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and DNA as Biological Markers of Exposure

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**TITLE:**

**ADDUCTION OF NITROAROMATIC COMPOUNDS WITH BLOOD PROTEINS AND DNA  
AS BIOLOGICAL MARKERS OF EXPOSURE.**

**I. INTRODUCTION:**

Environmental contamination of soil and ground water with residues of explosives, and the intermediates of explosive(s) synthesis and degradation products (nitramines and nitroaromatic compounds) are of particular concern to both the US Army as well as the US EPA. The presence of these pollutants in the environment is due to the discharge of waste water generated at the munitions production sites, load-and-package operations, and burning and detonation of explosives discarded during quality control checks. The most commonly observed nitroaromatic pollutants are 2, 4, 6-trinitrotoluene (TNT) and several of its by-products that are formed during TNT synthesis and degradation (dinitrotoluenes, trinitrobenzene and dinitrobenzenes). These products are typically degraded by microorganisms under anaerobic conditions, but the toxicological consequences of exposure to these by-products (e.g., nitroso compounds) are unknown. As a result the Army is currently unable to conduct definitive risk-based assessments to delineate the potential ecological impacts of these compounds.

1, 3, 5-Trinitro benzene (TNB) is a by-product of both trinitrotoluene (TNT) synthesis and photolysis. The complete mechanisms of TNB formation during TNT photolysis is unclear. However, Burlinson, et al., (1980), suggested that TNB is produced from decarboxylation of 2, 4, 6-trinitrobenzaldehyde, a major TNT photoproduct. These nitroaromatic compounds are found along with TNT as contaminants in ground water and soil in active and formerly active military installations. Several studies have shown that TNB undergoes biotransformation into 3,5-dinitroaniline (Burlinson, et al., 1980., Witchell, et al., 1982., McCormic, et al., 1976). Data on the toxicokinetics of TNB are sparse. Most of the information on its absorption, distribution and metabolism comes from studies that examined the formation of methemoglobin in rats and mice (Senczuk, et al., 1976; Watanabe, et al; 1976). Reddy and Gunnarson, (1993) and Chandra et al., (1995) have reported hematological changes and brain lesions in Fischer 344 rats treated with TNB. Recently we have determined subacute, subchronic and chronic toxic effects of TNB in Fischer 344 rats. Dietary administration of TNB(50-1200 ppm diet) for 14 days has been shown to induce hematological changes such as methemoglobinemia, decreased hemoglobin, red cell count and hematocrit, and toxic effects on brain, spleen, kidney and testes in rodents ( Reddy et.

al. 1996a). From the chronic 2 year study (50-300 ppm diet) a no observed adverse effect level (NOAEL) of 2.68 mg /day/kg BW and a low observed adverse effect level (LOAEL) of 13.31 mg/day/kg BW based on hematological, and renal effects (Reddy et al. 1996b). TNB has been shown to be genotoxic in several *Salmonella* test strains (TA 98, TA 100, TA 1535, TA 1537, TA 1538) at concentrations 0.1 to 100 u g/plate (McGregor, et al., 1980, Spangord, et al., 1982). TNB was also reported to be toxic to fresh water aquatic organisms such as Fathead minnow and Water flea. (van der Schalie, 1983).

In the present study, we demonstrated that TNB forms adducts with blood proteins and tissue DNA. Ultimately, blood protein adducts may be used as surrogates for target tissue doses (DNA-carcinogen adducts), hence, protein adducts can serve as biological markers for monitoring human and ecological exposure of TNB or other nitroaromatic compounds that are prevalent in the environment.

1,3-Dinitrobenzene (DNB) has been used to make explosives and certain dyes, as an intermediate in the synthesis of organic chemicals and in the manufacture of plastics. It is formed as a by-product during the manufacture of the explosive, 2, 4, 6-trinitrotoluene (TNT). DNB is also formed through the photochemical oxidative degradation of 2, 4-dinitrotoluene a by-product released



into the environment during TNT production (Spangford et al., 1980). DNB has been detected as an environmental contaminant of ground water and soil near TNT production waste disposal sites, certain Army installations and at certain superfund sites. DNB has been shown to be genotoxic in bacterial systems and fresh water aquatic organisms (McGregor et al., 1980, van der Schalie, 1983), and form adducts with blood proteins and tissue DNA (Reddy et al., 1995). It has been shown to induce hematological changes such as methemoglobinemia, decreased hemoglobin, red cell count and hematocrit, and toxic effects on brain, spleen, kidney and testes in rodents (Cody et. al. 1981, Goldstein and Rickert, 1985, Linder et. al. 1986). Humans exposed to DNB showed symptoms of poisoning which include headache, nausea, diarrhea, fever, decreased blood pressure, cyanosis, kidney malfunction and hepatomegaly (Beritic 1956). We have conducted subchronic toxic effects of DNB in Fischer 344 rats and showed a NOAEL of 0.06 mg DNB/day/ kg BW and a LOAEL of 0.35 mg/day/kg BW based on hematological effects ( Reddy et. al., 1995a). In the present study, we showed that DNB forms adducts with blood proteins and tissue DNA. The blood protein adducts have been used as surrogates for target tissue doses (DNA-carcinogen adducts), hence, protein adducts can serve as biological markers for monitoring human and ecological exposure of DNB or

other nitroaromatic compounds that are prevalent in the environment.

N-methyl-N,2,4,6-tetranitroaniline (Tetryl) is a man-made nitroaromatic chemical that has been used as an explosive in detonators, primers, and as a detonating agent for other less sensitive high explosives, and as a booster charge for military devices. Currently tetryl is neither manufactured nor used in the United States. However, the presence of significant amounts of tetryl has been detected in the soil and in water near military installations and Army ammunition plants where it was manufactured or stored. Erosion of, and movement through, contaminated soils at these sites may release tetryl to surface and underground water (Ryon, et. al., 1984).

