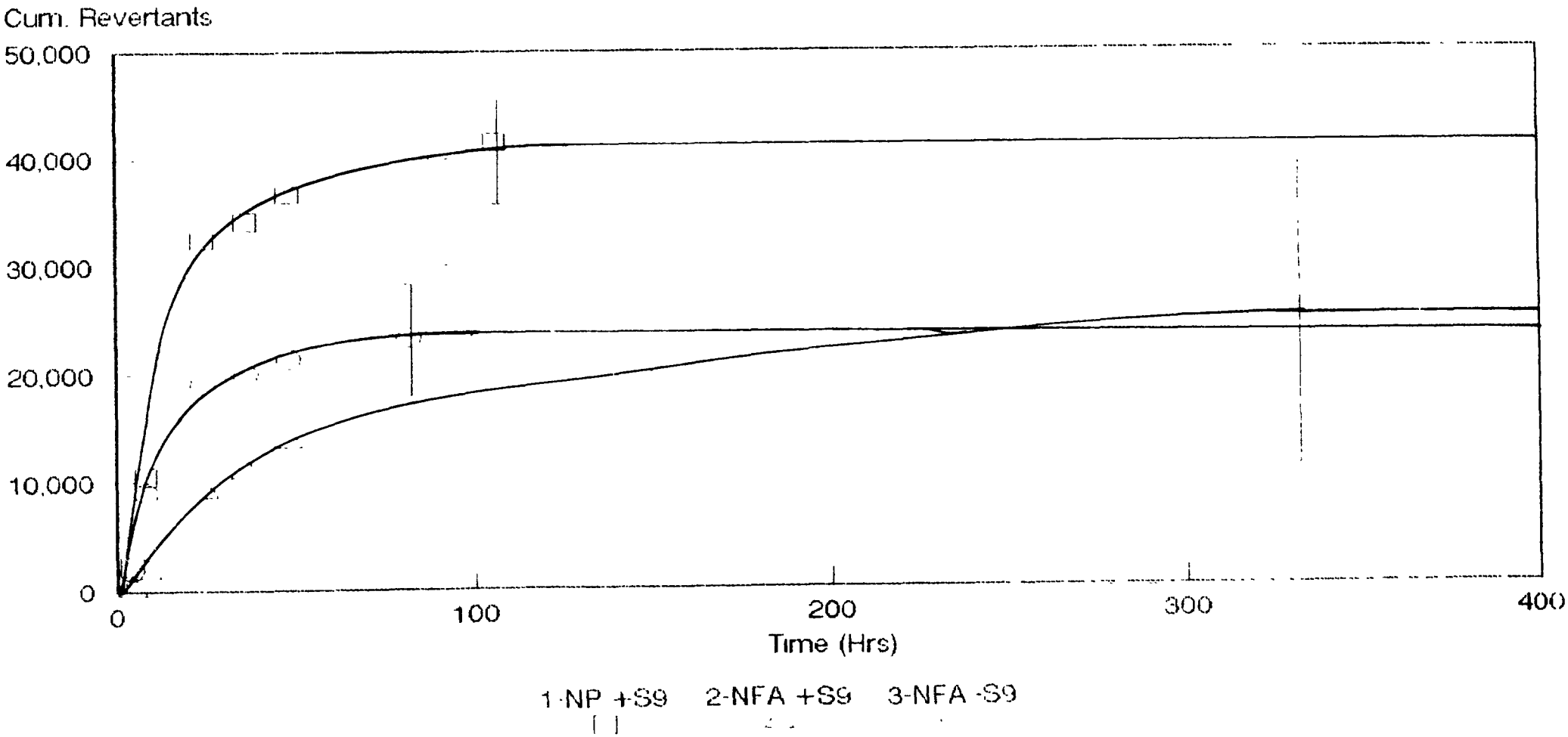

Figure 16

48 Hr Cumulative Revertant Plot's Values

Fig 7, 1 mg Dose verses 2 mg Dose

	1-NP(1mg) +S9	1-NP(2mg) +S9	2-NFA(1mg) +S9	2-NFA(2mg) +S9	3-NFA(1mg) S9	3 NFA(2mg) S9
4 Hours		2 216		208 + 200		1,872 + 900
8 Hours	2 436 + 1,600	10 492	1 080 + 680	1,768 + 1 500	2 000 + 800	8,992 + 4 500
24 Hours	9 880 + 4 100	32,428 + 450	3,066 + 1,390	9 160 + 4 600	4 936 + 3 500	19 088 + 7,220
36 Hours	11 194 + 4 300	34,168 + 450	3,608 + 1,390	10 948 + 5000	4 936 + 3 500	19 988 + 7,460
48 Hours		36,628 + 1 200		13 768 + 5610		21 320 + 7,540

Table 1

48 Hr Cumulative Revertant Plot's Values

Fig 8, 1 mg Doses of 1-NP, 2-NFA, & 3-NFA

	1-NP -S9	1-NP +S9	2-NFA -S9	2-NFA +S9	3-NFA -S9	3-NFA +S9
8 Hours	784 + 350	2,436 + 1,600	1,224 + 900	1,080 + 680	2,000 + 800	804 + 600
24 Hours	1,464 + 502	9,880 + 4,100	2,948 + 1,400	3,056 + 1,390	4,936 + 3,500	1,397 + 1,200
36 Hours	1,608 + 502	11,164 + 4,300	3,224 + 1,400	3,608 + 1 390	4,936 + 3,500	1,397 + 1,200

Table 2

48 Hr Cumulative Revertant Plot for 2 mg Doses

Fig 9, Values and Errors

	1-NP -S9	1-NP +S9	2-NFA +S9	2-NFA -S9	3-NFA -S9	3 NFA +S9
4 Hours	480	2 216	208 + 200	316 + 300	1,872 + 900	1 708
8 Hours	3 116	10 492	1,768 + 1,500	560 + 300	8 992 + 4 500	4 232
24 Hours	13 436 + 2,880	32 428 + 448	9 160 + 4,500	3,680 + 2 700	19 088 + 7 220	9 144 + 2 500
36 Hours	14 132 + 2,880	34,168 + 450	10,948 + 5 000	4 268 + 2,900	19 998 + 7,460	9 408 + 2 500
48 Hours	14 744 + 2 880	36 628 + 1 200	13,768 + 5,670		21 320 + 7 540	9,684 + 2 500

Table 3

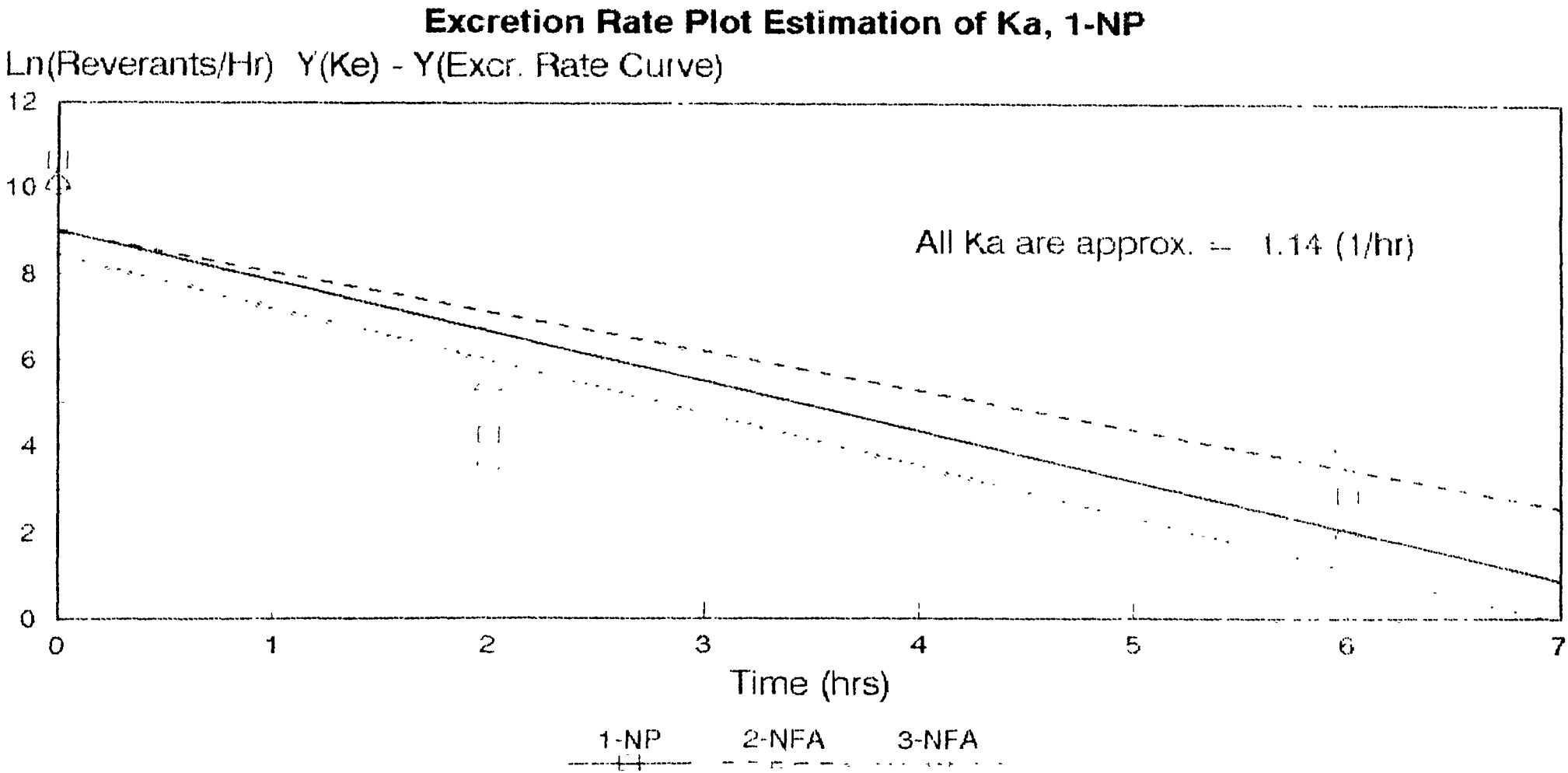
Excretion Rate Plot Estimation of Ka, 1-NP
Ln(Reverants/Hr) Y(Ke) - Y(Excr. Rate Curve)
12
10
8
6
4
2
0
All Ka are approx. = 1.14 (1/hr)
0
1
2
3
4
5
6
7
Time (hrs)
1-NP
2-NFA
3-NFA

Figure 17

TABLE 4

QUALITY ASSURANCE OF SAMPLES

Compound Injected	Amount (mg)	# Acceptable Rats	Unacceptable Rats and Reasons
1-NP	2 mg	5	2 died 2 poor physical health very ill 1 inadequate injection
1-NP	1 mg	2	1 inadequate injection
2-NFA	2 mg	2	1 poor physical health Obstructed/bloated intestine
	1 mg	2	1 poor physical health Abnormal looking intestine
3-NFA	2 mg	2	1 poor physical health Internal fluid in cavity
	1 mg	3	
Controls	2 mg	4	2 died
	1 mg	5	1 died
All	0.5 mg	0	11 Dosed too low for Mutagenic analysis

TABLE 5

LINEAR REGRESSION VALUES FOR REVERTANT VS DOSE PLOTS

2 MG DOSE

COMPOUND	SAMPLE	0-4 HRS Rev/uL Sample	4-8 HRS Rev/uL Sample	8-24 HRS Rev/uL Sample 1	8-24 HRS Rev/uL Sample 2	24-36 HRS Rev/uL Sample	36-48 HRS Rev/uL Sample
1-NP	46-S9			3		0.74	0.57
1-NP	46+S9			8.00		1.92	1.90
1-NP	47-S9			3.65			0.17
1-NP	47+S9			6.47		1.30	0.94
1-NP	Avg -S9	2.14	3.57				
1-NP	Avg +S9	9.11	11.18				
2-NF	26-S9	0.27	0.17	0.45			0.22
2-NF	26+S9	0.19	0.41	1.09	1.67	0.94	1.50
2-NF	27-S9			1.65	1.11		0.28
2-NF	27+S9		1.76	1.67	3.74	1.61	0.85
3-NF	14-S9	1.15	1.07	1.98	0.83	1.11	0.81
3-NF	14+S9	0.59	0.62	0.84	0.34		0.44
3-NF	15-S9	0.53	3.01	2.03	1.29	0.25	0.12
3-NF	15+S9			0.80	1.11	0.15	
CNTRL	-S9	0.11	0.04	0.115	0.086	0.02	0.04
CNTRL	+S9	0.12	0.09	0.116	0.14	0.07	0.11

1 MG DOSE

COMPOUND	SAMPLE	0-8 HRS Rev./uL Sample	8-24 HRS Rev./uL Sample 1	8-24 HRS Rev/uL Sample 2	24-36 HRS Rev./uL Sample
1-NP	6-S9		0.19	0.19	0.17
1-NP	6+S9		2.48	2.36	1.36
1-NP	7-S9	0.40	0.25		0.17
1-NP	7+S9	1.25	1.40	1.80	1.60
2-NF	29-S9	1.00	0.36	0.24	0.10
2-NF	29+S9	0.90	0.45	0.28	0.25
2-NF	31-S9	0.30	0.28	0.32	
2-NF	31+S9	0.36	0.40	0.31	
3-NF	17-S9	0.59	1.37	0.83	
3-NF	17+S9	0.32	0.54	0.29	
3-NF	19-S9	0.89	0.33	0.30	0.02
3-NF	19+S9	0.39	0.19	0.14	0.09
CNTRL	-S9	0.01	0.04	0.05	0.01
CNTRL	+S9	0.09	0.13	0.12	0.05