Most of the information on health effects of tetryl is from studies on workers employed in military facilities during the World Wars (Bergman, 1952). Workers complained of coughs, fatigue, headaches, nosebleeds, nausea and/or vomiting due to occupational exposure of tetryl dust (Fischer and Murdock, 1946). Many workers who had skin contact with tetryl dust or tetryl-containing compounds also developed skin rashes which often included reddening, itching, swelling and peeling of the skin (Goh, 1984). Some workers were more sensitive to tetryl exposure and developed

allergies that often included severe asthma-like reactions.

Toxicity data on tetryl is limited (Timofievskaya, et al., 1973). In rats the oral LD50 was shown to be greater than 5 g/kg BW for combined sexes. Tetryl was not an irritant at 5 g/kg BW when applied to rabbit skin for 24 hours, severe eye irritation potentials in rabbits were noted (Fitzgerald, et al., 1992). Sensitization tests showed that tetryl is not prominent skin sensitizer (Fitzgerald, et al., 1992). Recently we have investigated the subchronic toxic effects of tetryl and showed a NOAEL of 13 mg/day/kg BW (Reddy et al.1995b). Tetryl has been shown to be genotoxic in the Salmonella mutagenesis assay (McGregor, et. al., 1980). In this study we have shown that tetryl forms adducts with blood proteins and tissue DNA in rats. These adducts can be used in exposure monitoring at contaminated sites as well as the sites that were subjected to remediation.

## **II. BACKGROUND:**

In the early 1970's Ehrenberg and his colleagues made a substantial improvement to the process of risk assessment by pointing out that the toxicologic effects of a pollutant are a function of the actual target tissue dose, as compared to the exposure (e.g., dose administered in mg/kg-day) They further demonstrated that for electrophilic compounds, the tissue dose

could be deduced from a knowledge of the level of adducts formed with cellular nucleophiles including macromolecules (e.g., DNA and protein), (Ehrenberg et al.1974).

Almost all genotoxic agents have been shown to form DNA adducts. The data from the available literature suggest that there is a strong correlation between specific DNA adducts and cancer development (Swenberg, et al., 1987, Poirier and Beland, 1994). The genotoxic agents that have the ability to form DNA adducts have been shown to bind to proteins and form protein adducts (Ehrenberg et al., 1974, Lutz, 1979). Ehrenberg and his associates suggested the use of hemoglobin adducts as a quantitative measure of DNA carcinogen adducts (Ehrenberg et al., 1974). This proposition has been successfully employed in predicting the occupational exposure of humans to ethylene and ethylene oxide (Segerback, 1983). A similar dose response relationship for adduct formation in mouse liver DNA and hemoglobin following topical application of benzo(a)pyrene (BP) has been reported (Shugart, 1985, 1986).

In the proposed study, the hypothesis that genotoxic agents bind to cellular DNA and proteins was investigated using TNB, DNB, tetryl.

### III. PURPOSE AND OBJECTIVES:

To investigate the potential of TNB, DNB and tetryl to form covalent adducts with target tissue DNA and blood proteins, using [<sup>14</sup>C] labeled TNB, DNB and tetryl and develop sensitive methods for the identification and quantitation of DNA and blood protein adducts of TNB, DNB and tetryl, and utilize these biomarkers (DNA and hemoglobin adducts) as dose monitors in humans and ecological exposure assessment of nitroaromatic compounds.

### IV. Materials and Methods:

#### A. Test Chemicals : TNB, DNB and tetryl.

TNB and tetryl was synthesised by Dr. W. Koppes, Naval Surface Warfare Center, Silver Spring, MD., and was supplied by U. S. Army Biomedical Research and Development Laboratory (USABRDL). The purity has been checked by USABRDL and further verified at our laboratory by High Performance Liquid Chromatography (HPLC) and found to be 99.67% (TNB) and 99.45% (tetryl). DNB was purchased from Fluka Chemical Corp. (Ronkonkoma, New York), and the purity was 99.15%. This was further confirmed by HPLC at our laboratory.

**B. Radioactive testchemicals :** [<sup>14</sup>C] ring labeled 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), and tetryl were prepared via custom synthesis by NEN-DuPont Radiochemicals. Radiochemical

purity for TNB, DNB, and tetryl was 97.7, 97.7, and 97.1 respectively. The specific activities (sp.act.) were 45, 65.4, and 70.80 mCi/mmol respectively for TNB, DNB and tetryl.

**C. Vehicle :** Dimethyl Sulfoxide (DMSO), 1ml/kg BW.

**D. Enzymes and other chemicals :** Enzymes required for hemoglobin or globin digestion (pepsin, trypsin, pronase), DNA isolation (Proteinase k, RNase, RNase T<sub>1</sub>) and for DNA enzymatic digestion (DNase 1 Phosphodiesterase) were purchased from Sigma or Worthington Chemical Company. Liquid scintillation cocktail required for scintillation counting and for HPLC radioisotope monitoring were purchased from Beckman/Fisher Scientific Company.

**E. Test Animals :** Male Fischer 344 rats 11-12 week old (150-200 gm). Fisher rats were selected, based on the Goldstein and Rickert (1984) study which showed that Fisher 344 rats are more responsive to nitrobenzene than B<sub>6</sub>C<sub>3</sub>F<sub>1</sub> mice. Rats 9-10 week old were purchased from Charles River Laboratories and after arrival they were kept in quarantine for 2 weeks and then transferred and housed in approved AAALAC quarters, in large polycarbonate cages (2 or 3/cage) with corn cob bedding and in climate controlled rooms with a 12 hr light-dark cycle. Food and water were given *ad libitum*.

**F. Dose administration :**

**TNB:** 225 mg/kg body weight (BW) (1.06 mmol, sp. act. 9.47 mCi/mmol), 45 mg/kg BW (0.21 mmol, sp. act. 45 mCi/mmol), 9 mg/kg BW (0.04 mmol, sp. act. 23.8 mCi/mmol).

**DNB:** 30 mg/kg BW (0.18 mmol, sp. act. 4 mCi/mmol), 6 mg/kg BW (0.04 mmol, sp. act. 65.4 mCi/mmol), and 1.2 mg/kg BW, (0.01 mmole, sp. act. 65.4 mCi/mmol).

**Tetryl:** 1200 mg/kg BW (4.2 mmol , sp. act. 1.48 mCi/mmol), 600 mg/kg BW (2.1 mmol, sp.act. 2.99 mCi/ mmol), 300 mg/ kg BW (1.05 mmol, sp.act. 5.87 mCi/mmol), 150 mg/kg BW (0.52 mmol, sp. act. 11.51 mCi/mmol).

Each radioactive chemical was mixed with an appropriate quantity of unlabeled test chemical and dissolved freshly in DMSO and was given via single oral intubation (1 ml/ kg BW).

**V. EXPERIMENTAL APPROACH :**

The following experiments were conducted to achieve our objectives.

The experimental details are as follows:

**TNB: Experiment 1. Determination of optimum time required for maximum adduct formation of TNB with blood proteins and DNA.** In this experiment twenty five male Fisher 344 rats were divided into five groups (five rats/group). Groups 1-5 were given [<sup>14</sup>C] labeled

TNB 225 mg/kg BW, (1.056 mmol, sp. act. 9.47mCi./ mmol), by oral gavage and sacrificed respectively at 8h, 1, 2, 4, 7 days after TNB administration. At the time of sacrifice, blood, liver, kidney, spleen and testes were collected. The optimum time required for maxium TNB adduction with blood proteins or tissue DNA was determined and the results are presented in appendix A.

**Experiment 2. Kinetics of TNB adducts formation with blood proteins and tissue DNA:** This experiment was designed to determine the effect of dose on adduct formation. This experiment consisted of 18 groups of 5 rats each. Groups 1 - 6 were given 225 mg/kg BW, (1.056 mmol, sp. act. 9.47 mCi./ mmol). Groups 7 - 12 were given 45 mg/kg BW (0.211 mmol, sp. act. 45 mCi/mmol); and groups 13 - 18 were given 10 mg/kg BW ( 0.042 mmol, sp. act. 23.8 mCi/mmol), and sacrificed at 1, 2, 4, 7, 14, 21 days after exposure and the data is presented in appendix A.

**Experiment 3. Determination of the stability and persistence of TNB adducts with blood proteins and tissue DNA:** In this experiment 30 rats were divided in to 6 groups of 5 rats each. All thirty rats were given 225 mg/kg BW, (1.056 mmol, 9.47 mCi/mmol). Groups 1-6 were sacrificed respectively at 7,14,28, 42, 56, and 70 days after a single oral administration of TNB. The data is shown in appendix A.



**DNB: Experiment 1. Determination of optimum time required for maximum adduct formation of TNB with blood proteins and DNA.** In this experiment forty male Fisher 344 rats were divided into eight groups (five rats/group). Groups 1-8 were given [<sup>14</sup>C] labeled DNB 30 mg/kg BW, (0.18 mmol, sp. act. 4 mCi/mmol), by single oral gavage and sacrificed respectively at 2 h, 8 h, 1, 2, 3, 4, 6, 8 days thereafter. At the time of sacrifice, blood, liver, kidney, spleen and testes were collected. The optimum time required for maximum DNB adduction with blood proteins or tissue DNA was determined. The data is shown in appendix B.

**Experiment 2. Kinetics of DNB adducts formation with blood proteins and tissue DNA:** This experiment was designed to determine the effect of dose on adduct formation. This experiment consisted of 18 groups of 5 rats each (ninety rats). Groups 1 - 6 were given 30 mg/kg BW (0.18 mmol, sp. act. 4 mCi./mmol). Groups 7 - 12 were given 6 mg/kg BW (0.04 mmol, sp. act. 65.4 mCi/mmol); and groups 13 - 18 were given 1.2 mg/kg BW (0.01 mmol, sp. act. 65.4 mCi/mmol). Groups 1, 7, 13 were sacrificed one day after treatment. Groups 2, 8, 14 were sacrificed after two days. Three days after exposure groups 3, 9, 15 were sacrificed. Groups 4, 10, 16 were sacrificed four days after treatment. Similarly, groups 5, 11, 17 and 6, 12, 18 were sacrificed six and eight days post

treatment. At the time of sacrifice blood and tissue samples (liver, kidney, spleen, and testes) were collected for further analysis. The representative data is presented in appendix B.

**Experiment 3. Determination of the stability and persistence of**

**DNB adducts with blood proteins and tissue DNA:** In this experiment 50 rats were given 30 mg/kg BW (0.18 mmol, sp.act. 4.0 mCi/ mmol), and divided into 10 groups of five rats each. Groups 1-10 were sacrificed respectively at 8 h, 1, 2, 4, 7, 14, 28, 42, 56, and 70 days after a single oral administration of DNB. The stability and persistence of DNB-hemoglobin and DNB-DNA adducts were determined. The results are shown in appendix B.

**Experiment 4. Determination of the steady state level of adduct**

**formation:** This experiment consisted of forty rats (eight groups of five rats each). Groups 1 and 2 were given a single oral gavage (15 mg DNB/kg BW) and sacrificed 7 and 14 days after DNB treatment. Group 3 was given 15 mg DNB/kg BW daily for 7 days and sacrificed. Group 4 received 15 mg DNB/kg BW daily for 14 days and then sacrificed. Groups 5 and 6 received 1.07 mg DNB/kg BW daily for 7 and 14 days and were sacrificed (the same total dose as in Group 1 or Group 2 but, the dose was subdivided into fourteen equal doses and was given daily for seven (group 5), or fourteen days (group 6) and sacrificed 16 hours after the last dose). Groups 7 and 8 were

given 6 mg DNB/kg BW daily for 7 and 14 days and sacrificed 16 hours after the last dose. Blood and tissue samples were processed for adduct analysis. The data is shown in appendix B.