TABLE 6

Linear Regression Coefficients
For
Revertant/Dose Plots' Regression Lines

Compound	0-4 hrs	4-8 hrs	8-24 hrs	24-36 hrs	36-48 hrs	Avg L R.coeff.
2 MG DOSE						
1NP 46 -S9						
1NP 46 +S9	0.98	0.99	0.80	1.00	0.98	0.95
1NP 47 -S9		1.00	0.98		0.86	0.95
1NP 47 +S9	0.97	0.99	0.89	0.79	0.66	0.86
2NF 26 -S9		0.96	0.97		0.94	0.96
2NF 26 +S9		0.97	0.96	0.94	0.97	0.96
2NF 27 -S9	0.92		0.98		0.73	0.88
2NF 27 +S9	0.68	0.98	0.99	0.99	0.99	0.93
3NF 14 -S9	0.92	0.98	0.98	0.96	0.87	0.94
3NF 14 +S9	0.93	0.96	0.99		0.91	0.95
3NF 15 -S9	0.53	0.96	0.87	0.93	0.98	0.85
3NF 15 +S9			0.88	0.66		0.77
CTRL -S9	0.63	0.96	0.74	0.16	0.50	0.60
CTRL +S9		0.64	0.64	0.40	0.84	0.63
Avg Coeff.	0.85	0.98	0.94	0.90	0.89	

1 MG DOSE	0-8 HRS	8-24 HRS	24-36 HRS	Avg L.R.coeff.
1NP 6 -S9		0.95	0.81	0.88
1NP 6 +S9		0.95	0.92	0.94
1NP 7 -S9	0.99	0.85	0.81	0.88
1NP 7 +S9	0.98	0.93	0.92	0.94
2NF 29 -S9	0.99	0.96	0.60	0.85
2NF 29 +S9	0.99	0.91	0.92	0.94
2NF 31 -S9	0.98	0.95		0.97
2NF 31 +S9	0.98	0.95		0.97
3NF 17 -S9	0.96	0.92		0.94
3NF 17 +S9	0.97	0.95		0.96
3NF 19 -S9	0.93	0.90	0.26	0.70
3NF 19 +S9	0.98	0.86	0.65	0.83
CNTRL -S9	0.15	0.25	0.33	0.24
CNTRL +S9	0.74	0.80	0.33	0.62
Avg L.R.Coefficient	0.97	0.92	0.74	

Overall Avg L.R. Coeff.= 0.90

TABLE 7

REVERTANTS/HOUR RATE FOR ALL SAMPLES

2 MG DOSE

Hrs	1-NP 46-S9	1-NP 46+S9	1-NP 47-S9	1-NP 47+S9	1-NP COMB-S9	1-NP COMB+S9	CNTRL-S9	CNTRL+S9
0	0	0	0	0	0	0	0	0
4					120.5	554.38	13.25	15
8					658.88	2069.25	10.5	27
24	515.63	1351.08	774.72	1391.39	645.17	1371.23	20.73	26.4
36				146.46	58.23	145.21	3.44	16.04
48		187.08	31.25	223.75	51.25	205.42	18.33	50.42

Hrs	2-NFA 26-S9	2-NFA 26+S9	2-NFA 27-S9	2-NFA 27+S9	3-NFA 14-S9	3-NFA 14+S9	3-NFA 15-S9	15+S9
0	0	0	0	0	0	0	0	0
4	155.5	103.75			849.25	427.5	86.13	
8	61.75	147.25		633	1126.38	631.75	2435.13	
24	88.78	318.6	302.71	607.58	506.15	194.1	757.29	421.26
36		179.79		118.13	89.06		59.81	21.46
48	35.67	274.58	63.33	197.5	116.67	22.92	107.33	

1 MG DOSE

Hrs	1-NP 6-S9	1-NP 6+S9	1-NP 7-S9	1-NP 7+S9	2-NFA 29-S9	2-NFA 29+S9	CNTRL+S9
0	0	0	0	0	0	0	0
8			195.25	608.88	235.25	197.63	16.13
24	25.04	468.67	49	302	153	160.13	46.69
32	11.67	97.33	11.67	117.33	23.13	46.5	16
24	45.88	689.5		402	99.35	81.96	

Hrs	2-NFA 31-S9	2-NFA 31+S9	3-NFA 17-S9	3-NFA 17+S9	3-NFA 19-S9	3-NFA 19+S9	CNTRL-S9
0	0	0	0	0	0	0	0
8	71.5	73.88	108.38	43.88	392.69	156.94	2.25
24	81.03	87.78	350.41	95.44	93.14	12.7	15.38
32					0	0	2.5
24	96.5	56.84	206.97	28.23	84.94	0	

V. DISCUSSION

Investigating *in vivo* metabolism via a small scale study is inherently prone to individual animal variations. Averaging only two values is insufficient data to do statistical analysis, thus a graphical range method was used to provide the most reliable and understandable presentation. Animals do not consistently urinate which generates large standard deviations when averaging single collection periods although over the entire collection period individual rat's results are similar. Two rats injected with 2 mg of 3-NFA produced revertant excretion amounts within 25% of each other, but during specific collection periods they varied up to 50%. The outcome is an overall error range of 50%, double the actual difference. Accordingly more value may be placed on the best fit lines than normally expected.

V. A. Urinary Mutagenicity

In the first 48 hours after injection, 1-NP injected rats' cumulative urinary mutagenicity was twice 3-NFA's direct mutagenicity, five times 3-NFA's indirect mutagenicity, and triple 2-NFA's indirect mutagenicity. Estimated total revertant amounts excreted by 1-NP injected rats remained the highest although matched by the upper range of 2-NFA injected rat's total amount while 3-NFA injected rat's amount remained equal to 2-NFA rat's median mutagenicity amount, as shown in Fig. 16.

3-NFA injected rat's maximum urinary mutagenicity excretion rate occurred in the initial phases 4-10 hrs following injection, while 1-NP injected rat's occurred slightly after during the 7-12 hrs following injection, and 2-NFA injected rats had the slowest and most consistent rate lasting a full day. This coincides with previous research results showing peak urinary mutagenicity from 1-NP metabolism occurs in the 8 - 12 hr period after injection and is dependent on S9 activation (Ball, *et al* 84; Ball & King, 85). Representing the middle of the road, 1-NP's kinetics are bordered above by 3-NFA's fast kinetic rates and below by 2-NFA's slow kinetic rates. All three compounds had similar Ka constants suggesting the Ka measured may have been also representative of the acetone carrier rather than just the compounds' structure.

Stocking (89) suggested 2-NFA to be less mutagenic than 3-NFA due to its slower and inefficient bacterial metabolism. Indeed, 2-NFA's slower metabolism generates a slower mutagenic production rate, however, the duration of active mutagenic urinary metabolites being excreted is much longer than for 1-NP. The result being a total revertant output potentially as large as 1-NP and at least as large as 3-NFA's amount.

V. B. Kinetic Relationship to Mutagenicity

The resulting mutagenicity of each compound may be directly related to the reciprocal dependency between the metabolism process and the kinetics involved. 1-NP has been described in the Literature Review

as relying strongly on nitroreduction and enterohepatic circulation for acetylation in the liver. Our study's results show urinary mutagenicity from 1-NP metabolism behaved as establish by several preceding studies (Ball, *et al* 84; Ball and King, 85; Bond, *et al* 85; Howard, *et al* 85) and thus underwent predicted pathways and kinetics.

The revertant excretion rate plot profile and this study's analysis strongly suggest the overall excretion of 2-NFA's mutagenic metabolites is significantly slower than 1-NP's and apparently slower than 3-NFA's. *In vitro* research has shown 3-NFA is metabolized in 6 hrs while 2-NFA takes up to 24 hrs (Stocking, 89). 2-NFA's slower metabolism may be counterbalanced by its longer biological half life and subsequent increased probability of metabolism. The longer half life may result from slow partitioning of 2-NFA and its metabolites *in vivo* possibly due to structural differences discussed later. Stocking (89) found 3-NFA to efficiently produce only 3-AFA via nitroreduction, while 2-NFA was reduced to 2-AFA which could undergo acetylation to 2-NAAFA. 2-NFA's longer biological half life and greater opportunity for metabolism may increase its ability to form both 2-AFA and its metabolite 2-NAAFA. 2-NAAFA may cause the indirect acting mutagenicity found from 2-NFA metabolism similar to 1-NAAP production from 1-NP. 2-AFA, like its isomer 3-AFA, may be responsible for the direct acting mutagenicity.

Moreover, 3-NFA had the fastest revertant excretion rate averaging 40% greater than 1-NP's rate and 400% faster than 2-NFA's rate. This high revertant excretion rate for 3-NFA results may be indicative of two situations. There may have been insufficient enterohepatic circulation of the compound for acetylation metabolism to produce the secondary mutagen species exhibited by 1-NP and 2-NFA. The second and more probable situation is acetylation of 3-NFA may simply not produce an active mutagenic species. Ball (*et al* 85; *et al* 86) did show further metabolism of 3-NFA's metabolites *in vitro* detoxifies them, as shown even in this study where the exogenous metabolism by S9 halved their total mutagenicity. 3-NFA's high mutagenicity excretion rate during the first 8 hrs was comparable to 1-NP's rate. 1-NP is known to produce phenolic and dihydrodiol metabolites during initial hours of excretion (Ball *et al* 84; Ball *et al* 85). 3-NFA mutagenic species' immediate excretion and direct acting mutagenicity correlates with *in vitro* results showing 3-NFA relies heavily on O-esterification producing epoxide intermediates (Zielinska, *et al* 87; Consolo, *et al* 89). This may be evidence the initial and majority of its mutagenic urinary metabolites are phenols and dihydrodiols. 3-NFA mutagenic metabolites' fast excretion rate with inactivation of mutagenicity by further metabolism, as seen with S9, also complies with *in vitro* research (Howard, *et al* 88; Consolo, *et al* 89) discussed earlier in the Literature Review.

3-NFA metabolites may be excreted at a rate similar to 2-NFA, but detoxification by further metabolism allows the revertant excretion rate to provide a misconception of fast excretion kinetics. Indeed *in vitro* results suggested 3-NFA to be more mutagenic than both 2-NFA and 1-NP (Stocking, 89; Consolo, *et al* 89). Considering that 3-NFA's cumulative mutagenicity could be only half of 2-NFA's amount, it is probable the revertant excretion rate actually represents the detoxifying metabolism rate of 3-NFA's mutagenic metabolites as they enter the enterohepatic circulation and undergo additional metabolism. Without the ability to account for the total dose excreted this theory can not be sufficiently evaluated.

The differing excretion kinetics may signify differing substrate binding affinities of 2-NFA and 3-NFA. The binding may be stronger for 3-NFA promoting metabolism or be stronger for 2-NFA delaying metabolism and lengthening the biological half life of mutagens or their precursors. This complements Stocking's (89) second implication that the NFAs' nitro-groups may structurally affect nitroreductase's availability to the active site according to Vance and Levin's (84) research. Whether the mechanism is to catalyze nitroreduction, catalyze esterification, or act as a competitor remains obscured. This structural theory may also be relevant to the metabolism mechanism if acetylation of 3-NFA truly does not produce active mutagenic metabolites and acetylation of 2-NFA does result in mutagenic species.

V. C. Dose Relationship to Mutagenicity

All three compounds appear to exhibit a nonproportional rise between indirect acting mutagens (S9 activated) and direct acting mutagens with increasing dose. The general observation is the predominant mutagenic metabolites of each specific compound received a disproportionate increase with increasing dose. This observation is not caused by assay bias but appears to be truly dose dependent since the nonproportional increase occurred to 3-NFA's direct acting mutagenic metabolites and 1-NP's and 2-NFA's indirect acting mutagens. Doubling the 2-NFA dose seems to increase the indirect acting mutagens more. Stocking's (89) suggested a saturable process existed in the bacterial strains metabolism of 2-NFA which may or may not be related to this apparent event *in vivo*. A second possibility is the higher dose allows a higher probability of enterohepatic circulation of products. This would account for 2-NFA's equal quantities of indirect and direct mutagens initially with the low dose but, then with increased dose an increased percentage of compound undergoing reduction and acetylation seemingly produces more indirect mutagenicity.

I suggest 2-NFA and 3-NFA are truly similar in their metabolism pathways and mass excretion rates. Their main difference seems to be in the metabolism mechanism and kinetics which allows production of the potent NAAF metabolites by nitroreduction and acetylation. 3-NFA's initial oxidative metabolites are easily detoxified by additional

metabolism while 2-NFA, like 1-NP, produces a variety of mutagenic species by additional metabolism introduced by the enterohepatic circulation. Contrary to *in vitro* research, 2-NFA appears to present a higher mutagenicity dose *in vivo* similar to 1-NP while 3-NFA provides a comparatively small *in vivo* mutagen dose due to the *in vivo* metabolism system's detoxifying action.

V. D. Conclusions

This preliminary comparison of urinary mutagenicity of 1-NP, 2-NFA, and 3-NFA provides strong evidence:

1. Not all nitro-PAHs are metabolized to similar mutagenic species
2. Revertant excretion rates of 3-NFA > 1-NP >> 2-NFA
3. Cumulative urinary mutagenicity from 1-NP $\geq$ 2-NFA $\geq$ 3-NFA
4. 3-NFA urinary mutagenicity is direct acting and decreased by additional metabolism.
5. 1-NP, 2-NFA, and 3-NFA urinary mutagen excretion is formation rate limited.

V. E. Recommendations For Future Research

The mutagenicity dependence on kinetics seen in this study by 2-NFA and 3-NFA has been difficult to analyze completely. Elucidation of these kinetics is needed to better understand not only how mutagenic is a compound but, its actual *in vivo* mutagen dose. Future experiments should be geared to thoroughly study and compare structural, *in vitro*, and any other *in vivo* studies to fully grasp any relationship related to their kinetic mechanisms. Researchers continuing the risk assessment of nitro-PAHs may next identify the nitro-PAH characteristics controlling the metabolism pathways and resulting biological exposure to mutagens.

I recommend repeating this study using radiolabled compounds at 1 mg to 3 mg range doses, to track and identify the urinary metabolites and their relationship in metabolism/excretion kinetics. Better metabolite excretion rate plots are necessary requiring extension of the collection period out to three days with at least four animals per dose per chemical. Collection of urine during the initial hrs after injection at three 4 hour intervals or two 6 hour intervals will better characterize the absorption/formation part of the excretion rate plot. The compound radiolabels should quantify urinary metabolite amounts

along with mutagenicity to better characterize potency. The percentages of the initial dose of parent compound excreted over time should allow investigation into the relationship between metabolism and resulting mutagenicity and the associated kinetics. The primary metabolites of 3-NFA and 2-NFA responsible for the direct and indirect mutagenicity need to be identified and their formation rates determined.