**Tetryl: Experiment 1: Kinetics of adduct formation (Effect of tetryl dose on blood proteins and DNA adduct formation).** In this experiment 100 rats were divided into 20 groups of five rats each. Groups 1-5 were administered with 1200 mg tetryl/kg BW (4.18 mmol, sp.act. 1.47 mCi/mmol), as single oral dose and sacrificed respectively at 8 h, 1, 2, 4, 8 days post treatment. Groups 6-10 were gavaged with 600 mg tetryl/kg BW (2.1 mmol, sp.act. 3 mCi/mmol), as single oral dose and sacrificed respectively at 8 h, 1, 2, 4, and 8 days after treatment. Similarly, groups 11-15 were given 300 mg tetryl/kg BW (1.05 mmol, sp.act. 6 mCi/mmol) as single oral dose and sacrificed respectively according to the above schedule. Groups 16-20 were gavaged with 150 mg tetryl/kg BW (0.52 mmol, sp.act. 11.5 mCi/mmol) and sacrificed according to the schedule described above (8 h, day 1, 2, 4, 6, and 8). During sacrifice blood and tissues were collected from all the rats for adduct analysis. The data is presented in appendix C.

**Experiment 2. Determination of the stability and persistence of tetryl adducts with blood proteins and tissue DNA:** In this experiment 30 rats were given 600 mg tetryl/kg BW (2.1 mmol,

sp.act. 2.99 mCi/mmol) as single oral dose and divided in to 6 groups of 5 rats each. Groups 1-6 were sacrificed respectively at 7, 14, 28, 42, 56, and 70 days post treatment. Blood and tissue samples were collected at necropsy for adduct analysis. The data is presented in appendix C.

**Experiment 3. Determination of the steady state level of adduct formation:** This experiment consisted of six groups of five rats each. Groups 1 and 2 was given a single oral gavage 300 mg/kg BW, (1.05 mmol, 6 mCi/ mmol). Group 1 was sacrificed after 7 days and group 2 was sacrificed after 14 days. Group 3 was given the same total dose as in group 1 but, the dose was divided into 14 equal doses and was given daily for 7 days and sacrificed 16 hours after the last dose. Group 4 was given the same total dose (as in grps. 1, 2) but the doses were divided into 14 equal doses and were given daily for 14 days and sacrificed 16 hours after the last dose. Groups 5 and 6 were given 300 mg/kg BW (1.05 mmol, sp.act. 6 mCi/mmol), daily for 7 days (group 5) and 14 days (group 6) and were sacrificed. Blood and tissue samples were collected for adduct analysis to determine the steady state levels. The data is presented in appendix C.

#### **VI. BLOOD AND TISSUE COLLECTION :**

At preselected sacrifice time points rats (from all experiments

TNB, DNB, or tetryl treated) were anesthetized with Nembutal (pentobarbital, 60 mg/kg BW). Blood was drawn by heart puncture into tubes containing 0.05 M sodium citrate or EDTA as anticoagulant. Soon after blood collection, liver, kidney, testes, and spleen were quickly excised, blotted, weighed and dropped in liquid N<sub>2</sub> and kept at -80°C until further analysis.

#### VII. FRACTIONATION OF BLOOD INTO REDBLOOD CELLS, PLASMA AND

**SERUM:** The blood fractionation protocol was shown in Fig. 1.

10 ul of whole blood was counted and the fraction of total radioactivity associated with the blood was determined. The remaining blood was centrifuged at 2000 xg for 10 min. Plasma was separated and transferred into freshly labelled tubes and 10 ul of plasma was counted for the determination of that fraction of radioactivity associated with plasma. The remaining plasma was frozen at -80°C. The packed RBC are stored at 20°C after mixing with 2 volumes of glycol-citrate solution (60 g. sodium citrate 2H<sub>2</sub>O/L, pH 8.4 containing 40% ethylene glycol).

#### VIII. PREPARATION OF ALBUMIN AND GLOBULIN FROM PLASMA AND

**DETERMINATION OF TEST CHEMICAL(S) (TNB, DNB OR TETRYL) BINDING TO GLOBULINS AND ALBUMIN (see FIG 1).** The procedure described by

Bechtold (1992) was followed. Briefly, one volume of plasma was mixed with equal volume of 0.5 M Calcium Chloride and left at room temperature for four hours and the precipitated fibrin was removed by centrifugation. Fibrin free supernatant (serum) was diluted twice with saline. To the diluted serum 45 volumes of HCL-ethanol reagent (1 ml of concentrated HCl per 600 ml of absolute ethanol) are added drop by drop with constant stirring. After incubation at 37°C for 30 min the solution is centrifuged at 3000 x g for 5 min and the supernatant (containing albumin) is separated from the precipitate (globulins). Globulin precipitate was mixed with 25 volumes of Bloor's solvent (ethanol: ethyl ether, 3:1) and the mixture was incubated at 37°C for 5 min with occasional shaking. The samples were cooled and centrifuged at 3000 x g for 5 min and the supernatant was discarded. The pellet was washed once with ethyl ether and, after careful removal and draining of the ethyl ether, the globulin pellet was dissolved in an appropriate volume of 0.05N NaOH and an aliquot was counted using liquid scintillation counter, and the specific activity was determined (dpm/mg globulin), and the data was expressed as picomoles of TNB, DNB or tetryl/mg globulin. Protein determination was done according to Lowry et al., (1951).

#### **IX. DETERMINATION OF ALBUMIN ADDUCTION WITH TEST CHEMICALS**

**(TNB, DNB, or tetryl):** One volume of supernatant (containing albumin; see above) was mixed with 0.1 volume of sodium acetate-ethanol solution (0.2 M sodium acetate in absolute ethanol) by slowly adding the latter while stirring. After allowing the mixture to stand at room temperature for 10 min, it was centrifuged at 3000 x g for 5 min and the supernatant was decanted and stored as albumin soup. The tube containing albumin precipitate is then allowed to drain completely and the pellet was dissolved in an appropriate volume of distilled water. Albumin binding with test chemicals was then calculated after quantitation of protein (Lowry et al. 1951), and the radioactivity from the albumin solution and expressed as picomoles of TNB, DNB or tetryl/ mg albumin. Albumin supernatant was pooled concentrated using speed vac. (Savant), and was stored as albumin soup at -80°C.

#### **X. DETERMINATION OF HEMOGLOBIN ADDUCTS WITH TEST CHEMICALS:**

**Hemoglobin:** Essentially the procedure described by Shugart (1986) was used with a slight modification. The RBC suspension stored at -20°C in glycerol citrate solution is thawed and centrifuged at 3000 x g for 10 min. The supernatant was discarded and the RBC pellet was washed twice with ice cold saline and the washings were discarded. Five volumes of ice cold tris buffer (0.2 M, pH 9.2) is

added to the RBC pellet and sonicated for 30 seconds to lyse the RBCs, allowed to sit on ice for 20 minutes and centrifuged at 25000 x g at 4°C for 20 minutes. Supernatant hemoglobin is transferred, labeled as hemogloin. The lysing process was repeated at least three times until the membrane pellet is free of hemoglobin. All hemoglobin supernatants were pooled and stored. The hemoglobin content was determined using Sigma Kit #525. This quantitation is based on the determination of cyanomethemoglobin (Riggs 1981). For specific activity determination (pmol/mg hemoglobin), a known aliquot of hemoglobin supernatant is decolorized with 6N NaOH and tertbutyl hydroperoxide at 60°C for one hour. Then 10 ml of scintillation cocktail is added and the radioactivity is determined and expressed as DPM or pmol TNB, DNB or tetryl per mg hemoglobin.

#### **XI. ISOLATION OF GLOBIN FROM HEMOGLOBIN AND DETERMINATION OF**

**GLOBIN ADDUCTS WITH TEST CHEMICALS:** The procedure described by Shugart (1986) as modified in our laboratory was used. Pooled hemoglobin solution was diluted with 3 volumes of water (to bring tris concentration to 0.05M) and added dropwise into 9 volumes of ice cold acetone containing 5 mM HCl. The precipitated globin is removed by centrifugation 3000 x g for 10 min. The globin pellet is washed once with acetone HCl and resuspended in 5 ml water and



reprecipitated as above by adding drop wise with constant stirring into 9 volumes of ice cold acetone-HCl. The precipitated globin was centrifuged at 10,000 X g for 10 min. and the pelleted globin was washed with 20 ml acetone-HCl twice and with 20 ml of 95% ethanol twice, and dried under a stream of nitrogen gas (N<sub>2</sub>) and the powdered globin was stored at -20°C. The specific activity is determined by dissolving a fraction of powdered globin in water and by counting an aliquot for radioactivity (DPM/ml) and determining the globin concentration (mg/ml). The values were expressed as before (picomoles of test chemical/ mg globin). The hemoglobin soup (the decanted soup after globin precipitation) was pooled concentrated and stored for determining the possible labile radioactivity during globin precipitation.

**XIII. ISOLATION OF TISSUE DNA:** For the isolation of DNA, the procedure described by Kunnath and Locker (1982) was used. Briefly, the frozen tissue (0.5 to 1.0 g) WAS homogenized with polytron (Brinkman) homogenizer in 20 volumes of NTE buffer (150 mM NaCl, 10 mM Tris and 2 mM EDTA, pH 7.6) for 10 seconds. The homogenate is centrifuged at 1500 x g for 15 minutes. The bottom nuclear pellet is resuspended in 10 volumes of NTE buffer and SDS to a final concentration of 1%. Then Protinase K (500 µg/ml was added incubated at 37°C for 12 hours, then extracted with equal

volumes of TNE buffer saturated phenol: chloroform: isoamylalcohol mixture (25:24:1 v/v/v) pH 7.5 in 50 ml polypropylene tubes with tightly fitted tops, and centrifuged at 10,000 x g for 15 minutes. The top aqueous phase was transferred into fresh tubes and the phenol:chloroform:isoamylalcohol extraction was repeated until no protein was seen in the interphase (at least 3 extractions). To the aqueous layer after final extraction, 0.1 volumes of 2 M potassium acetate and 2.5 volumes of cold ethanol was added slowly from the tube sides and the precipitated DNA was spooled out and rinsed sequentially with 70% ethanol, 100% ethanol, and ether. The DNA pellet was then resuspended in 10 ml TNE buffer; 100  $\mu$ g/ml RNAase and 50 units/ml RNAase T<sub>1</sub> is added; incubated at 37°C for 1 hour; 500  $\mu$ g/ml Proteinase K added and then incubated for an additional 1 hour. The solution was then re-extracted with phenol: chloroform:isoamyl alcohol. DNA was reprecipitated with ethanol and rinsed as before. The dried DNA pellet was resuspended in distilled water overnight and the concentration and purity was estimated spectrometrically using the reference value of 20 absorbance units at 260 nm per mg DNA. The purity is checked by optical density ratio at 260/280. The value of 1.8 to 2 is considered pure preparation. Impure DNA preparations due to contamination with RNA, protein or phenol were further purified by

enzyme treatment (Rnase and Protease) and phenol: chloroform extraction (Optical density less than 1.8 or more than 2.0 was considered as an impure preparation and subjected to the above reprecipitation steps.

**XIII. QUANTITATION OF DNA ADDUCTS FORMED WITH TEST CHEMICALS:**

A portion of the DNA solution (2 ml) was hydrolyzed with 0.1 N HCl at 60°C for 45 minutes. Portions of DNA hydrolysate (1-2 ml) was used for counting for DNA in scintillation counter to determine dpm/ml and an aliquot for the quantitation of DNA by the diamino benzoic acid method (Kissane and Robbins, 1958). The data were expressed as picomoles of test chemical bound/mg DNA.