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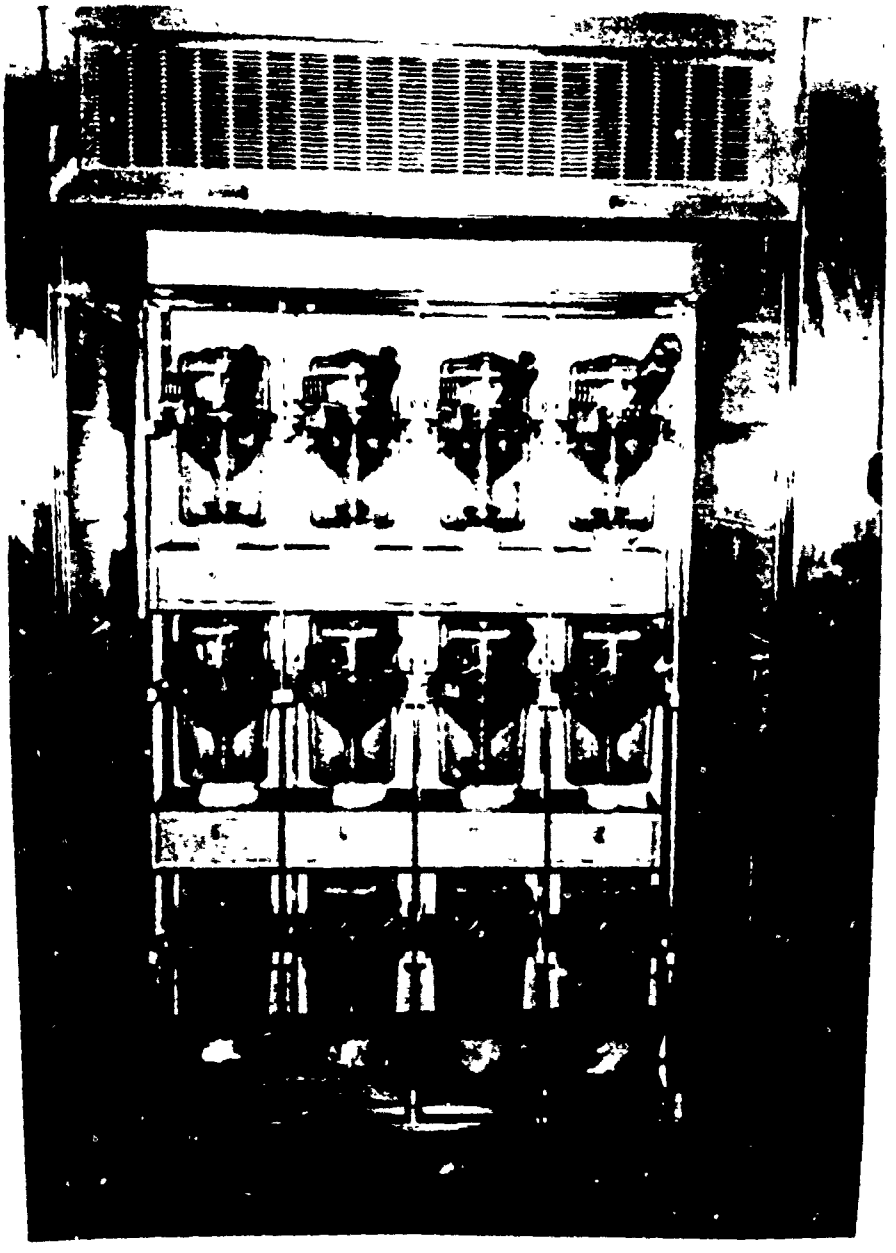

Figure 18. Metabolism cage and dry ice box apparatus for collection and immediate freezing of the rat urine. Located in isolation booth in Perryhill Bldg, UNC.

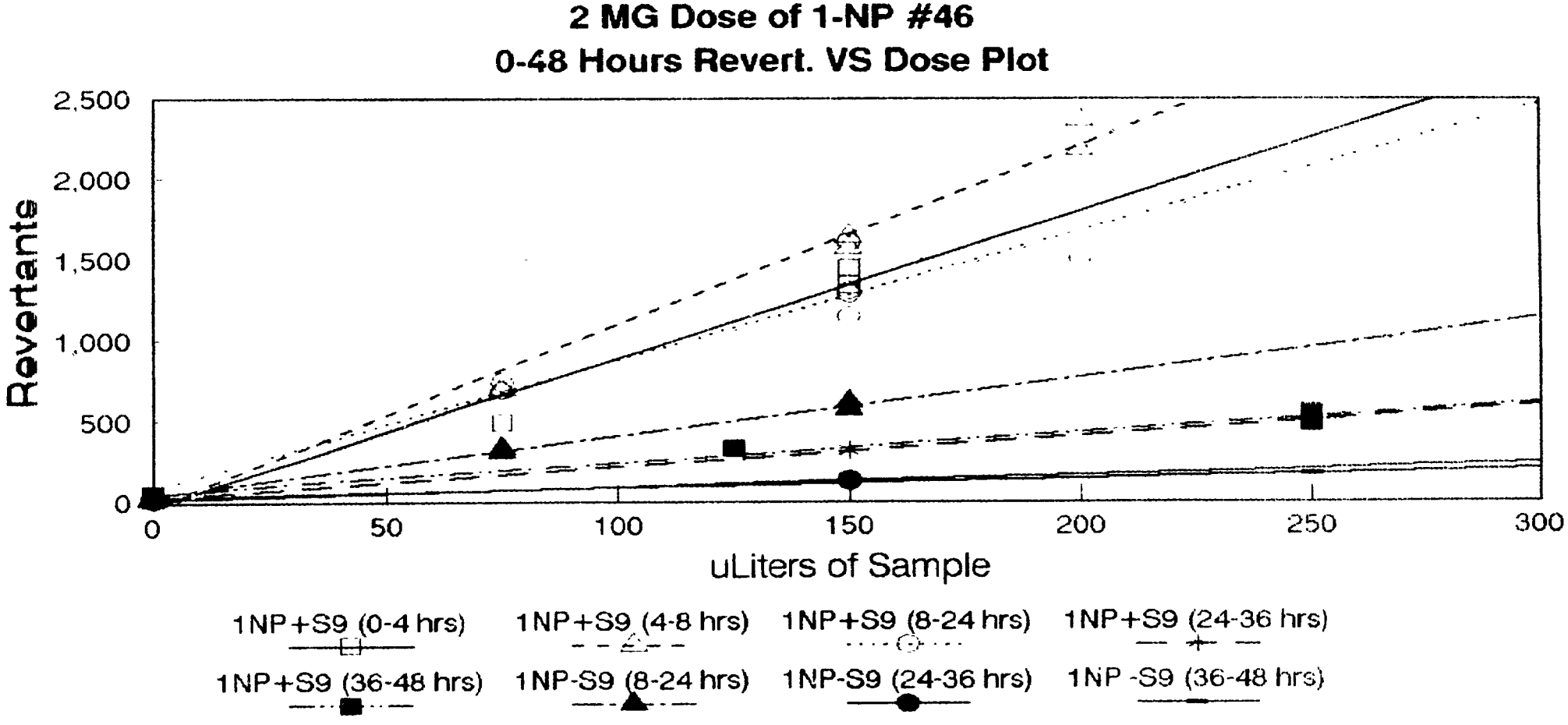
2 MG Dose of 1-NP #46
0-48 Hours Revert. VS Dose Plot
Revertants
2,500
2,000
1,500
1,000
500
0
0
50
100
150
200
250
300
uLiters of Sample
1NP+S9 (0-4 hrs)
1NP+S9 (4-8 hrs)
1NP+S9 (8-24 hrs)
1NP+S9 (24-36 hrs)
1NP+S9 (36-48 hrs)
1NP-S9 (8-24 hrs)
1NP-S9 (24-36 hrs)
1NP -S9 (36-48 hrs)

Figure 19

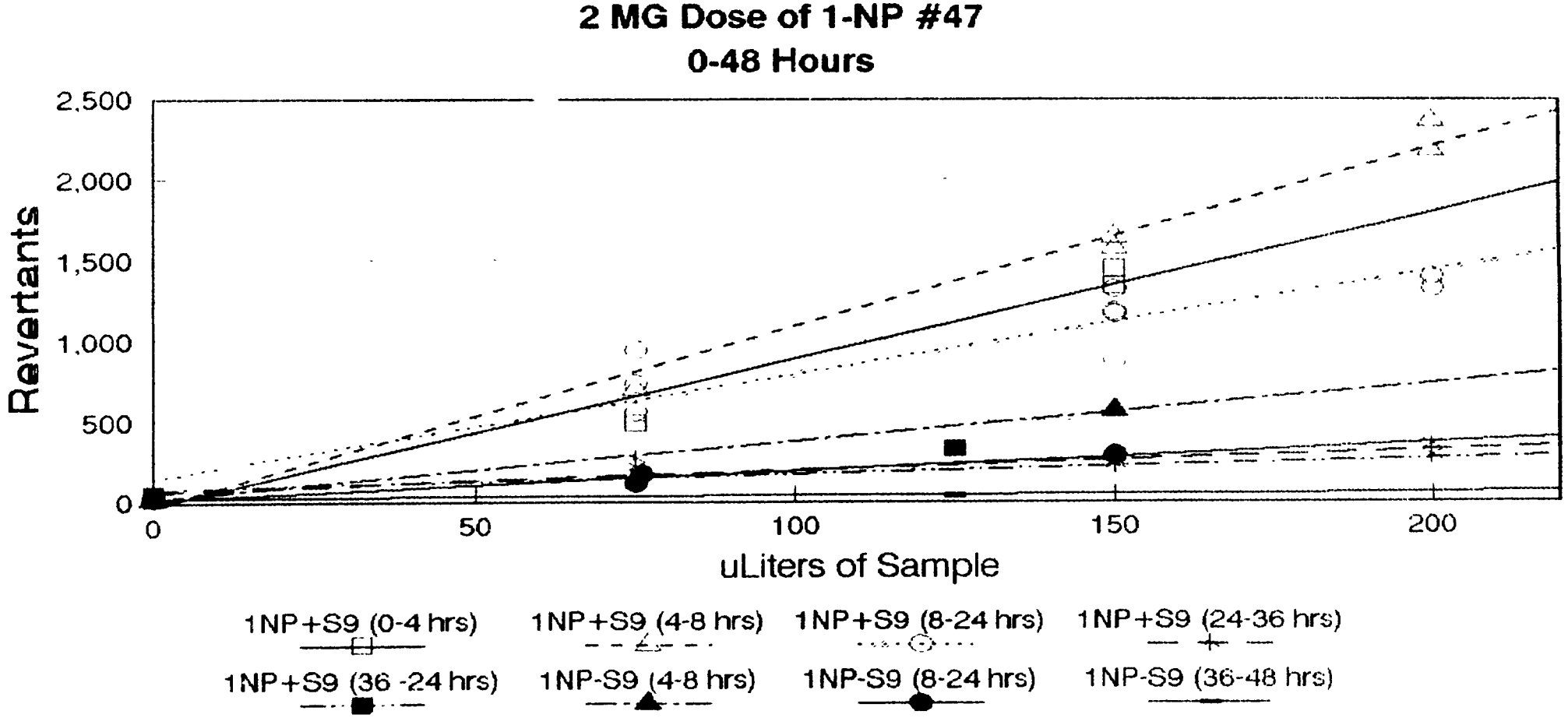
2 MG Dose of 1-NP #47
0-48 Hours
Revertants
2,500
2,000
1,500
1,000
500
0
0
50
100
150
200
uLiters of Sample
1NP+S9 (0-4 hrs)
1NP+S9 (4-8 hrs)
1NP+S9 (8-24 hrs)
1NP+S9 (24-36 hrs)
1NP+S9 (36 -24 hrs)
1NP-S9 (4-8 hrs)
1NP-S9 (8-24 hrs)
1NP-S9 (36-48 hrs)

Figure 20

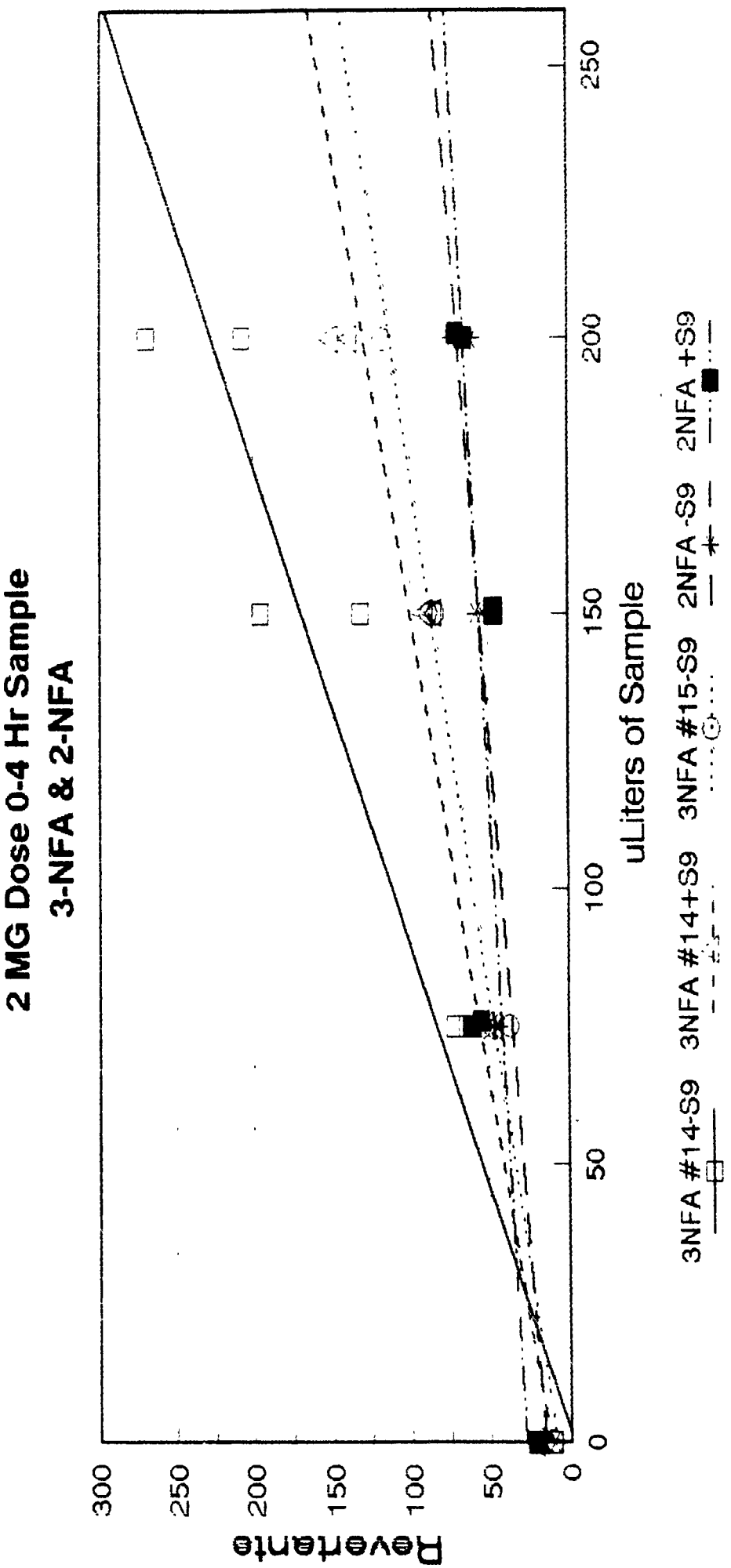
2 MG Dose 0-4 Hr Sample
3-NFA & 2-NFA
Revertants
uLiters of Sample
0
50
100
150
200
250
300
3NFA #14-S9
3NFA #14+S9
3NFA #15-S9
2NFA -S9
2NFA +S9