**XIV. IDENTIFICATION AND QUANTITATION OF BLOOD PROTEIN ADDUCTS:**

Albumin, hemoglobin or globin isolated from rats treated with TNB DNB or tetryl was digested enzymatically (Turesky, et al., 1987) or hydrolysed with 1 N Hcl (Suzuki, et al., 1989) or with 1 N sodium hydroxide (Cheever et al., 1992). Hemoglobin digest or acid or alkaline hydrolysate was extracted with ether or water saturated ethyl acetate three times and the pooled organic extracts were dried reconstituted in methanol and separated on Waters 600E HPLC equipped with Berthhold HPLC radioactive monitor, which is interfaced with an Epson PC. The radioactive peaks were identified with the corresponding HPLC peaks. Further confirmation of the

adducted chemical was achieved by Co-chromatography with an appropriate standard(s)/ metabolite(s).

**XV. IDENTIFICATION AND QUANTITATION OF DNA ADDUCTS:**

DNA isolated from Liver, spleen, and kidney from rats treated with TNB, DNB, or tetryl was digested enzymatically with DNase alkalinephosphatase and, phosphodiesterase (Joyce and Daniel, 1982, Rojas and Alexander, 1986), and the DNA digest with modified and unmodified nucleosides were resolved on Waters 600E HPLC equipped with a Berthold HPLC radioactive monitor, which was interfaced with an Epson computer and the radioactivity was monitored via Berthold radioactive detector. When the radioactive counts were low (DNA adducts), 0.5 ml fractions were collected using a fraction collector and the radioactivity was determined and the corresponding chromatographic peaks were identified.

**XVI. STATISTICAL ANALYSIS:**

The following statistical methods were employed for Experiments 1-4.

Experiment 1,3 and 4 : A Non-parametric one-way analysis of variance with Alpha 0.05 was used. SAS procedures was used for the analysis. The Tukey multiple comparison test or a contrast between optimum time point and the adjacent time points was used.

Experiment 2: A Non-parametric two-way analysis of variance was

used (dose and time). The Tukey multiple comparison test was be used for each factor. SAS procedures were used on the main frame at EPA Cincinnati.

**XVII. RESULTS AND DISCUSSION:**

**TNB: The Figures and tables listed under TNB, are presented in Apendix A:**

Fischer Rats treated with [<sup>14</sup>C]TNB (single oral dose) formed stable adducts with blood proteins (albumin, globulins, hemoglobin/globin) and tissue (liver, kidney and spleen) DNA in a dose dependant manner and the results are shown in figures 2 to 6 and tables 1-5. Figure 1 describes the fractionation of whole blood into different fractions of interest (albumin, globulins and hemoglobin or globin). During albumin and globin precipitation significant amount of TNB radioactivity was lost and recovered in the serum supernitant fraction and in heme fraction (Table 1 ). This suggests that TNB adducts formed to albumin/hemoglobin are of two types. One of the types is acid labile and perhaps the adduct is formed through sulfonamide linkage and the second adduct type is not acid labile (may be covalently bound). Table 2 describes the clearance (decrease) of TNB radioactivity form plasma with time. Maxium radioactivity (adducts) was detected on day two with the high dose and for mid and low dose groups, the peak adduct

levels were reached by day one and by day two significant amount of radioactivity was cleared. Figure 2 depicts the formation and persistence albumin, globulins and globin. TNB adducts in both albumin and globin the adduct levels were maximum by day 2 while globulin adducts peaked by 24 hours. Both albumin and globulin radioactivity decreased (cleared) rapidly (about 90%) between day 2 and day 4. The TNB radioactivity was not detectable in globulins after one week and in the albumin after 2 weeks. The radioactivity associated with globin persisted up to 70 days (Table 3). In the albumin, the radioactivity decay pattern between day 4 and 7 was approximately parallel to the reported decay rate of the serum proteins (Schreiber et al 1971 ). The rapid decay during 48 and 96 hours suggest that the presence of TNB adducts (adduct load) destabilize and enhance the break down of the serum proteins (albumin and globulin molecule) faster than the reported normal half life (2.6 days for rat albumin) (Schreiber et al 1971, Skipper and Tannenbaum 1990, DeBord et al.,1992). The TNB radioactivity in the globin decreased at a slower rate and persisted throughout the life span of rat red blood cell (RBC) (60-65) days. But the decay pattern of radioactivity did not directly parallel to the anticipated loss of RBC due to aging, suggesting that the presence of TNB adducts in the globin destabilize the RBC membrane due to

adduct load, resulting in an accelerated lysis of the adducted RBC, and subsequent loss of radioactivity from the globin compartment or the adducted RBC are preferentially removed by processes in the spleen. Consistent with these kinetic observations, the acid lability of the radioactivity bound to hemoglobin varied with length of exposure time. Thus the percentage of TNB radioactivity dissociated from hemoglobin (i.e., radioactivity recovered in the heme supernatant (Fig.1) during globin precipitation from hemoglobin with cold acetone:HCl varied inversely with time. The observed percentages of acid-labile radioactivity on day 1, 2, 4, 7, 14, 28, and 42 were 50, 37, 24, 12, 10, 10 and 10% respectively (Table 1). Dose dependent formation of TNB-albumin and TNB-globin adducts are presented in figures 3 and 4. Analysis of albumin and globin samples for possible adducted metabolite(s) by HPLC (attached to Berthold HPLC-radioactive detector) was unsuccessful. It has been shown that 3,5-dinitroaniline as the major metabolite in earthworms and germinating Oat and Lettuce plants (Reddy et al. 1994). In another study, triaminobenzene has been identified as a metabolite in the urine and feces of small rodents such as mice (*Mus musculus*), white footed mouse (*Peromyscus leucopus*), short tailed Shrew (*Cryptotis parva*), and vole (*Microtus canicaudus*) (Reddy et al. unpublished data). Triaminobenzene has also been

identified as the hemoglobin adduct in shrew blood treated with TNB and it has been identified as a cystine adduct of hemoglobin (formed via sulfonamide linkage, which is acid/ alkali labile) (Pinorini-Godly, et al. 1996). In the present study, none of the above metabolites namely, 3,5-dinitroaniline, triacetamidobenzene or triaminobenzene were detected as aducts/metabolites in the hemoglobin. Several known metabolites of TNB namely 3,5-diaminonitrobenzene, 3,5-diaminoacetanilide, 3,5-dinitroacetanilide, 3,5-diacetamidonitrobenzene, 3-amono-5-nitroacetanilide, 1,3,5-triacetamidobenzene, were synthesised commercially and supplied by Dr. Gunda Reddy of USA BRDL, for adduct identification. All these metabolites were subjected to HPLC. None of the synthesized metabolites co-elute with the radioactivity peaks from albumin soup, heme soup, or hemogloin hydrolysate and no further analysis was done on the unknown radioactive peaks.

Formation of TNB-DNA adducts with liver, Kidney and spleen are presented in figurs 5-6 and tables 4-6. The liver DNA adduct levels, in the high and mid dose groups reached the maxium by 2 days (125 and 32 p moles/mg DNA) and the radioactivity decreased gradually with time. In the low dose group optimum adduct levels were reached by 24 hours (9.94 p moles/mg DNA, Table 4, Figure 5). At the end of four weeks the radioactivity associated with liver



DNA in high, mid and low dose groups respectively were 24, 21, and 10% (of the maximum adduct level). No detectable radioactivity was present in mid and low dose groups after 6 weeks. In the high dose group 10% of the radioactivity in the liver DNA was intact after 10 weeks (Table 4). In the kidney the pattern was similar. The kidney DNA adduct levels were 79, 29, and 2 p moles/mg DNA respectively for high, mid and low dose TNB treatment groups (Table 5). At the end of four weeks the radioactivity associated with kidney DNA in high, mid and low dose groups respectively were 28, 14, and 50% (of the maximum adduct level). No detectable radioactivity was present in mid and low dose groups after 6 weeks. About 10% of the radioactivity in the kidney was intact at the end of 10 weeks (Table 5, Figure 6).

In the spleen formation of DNA adducts appears to be bi-phasic. Adduct levels peaked at two different intervals in rats treated with high dose. For example, first peak adduct levels were detected on day 4 (27 p moles/mg DNA), with a marginal decrease in adduct levels by day seven (25 p moles /mg DNA). The second peak appeared on day 14 (39 p moles/ mg DNA) and gradually decreased with time. At the end of 70 days 40 % of the TNB radioactivity was intact in the DNA. In the mid and low dose groups the maximum adduct levels were seen on day 1 (6 and 2 p moles/ mg DNA), and the

adduct levels decreased with time. In both groups no radioactivity was detected in spleen DNA 28 days after exposure (Table 6). HPLC separation of standard mixture of four DNA nucleosides namely, deoxycytidine, deoxy guanosine, deoxy thymidine, and deoxy adenosine are shown in Figure 7. The gradient conditions, solvent systems and other chromatographic conditions used for DNA hydrolysates and standard mixtures were also presented in figure 7.

The resolution of three synthetic metabolites (1,3,5-triacetamidobenzene, 3-amino-5-nitroacetanilide and 3,5-dinitroaniline) by HPLC is presented in figure 8. HPLC separation of DNA nucleosides and synthetic metabolite mixture are presented in Figure 9. It is clear from the chromatography that all the seven components have base line resolution. HPLC analysis of liver DNA hydrolysate from rats treated with TNB (enzymatic) is presented in figure 10. The retention times of these nucleosides were very similar to the standerds. In Figure 11 the recovery of radioactive counts from DNA hydrolysate collected as 1 ml fractions are plotted along with the four nucleosides. The solid lines represent the total radioactivity in those peaks eluted at 5 min (with 81 DPM), 15 min (with 980 DPM), 20 min (with 1230 DPM) and 28 min (with 110 DPM). From the chromatography it is apparent that the radioactive peak at 15 minutes is associated with deoxyguanosine and the peak

at 20 minutes is associated with deoxyadenosine suggesting that they are the adducts of deoxy guanosine and deoxyadenosine. But the retention times did not correspond to the retention times of the known metabolites. Hence, the two major adducted metabolites with 51% of the total injected radioactivity could not be identified. However, the minor metabolite (4%) whose radioactivity was eluted at 28 minutes was identified as 3,5-dinitroaniline and the radioactivity co-eluted during chromatography with 3,5-dinitroaniline. About 45% of the injected radioactivity into HPLC did not elute from the column. This suggests that TNB/metabolites form DNA adducts in rat liver. The pattern was similar with kidney and spleen DNA.

**CONCLUSIONS:** TNB forms stable adducts with albumin, globin and tissue DNA in rats. In globin, detectable amounts of radioactivity was present even 70 days after single exposure. The adducts could not be identified due to lack of appropriate synthetic standards. Once the adducts are identified, hemoglobin adducts can be used for exposure assessment. In the DNA isolated from liver, kidney and spleen from rats treated with TNB, 3,5-dinitroaniline has been identified as a minor metabolite. Similar to globin, two major adducts from DNA (hydrolysates) could not be identified due to lack of standards. Protein and DNA adducts appear to have promise as

dose monitors for nitroaromatic munitions and their by-products. However, further studies are needed for the development of chemically-based analytical methods for albumin/hemoglobin, and DNA adducts characterization, in order to use TNB-albumin or/hemoglobin or DNA adducts for exposure assessment.