Figure 21

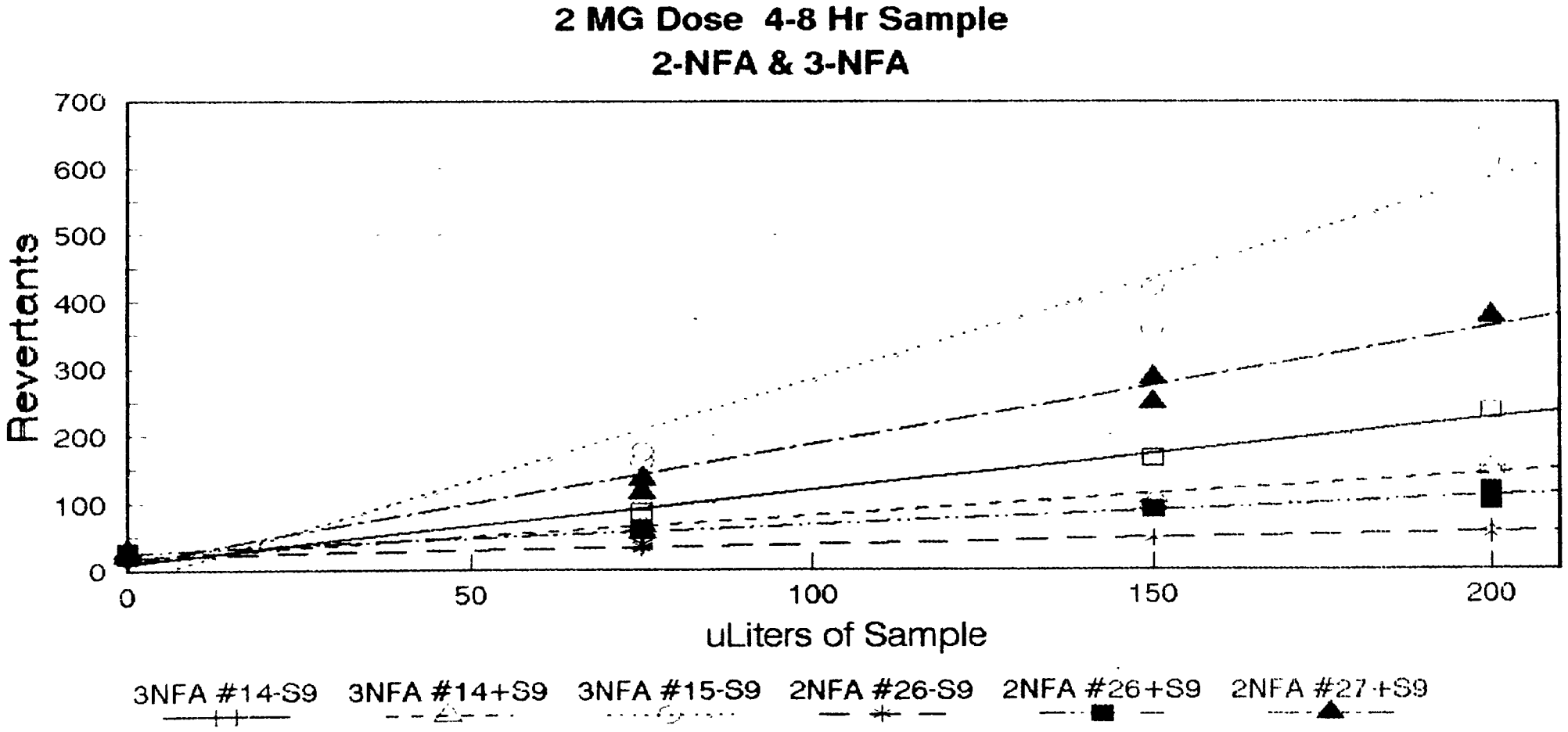
2 MG Dose 4-8 Hr Sample
2-NFA & 3-NFA
Revertants
700
600
500
400
300
200
100
0
0
50
100
150
200
uLiters of Sample
3NFA #14-S9
3NFA #14+S9
3NFA #15-S9
2NFA #26-S9
2NFA #26+S9
2NFA #27+S9

Figure 22

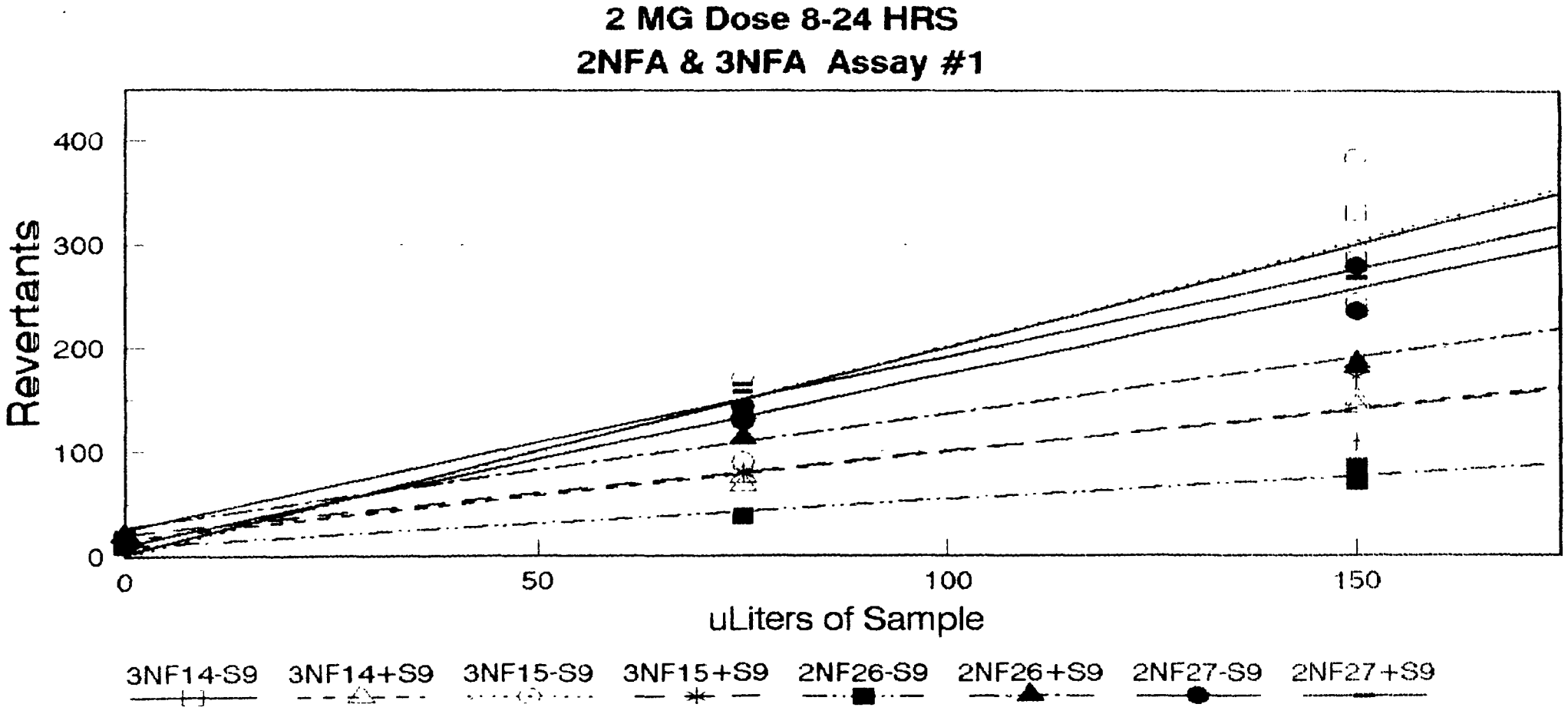
2 MG Dose 8-24 HRS
2NFA & 3NFA Assay #1
Revertants
400
300
200
100
0
0
50
100
150
uLiters of Sample
3NF14-S9
3NF14+S9
3NF15-S9
3NF15+S9
2NF26-S9
2NF26+S9
2NF27-S9
2NF27+S9

Figure 23

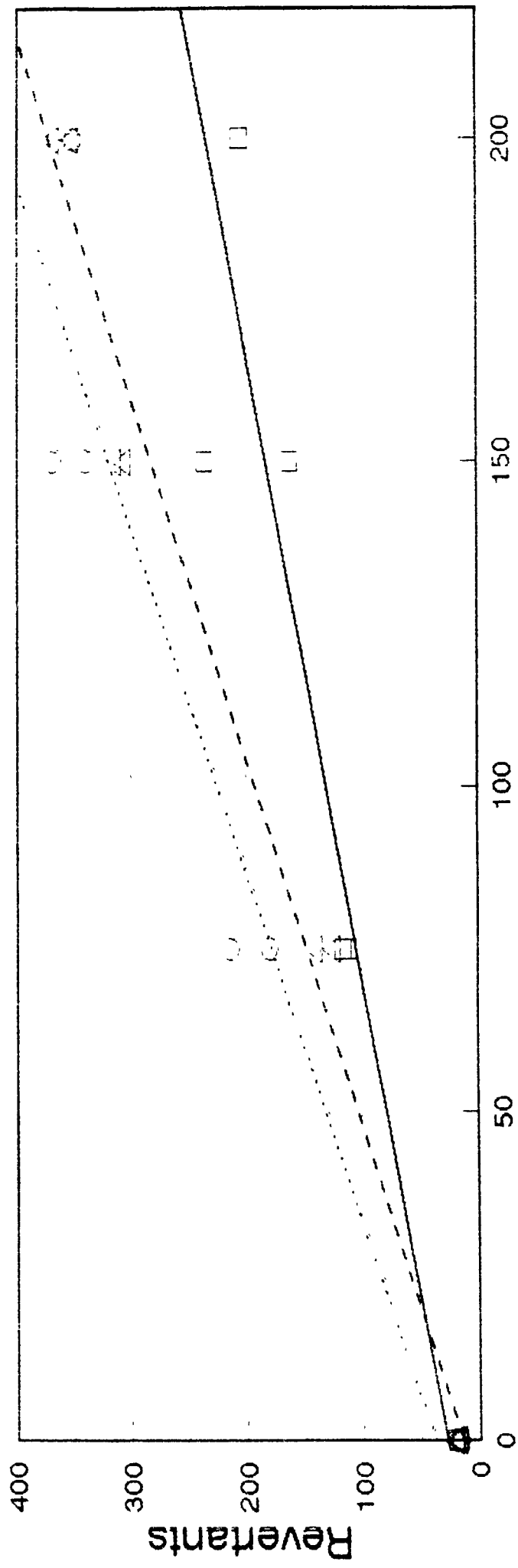
2 MG Dose 8-24 Hr Sample
2-NFA & 3-NFA Assay #2 Revertant vs Dose Graph
Revertants
400
300
200
100
0
0
50
100
150
200
uLiters of Sample
3NFA #14-S9
3NFA #15-S9
2NFA #27-S9

Figure 24

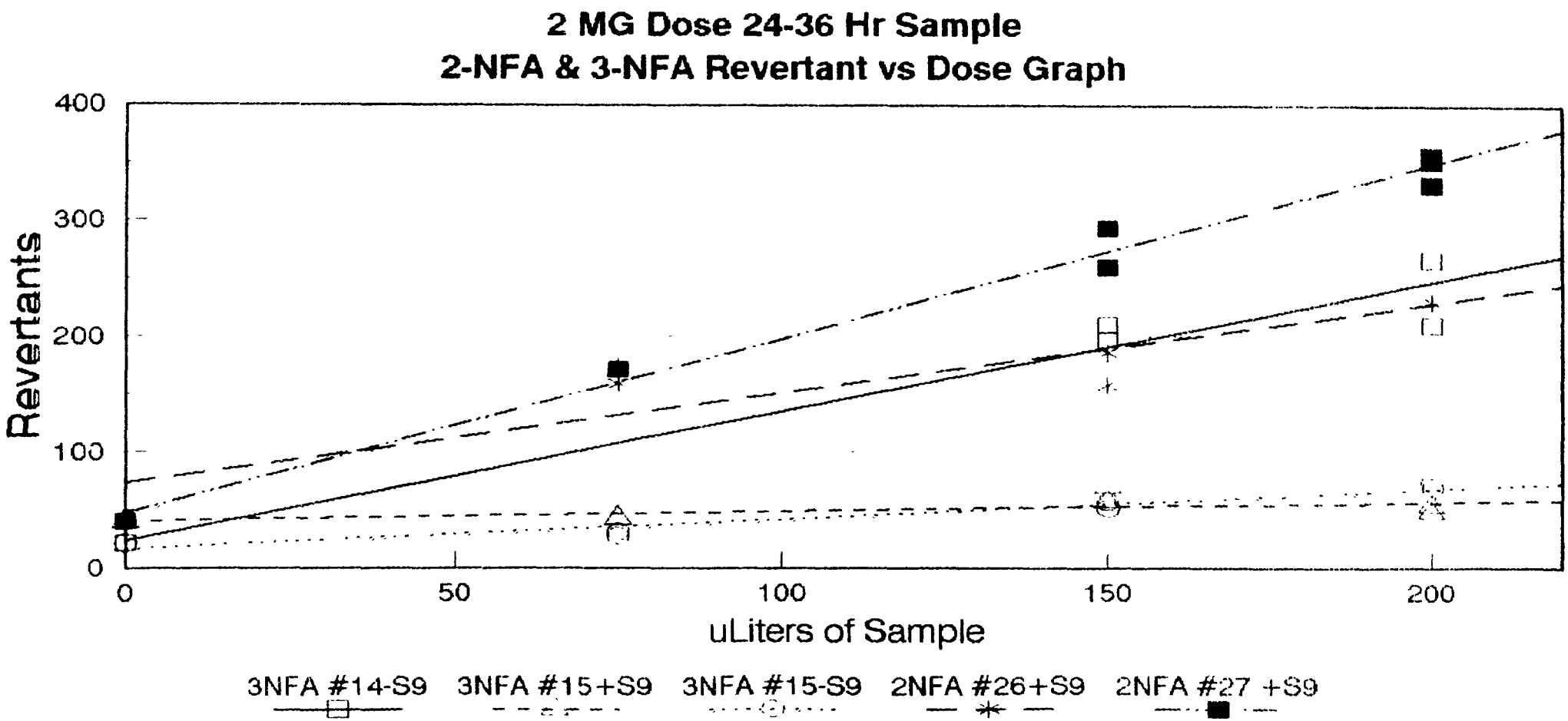
2 MG Dose 24-36 Hr Sample
2-NFA & 3-NFA Revertant vs Dose Graph
Revertants
400
300
200
100
0
0
50
100
150
200
uLiters of Sample
3NFA #14-S9
3NFA #15+S9
3NFA #15-S9
2NFA #26+S9
2NFA #27 +S9

Figure 25

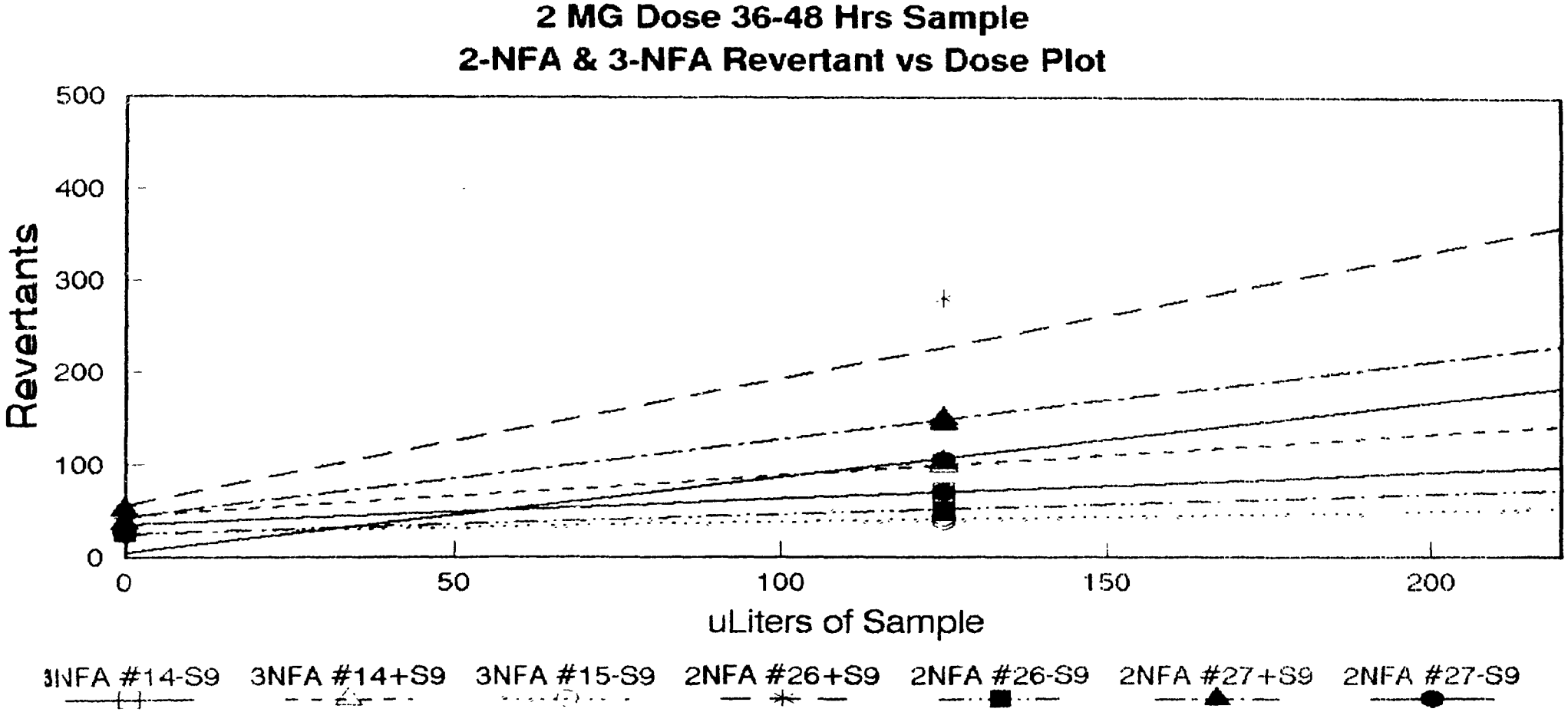
2 MG Dose 36-48 Hrs Sample
2-NFA & 3-NFA Revertant vs Dose Plot
Revertants
500
400
300
200
100
0
0
50
100
150
200
uLiters of Sample
3NFA #14-S9
3NFA #14+S9
3NFA #15-S9
2NFA #26+S9
2NFA #26-S9
2NFA #27+S9
2NFA #27-S9

Figure 26

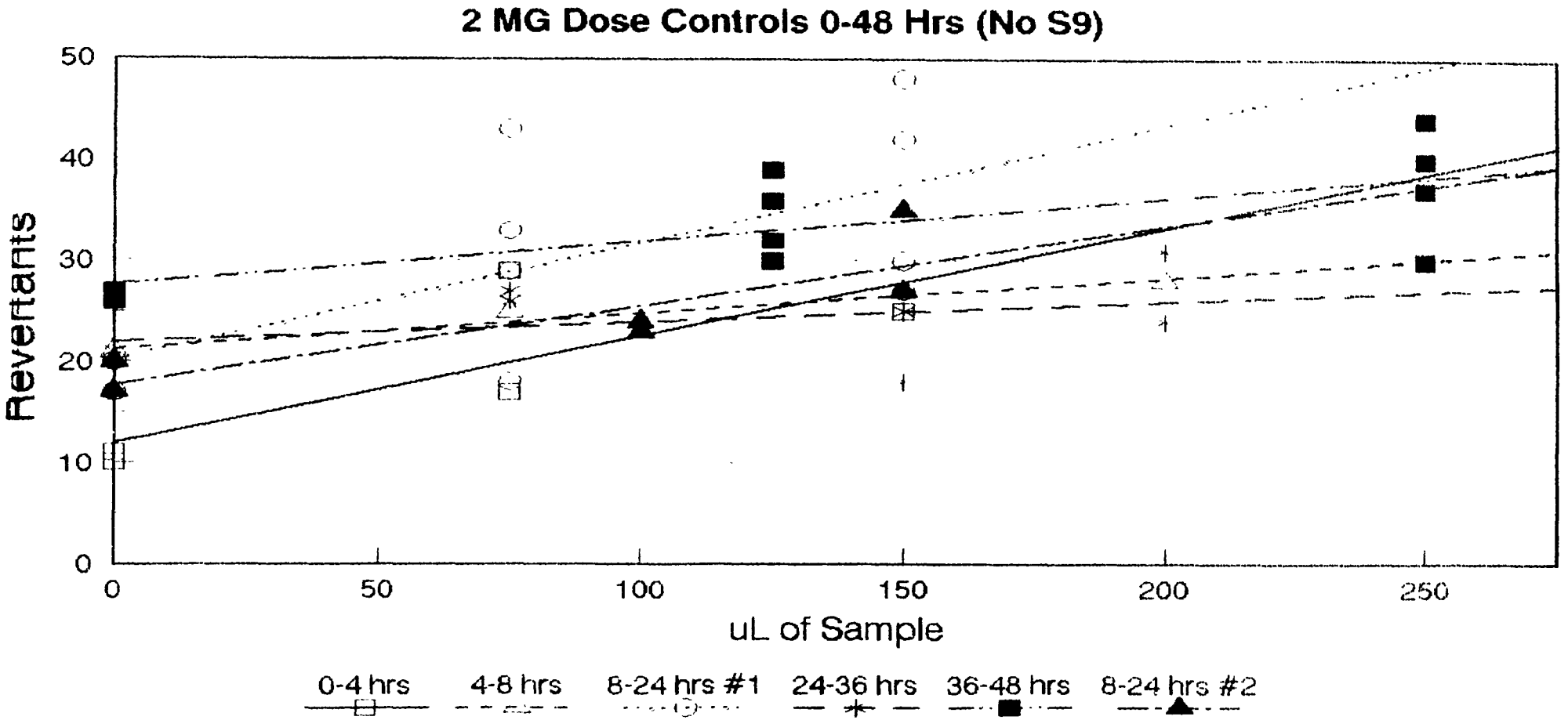
2 MG Dose Controls 0-48 Hrs (No S9)
Revertants
50
40
30
20
10
0
0
50
100
150
200
250
uL of Sample
0-4 hrs
4-8 hrs
8-24 hrs #1
24-36 hrs
36-48 hrs
8-24 hrs #2

Figure 27

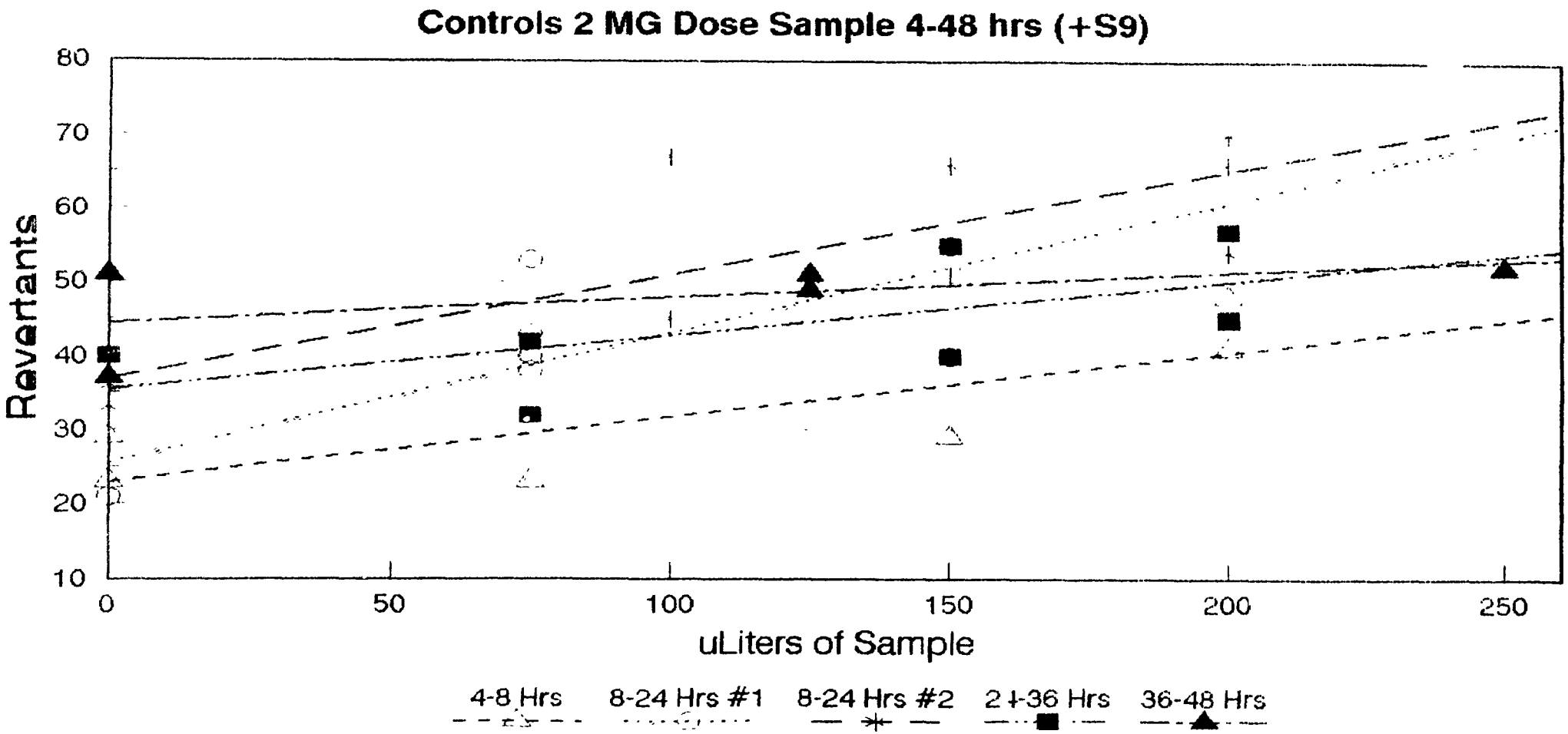
Controls 2 MG Dose Sample 4-48 hrs (+S9)
Revertants
80
70
60
50
40
30
20
10
0
50
100
150
200
250
uLiters of Sample
4-8 Hrs
8-24 Hrs #1
8-24 Hrs #2
24-36 Hrs
36-48 Hrs

Figure 28

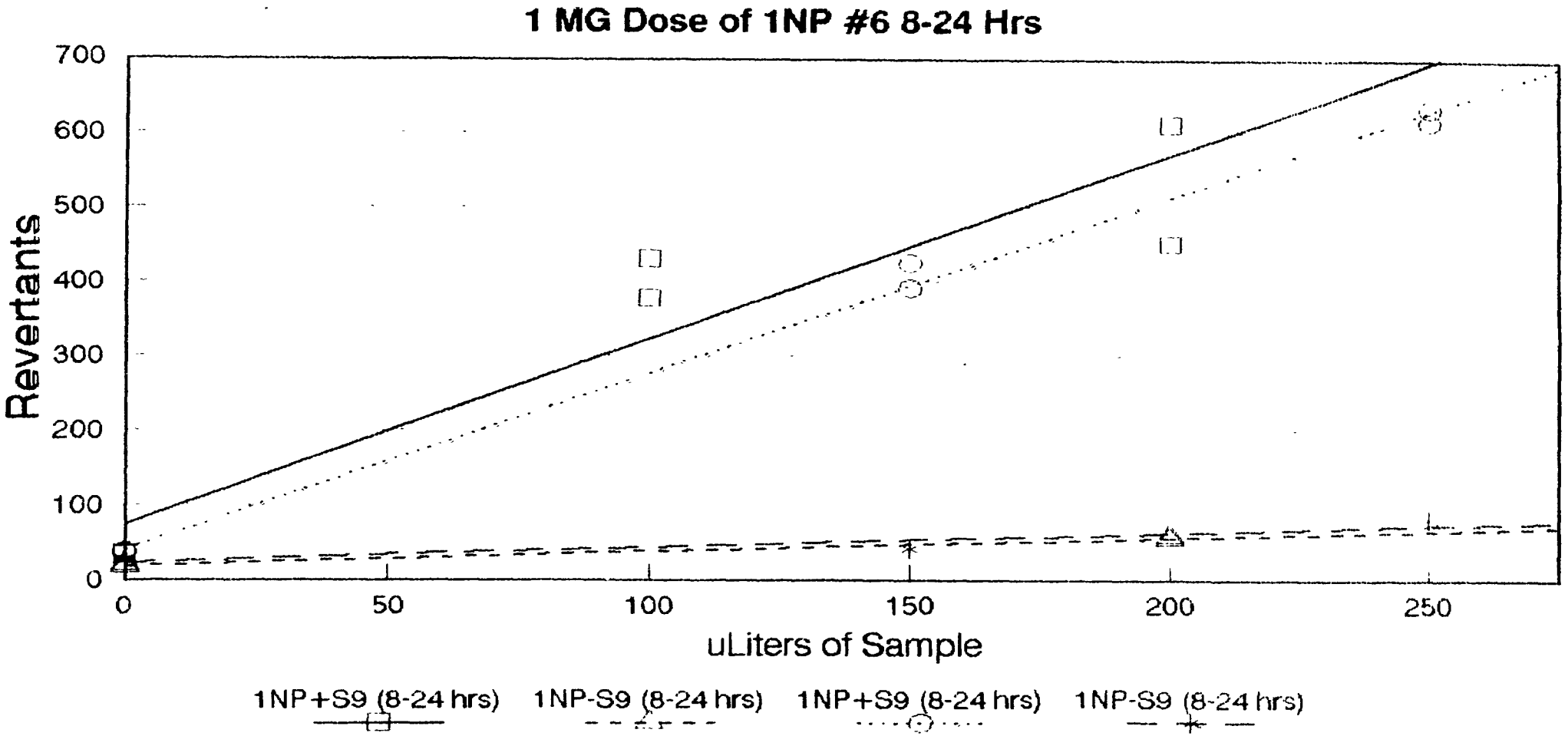
1 MG Dose of 1NP #6 8-24 Hrs
Revertants
700
600
500
400
300
200
100
0
0
50
100
150
200
250
uLiters of Sample
1NP+S9 (8-24 hrs)
1NP-S9 (8-24 hrs)
1NP+S9 (8-24 hrs)
1NP-S9 (8-24 hrs)

Figure 29

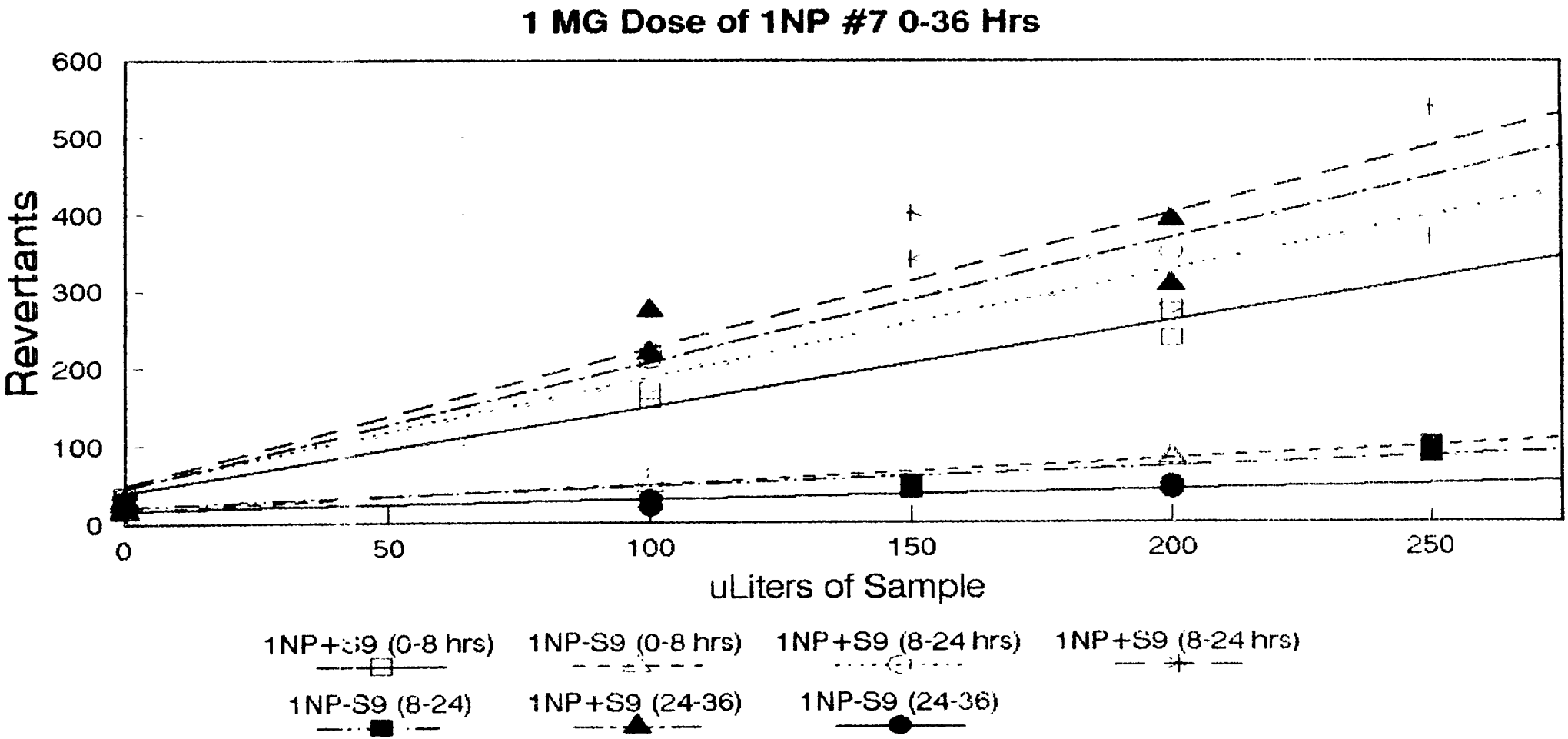
1 MG Dose of 1NP #7 0-36 Hrs
Revertants
600
500
400
300
200
100
0
0
50
100
150
200
250
uLiters of Sample
1NP+S9 (0-8 hrs)
1NP-S9 (0-8 hrs)
1NP+S9 (8-24 hrs)
1NP+S9 (8-24 hrs)
1NP-S9 (8-24)
1NP+S9 (24-36)
1NP-S9 (24-36)

Figure 30

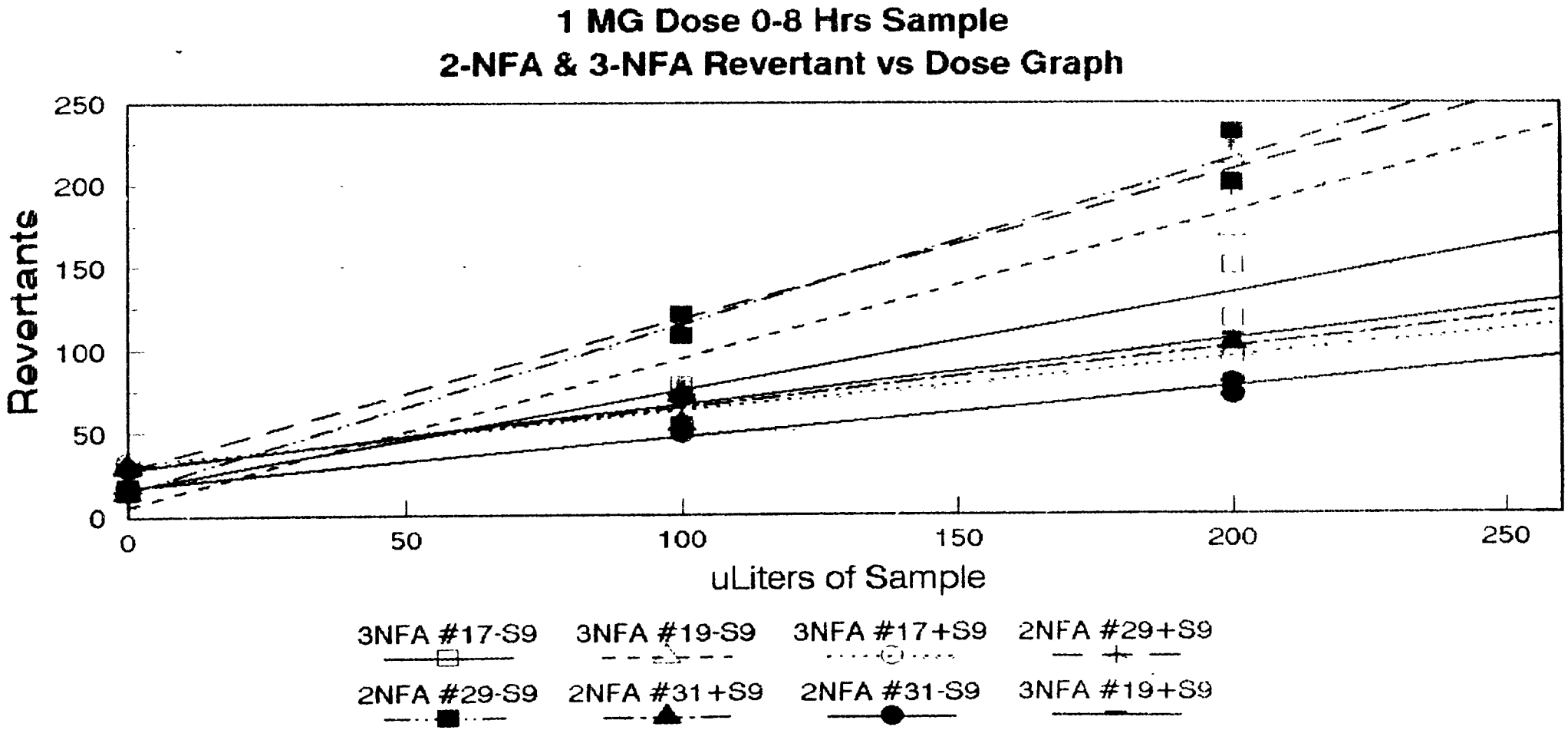
1 MG Dose 0-8 Hrs Sample
2-NFA & 3-NFA Revertant vs Dose Graph
Revertants
250
200
150
100
50
0
0
50
100
150
200
250
uLiters of Sample
3NFA #17-S9
3NFA #19-S9
3NFA #17+S9
2NFA #29+S9
2NFA #29-S9
2NFA #31+S9
2NFA #31-S9
3NFA #19+S9

Figure 31

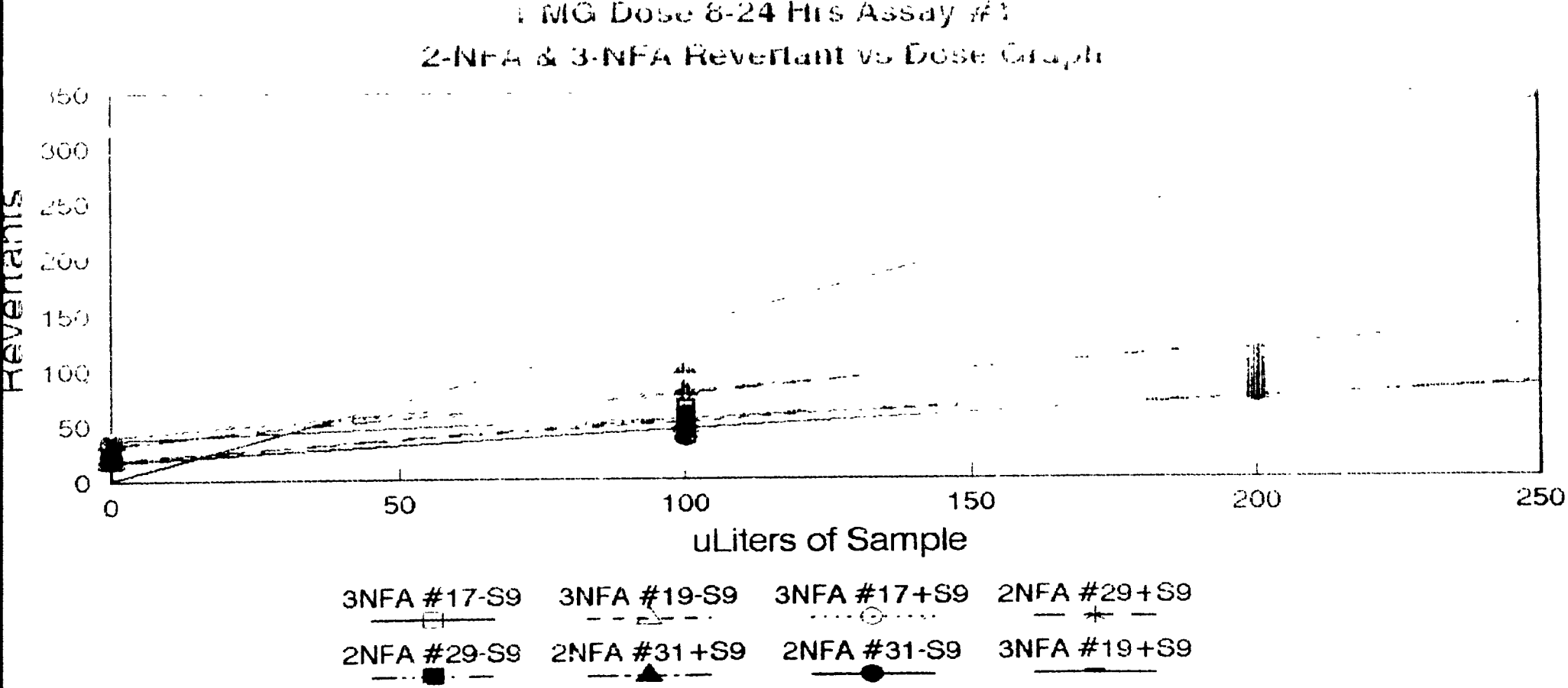
1 MG Dose 8-24 Hrs Assay #1
2-NFA & 3-NFA Revertant vs Dose Graph
Revertants
350
300
250
200
150
100
50
0
0
50
100
150
200
250
uLiters of Sample
3NFA #17-S9
3NFA #19-S9
3NFA #17+S9
2NFA #29+S9
2NFA #29-S9
2NFA #31+S9
2NFA #31-S9
3NFA #19+S9

Figure 32

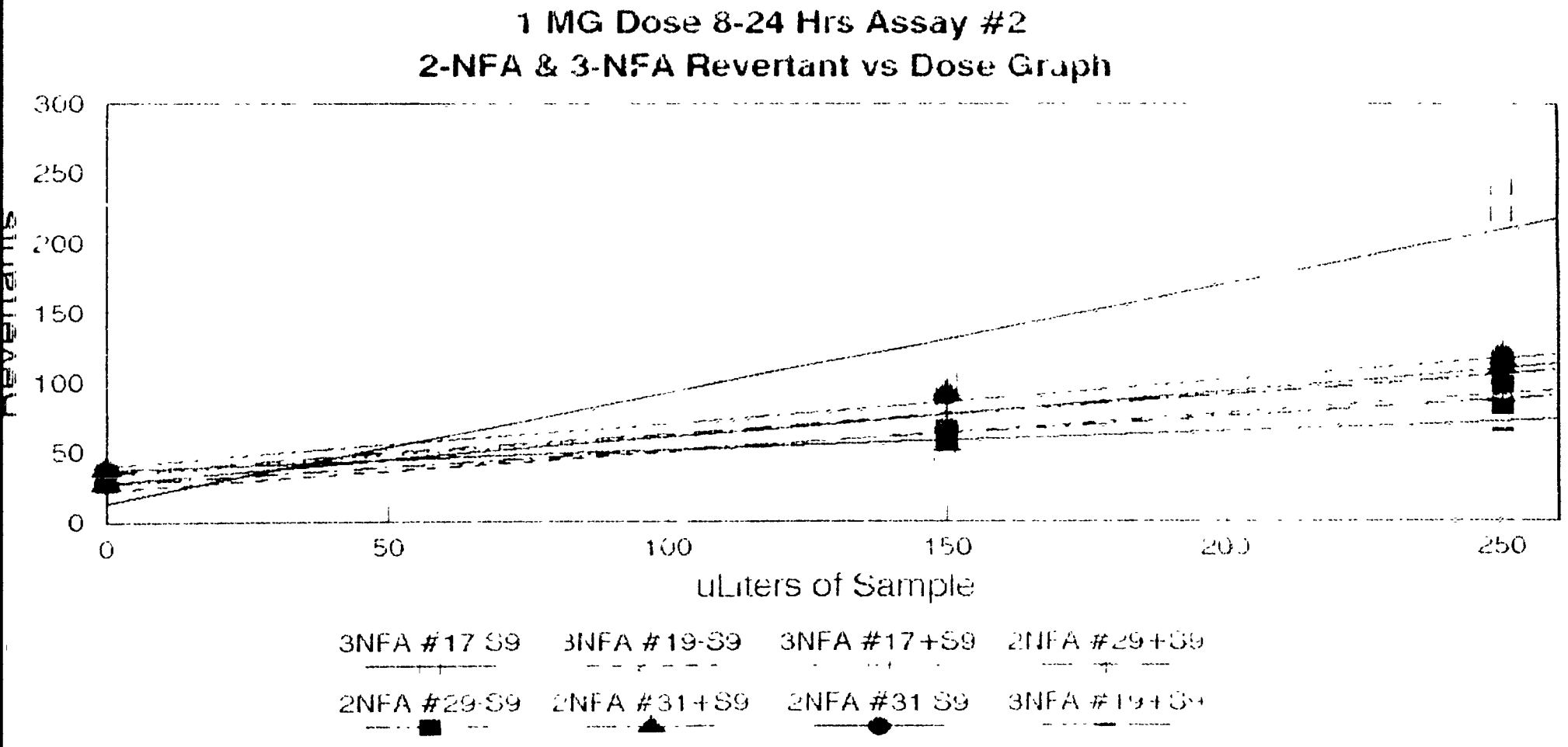
1 MG Dose 8-24 Hrs Assay #2
2-NFA & 3-NFA Revertant vs Dose Graph
Revertants
300
250
200
150
100
50
0
0
50
100
150
200
250
uLiters of Sample
3NFA #17 S9
3NFA #19-S9
3NFA #17+S9
2NFA #29+S9
2NFA #29-S9
2NFA #31+S9
2NFA #31 S9
3NFA #19+S9

Figure 33

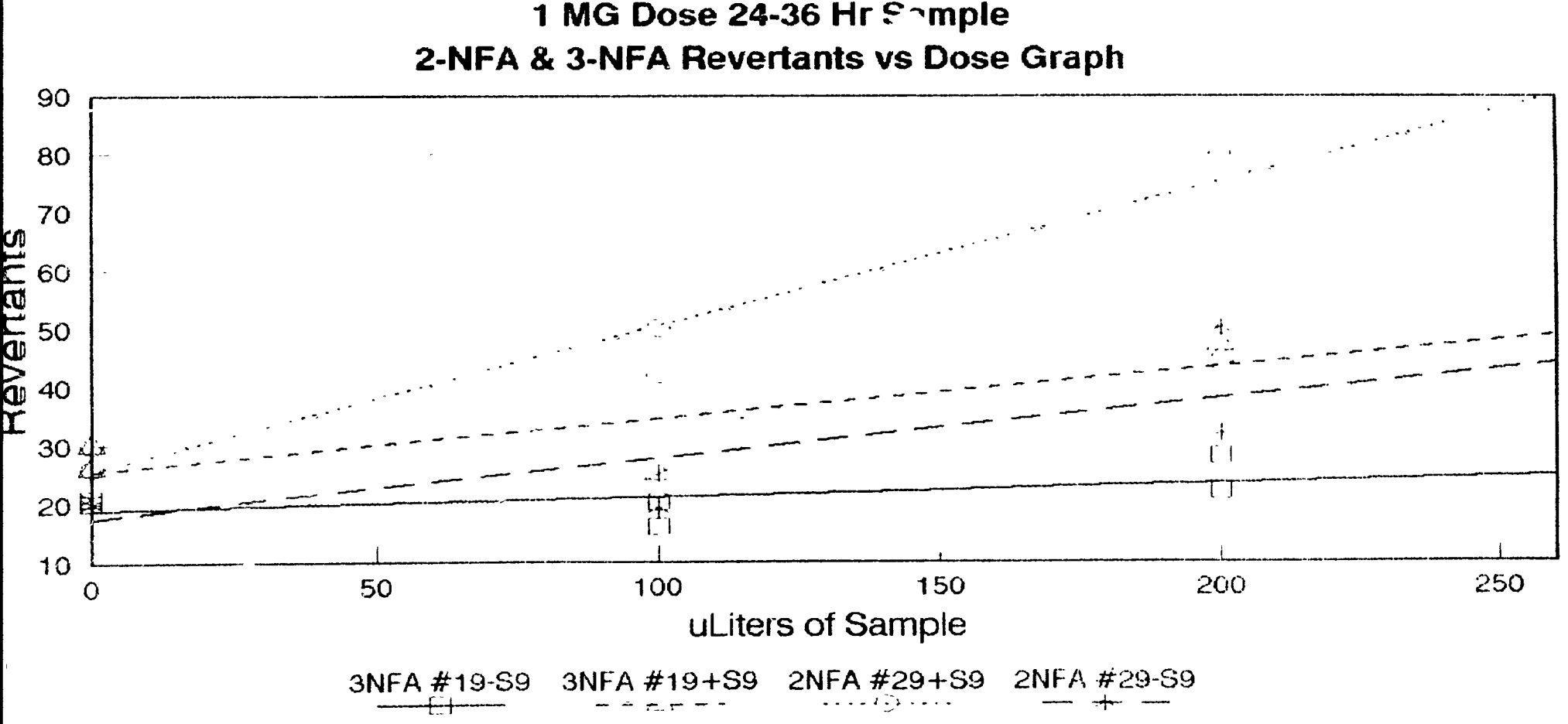
1 MG Dose 24-36 Hr Sample
2-NFA & 3-NFA Revertants vs Dose Graph
Revertants
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
uLiters of Sample
3NFA #19-S9
3NFA #19+S9
2NFA #29+S9
2NFA #29-S9

Figure 34

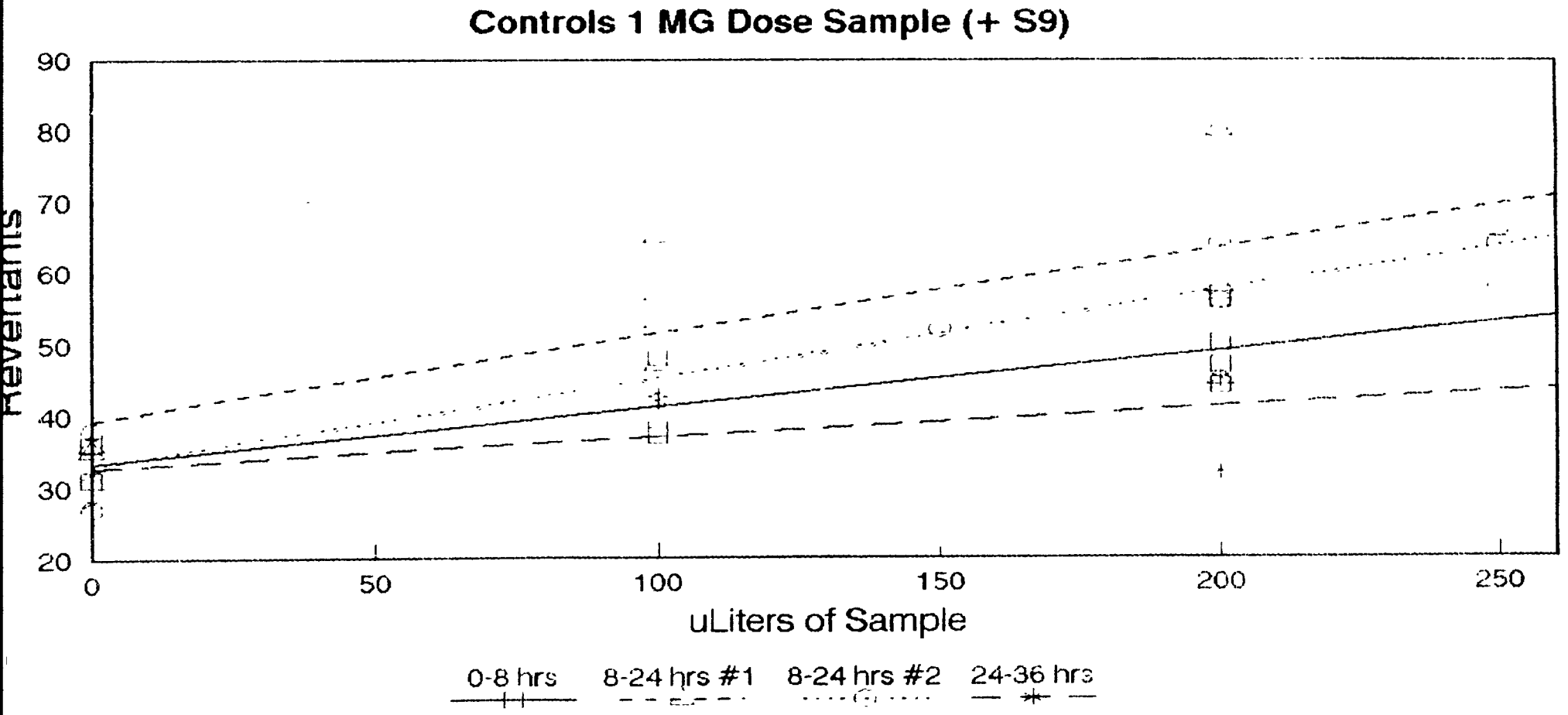
Controls 1 MG Dose Sample (+ S9)
Revertants
90
80
70
60
50
40
30
20
0
50
100
150
200
250
uLiters of Sample
0-8 hrs
8-24 hrs #1
8-24 hrs #2
24-36 hrs

Figure 35

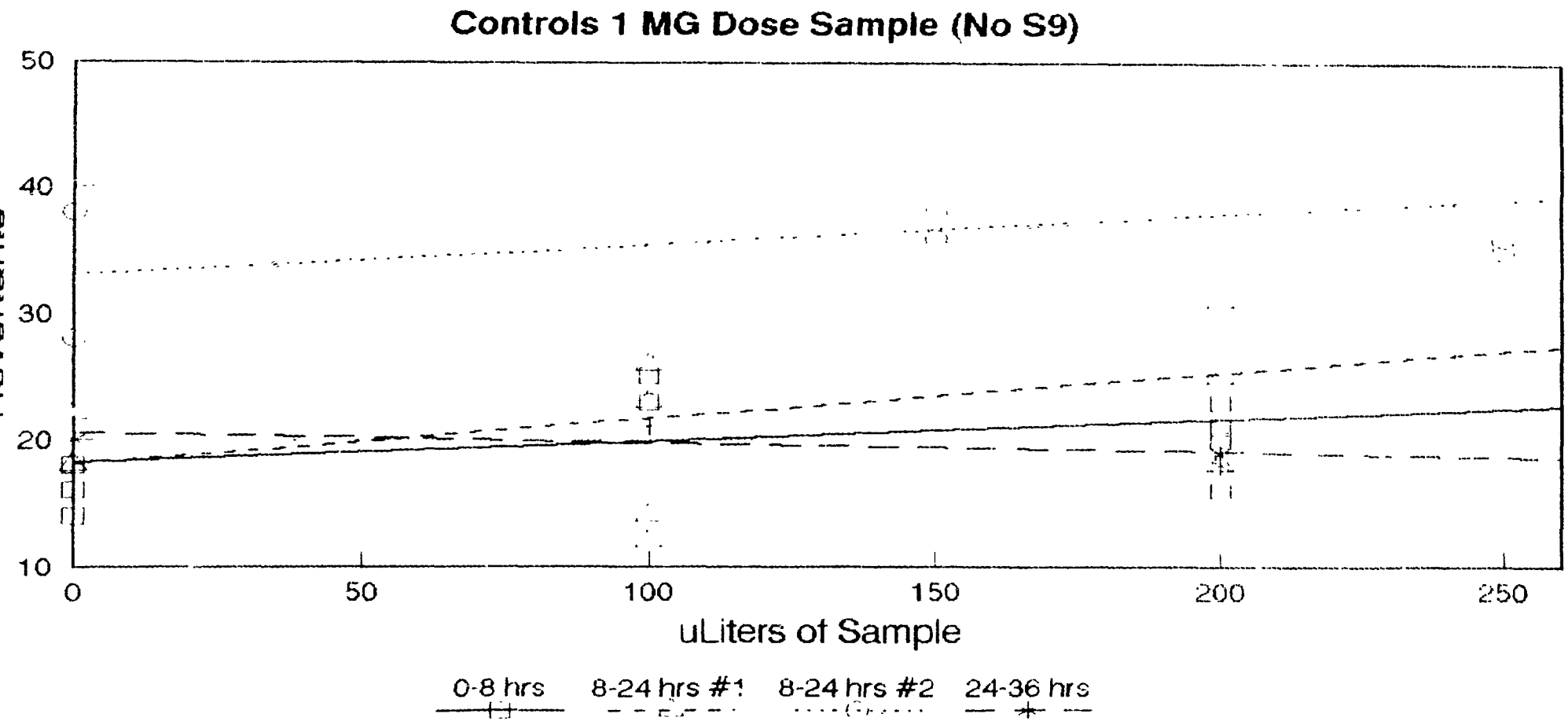
Controls 1 MG Dose Sample (No S9)
Revertants
50
40
30
20
10
0
50
100
150
200
250
uLiters of Sample
0-8 hrs
8-24 hrs #1
8-24 hrs #2
24-36 hrs

Figure 36

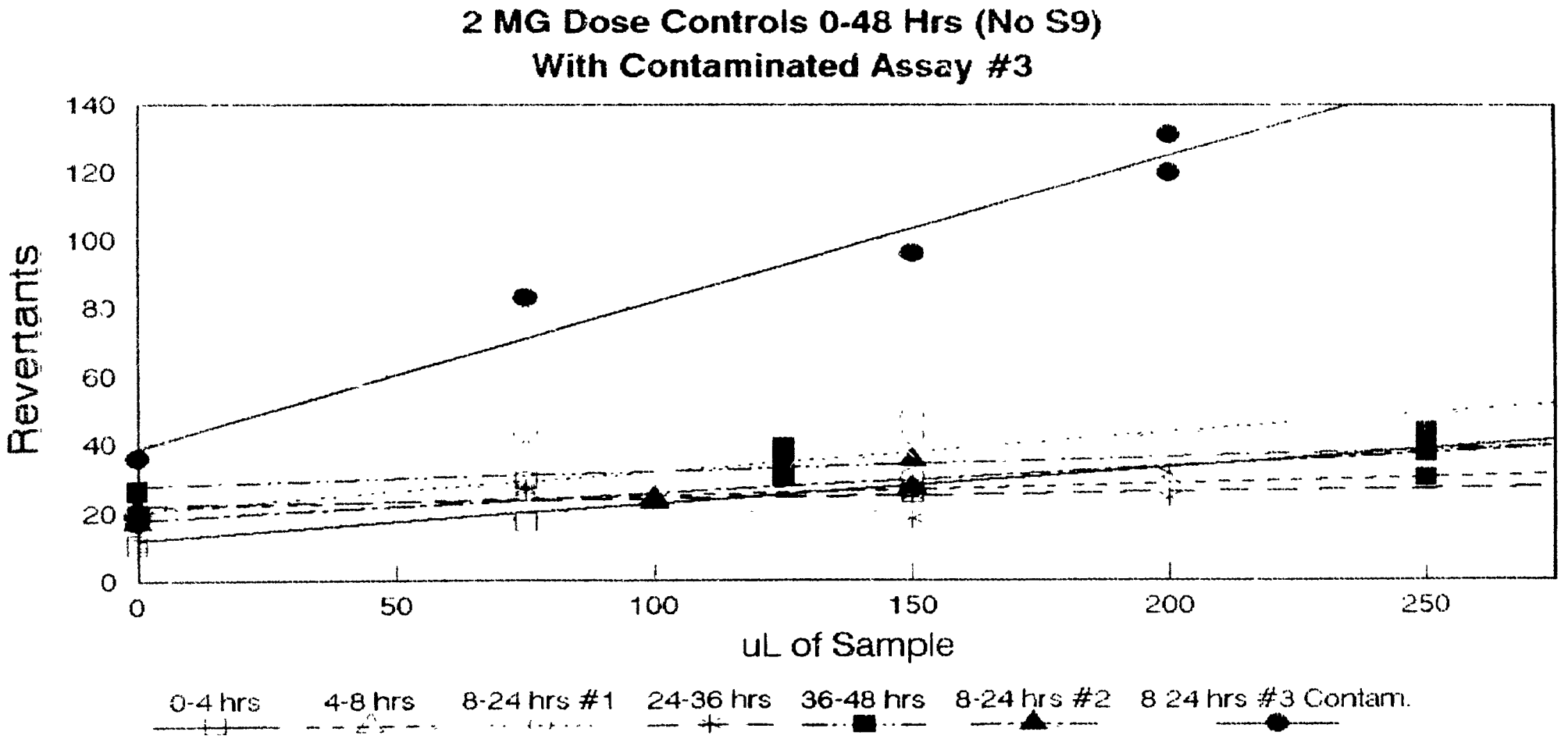
2 MG Dose Controls 0-48 Hrs (No S9)
With Contaminated Assay #3
Revertants
140
120
100
80
60
40
20
0
0
50
100
150
200
250
uL of Sample
0-4 hrs
4-8 hrs
8-24 hrs #1
24-36 hrs
36-48 hrs
8-24 hrs #2
8 24 hrs #3 Contam.

Figure 37

VII. B. Appendix B: Mathematics Used in Kinetic Analysis

VII. B. 1. Methods

VII. B. 1. i. Rates

Initial analysis of the data kinetics required calculating the slopes of excretion rate plots to determine the rates' and rate constants' highest and lowest possible values. The formula to determine any slope is: (Y2-Y1)/(X2-X1). The lines selected to determine the slopes from were those two lines which fit within the data's error range producing the steepest and the most shallow slopes. These two values were listed in the form; Average Slope $\pm$ Difference between the high or low slope's value from the average slope.

It is important to note the Y and X values used were adjusted to reflect their true meaning. In other words, the average revertant excretion rate during the 36 to 48 hour period was associated with the median time value of 42 hours rather than the end time value of 48 hours. This specific example was used to provide the first value of the terminal rate estimate where the second value was the X intercept determined graphically as shown in Appendix A.

VII. B. 1. ii. AUC and Cumulative Revertant Counts

Once the average revertant excretion rate was determined, it was multiplied by the number of hours from the 48 hour mark to the X intercept values determined graphically. This produced an average terminal end cumulative revertant count with a $\pm$ value as described in the previous paragraph. These two values were added separately to the previously determined 48 Hr cumulative revertant count to produce a total cumulative revertant count, again in the average value form with a $\pm$ error value. This is the area under the curve (AUC).

VII. B. 1. iii. Excretion Rate Constant Ke

The AUC value was then used to produce a Sigma Minus Plot via subtracting successive cumulative revertant amounts over increasing time from the AUC value producing a Revertants-Left-to-be-Excreted vs Time chart. This is the Sigma Minus Plot. The AUC number is therefore the Y intercept where X = 0. Again, the highest and lowest slopes outlined the range of excretion rate constants determined from this plot.

VII. B. 1. iv Absorption/Formation Rate Constant Ka

Once, the excretion rate constant was determined, this slope produced a line from which was subtracted the corresponding Y or Revertant Values on the initial side of the Excretion Rate Plot's curve.
[(Y of Ke) -(Y of initial curve)]= (Y of Ka)
The resulting difference produced a line with a slope representative of the absorption/formation rate constant, Ka, sjown in Fig. 17.

VII. B. 2. Numerical Calculations and Data

VII. B. 2. i. Terminal Tail Revertant Rate Estimation

1-NP

High Value: (205 revert/hr)*(1/2)*(120 hrs- 42 hrs)= 7,995 revert.
7,995 revert)/(120 hr - 42 hr)= 102.5 rev/hr
102.5 rev/hr*(120 hr- 48 hr)= 7,380 revert in terminal tail

Low Value: (145 revert/hr)*(1/2)*(80 hrs- 30 hrs)= 3,625 revert.
(3,625 revert)/(80 hr - 30 hr)= 72.5 rev/hr
72.5 rev/hr*(80 hr- 48 hr)= 2,320 revert in terminal tail

Avg Value: 4,850 terminal tail revert $\pm$ 2,530 revert

2-NFA

High Value: (235 revert/hr)*(1/2)*(220 hrs- 42 hrs)= 20,959 revert.
(20,959 revert)/(220 hr - 42 hr)= 117 rev/hr
117 rev/hr*(220 hr- 48 hr)= 20,252 revert in terminal tail

Low Value: (148 revert/hr)*(1/2)*(90 hrs- 30 hrs)= 4,440 revert.
4,440 revert)/(90 hr - 30 hr)= 74 rev/hr
74 rev/hr*(80 hr- 48 hr)= 3,180 revert in terminal tail

Avg Value: 11,680 terminal tail revert $\pm$ 8,500 revert

3-NFA

High Value: (111 revert/hr)*(1/2)*(110 hrs- 42 hrs)= 3,774 revert.
(3,774 revert)/(110 hr - 42 hr)= 55.5 rev/hr
55.5 rev/hr*(110 hr- 48 hr)= 3,441 revert in terminal tail

Low Value: (74.5 revert/hr)*(1/2)*(60 hrs- 30 hrs)= 1,117 revert.
1,117 revert)/(60 hr - 30 hr)= 37 rev/hr
37 rev/hr*(60 hr- 48 hr)= 447 revert in terminal tail

Avg Value: 1,944 terminal tail revert ± 1,500 revert

VII. B. 2. ii. Sigma Minus Plot (X' - X)

Time (Hrs)	Cumulative Revertants Remaining to be Excreted		
	1-NP	2-NFA	3-NFA
0	41,478 ± 3,700	25,448 ± 14,170	23,264 ± 4,000
4	39,262	25,240	21,390
8	30,986	23,680	14,272
24	9,050	16,288	4,176
36	7,310	14,500	3,276
48	4,850	11,680	1,944

VII. B. 2. iii Sigma Minus Plot Estimate of Ke

1-NP

Ke High [Ln{41478+3700}-ln{100}]/-80 = -0.076
Ke Low [Ln{41478-3700}-ln{100}]/-130 = -0.046
Ke avg = 0.061 ± 0.015 (1/hr)

2-NFA

Ke Best Fit [Ln{25448}-ln{100}]/-330 = -0.016
Ke High [Ln{25448+14170}-ln{100}]/-80 = -0.075
Ke avg = 0.016 ± 0.058 (1/hr)

3-NFA

Ke High [Ln{23264+4000}-ln{100}]/-50 = -0.112
Ke Low [Ln{23264-4000}-ln{100}]/-115 = -0.046
Ke avg = 0.079 ± 0.043 (1/hr)

VII. B. 2. iii Estimation of Ka From Ln. Excretion Rate Plot

Y=KX+I Where I is Y intercept = Ln(X')

1-NP

Time	Ln(Y of Ke)	-Ln(Y of Curve)	= Ln(Y of Ka)
0	10.63	0	10.63
4	10.57	6.3	4.25
8	10.5	7.63	2.86

Values Plotted in Figure 17

2-NFA

Time	Ln(Y of Ke)	-Ln(Y of Curve)	= Ln(Y of Ka)
0	10.1	0	10.1
4	10.08	4.6	5.44
8	9.7	5.97	4.04

Values Plotted in Figure 17

3-NFA

Time	Ln(Y of Ke)	-Ln(Y of Curve)	= Ln(Y of Ka)
0	10.05	0	10.05
4	9.7	6.15	3.58
8	9.4	7.48	1.93

Values Plotted in Figure 17