**DNB: The figures and tables listed Under DNB are presented in Appendix B.**

Fischer Rats treated with [ $^{14}\text{C}$ ]DNB (single oral dose) formed stable adducts with blood proteins (albumin, globulins, hemoglobin/globin) and tissue (liver, kidney and spleen) DNA in a dose dependant manner and the results are presented in figures 2-6 and tables 1 to 6. Figure 1 describes the fractionation of whole blood into albumin, globulins, hemoglobin/globin, albumin supernatant and globin supenatant (heme). Figure 2 shows the formation and loss of DNB radioactivity (adducts) in the serum in rats exposed (oral gavage) to 30 mg/ kg BW. Maxium adduct levels were seen at 2 h (936 p moles DNB/mg protein) and decreased rapidly to 525 p moles at 6 h and 483 p moles. by 8 h., and by 24 h only 23 p moles DNB was intact. After 8 days only trace amounts of radioactivity was present (1 p mole) in the serum, suggesting that rapid clearance may be due to adduct load. The Formation and persistence of DNB-albumin adducts are shown in Figure 3. The maximum adducts were

formed at 8 h (57 p moles DNB/mg protein) and gradually decreased with time. About 4% of the adducts were intact after 8 days. It appears that the decay is bi-phasic. A rapid decay during phase 1 (i.e., between 2 and 24 h) and a slow decay during second phase (i.e., between day 1 and 4). The high adduct load at earlier time points (2-24 h) appears to destabilize the protein and trigger the break down of the protein much faster than the normal half-life (2.66 days for albumin in rat) of albumin (Schreiberg, et al., 1971, Skipper and Tannenbaum, 1990, DeBord, et al. 1992). The adduct loss during day 1 and 4 may be due to the normal break down (half life 2.66 days) of adducted albumin, regardless of the adduct load. Similar to albumin peak, globulin adduct levels (7.5 p moles) were seen 8 h after exposure and gradually decreased with time and only trace amount of adducts (0.1 p mole) were intact after 8 days (figure 4). It is apparent from total serum data, that albumin and globulins (proteins that were isolated from serum) that a significant amount of radioactivity is lost during fractionation procedure, and the radioactivity is recovered in the albumin soup. This also suggests that DNB adducts in serum proteins are of two types and one type of the adduct is highly acid labile. The HPLC of albumin soup revealed the presence of 3-nitroaniline, a reductive metabolite of 3,5-dinitrobenzene (figure 12). Formation

and persistence of DNB hemoglobin adducts are shown in figure 5. Maximum hemoglobin adducts were seen at 8 h (303 p moles) and decreased rapidly to 122 p moles by 24 h. The loss of radioactivity was much slower between day 1 and day 8. About 40% of the radioactivity was intact on day 8. Similar to albumin, the loss of radioactivity in hemoglobin is bi-phasic. During the first phase (8-24 h) the adduct loss is much faster, followed by a slow decay between day 1-8. Faster adduct loss between 8 and 24 hours may be due the adduct load that trigger the break down of hemoglobin faster than the normal half life. When the hemoglobin ( from 8 h exposure) was fractionated into globin and heme, only 20% of the radioactivity remained intact in the globin fraction and 80 % of radioactivity was lost and recovered in the heme fraction (figure 6). Hemoglobin precipitation ( 24 h sample) into globin only 50 % radioactivity is lost and recovered in heme fraction. Globin retained 50% of the radioactivity. This also shows that in hemoglobin, two types of adducts are present. One type of adduct(s) is highly acid labile and the second type is stable and is not hydrolysed due to mild acid treatment. HPLC analysis of the radioactivity associated with the heme fraction revealed that the retention time of the radioactive peak was similar to 3-nitroaniline, and spiked nitroaniline coeluted with heme

radioactivity. Figure 7 shows the dose dependent formation of serum adducts with DNB. It is clear from the chart that adducts were formed in a dose dependent manner (19, 3.4 and 0.8 p moles DNB/mg protein respectively for 30, 6, and 1.2 mg/kg BW dose) and the adduct levels decreased with time. Dose dependent formation of DNB-albumin adducts are depicted in figure 8. It can be seen from the figure that the adducts were formed in a dose dependent manner (16, 3.3 and 0.7 p moles respectively for 30, 6, and 1.2 mg/ kg BW dose) and decreased with time. In Figure 9 the formation of globulin adducts with different doses of DNB is presented. Increased levels of adducts were formed with increase in dose and the adduct levels are decreased with time. Figure 10 shows the effect of DNB dose on hemoglobin adduct formation . It is clear from the figure that hemoglobin adduct levels are increased with dose. The adduct levels are decreased with time (for example on day 1 the adduct levels for 30 mg DNB dose was 136 p mole and on day 8 only 41 p moles were intact). Similarly, the dose dependent formation of globin-DNB adducts are presented in Figure 11. The pattern is very similar to that of hemoglobin. It also clear from the chart that globin adduct levels are about 50% less than hemoglobin and this decrease was due to the removal of radioactivity during globin precipitation from hemoglobin. Globin adducts are formed in a dose

dependent manner and adduct levels are decreased with time (for example on day 1 the adduct levels for 30 mg DNB dose was 52 p mole and on day 8 only 45% of the radioactivity was intact (23 p moles). In Figure 12, three different chromatograms representing standard 3-nitroaniline (NA) (UV detection at 254), organic extracts of hemoglobin acid hydrolysate or heme soup (C-14 radioactivity detection channel), and albumin soup (C-14 radioactivity detection channel) are presented. These three chromatograms were overlaid to show that the radioactive peak from heme soup (retention time 9.4 min with 98% of the injected radioactivity), and radioactivity peak from albumin soup (retention time 9.39 min, with 80% of the injected radioactivity) they are very similar to NA (HPLC retention time 9.4 min). The HPLC conditions are described in the figure 12. Table 2 describes the effect of single dose vs multiple doses and multiple divided doses. When rats were given single oral dose (15 mg/kg BW) and sacrificed after 7 days 33, and 12, p moles of DNB adducts detected in globin and albumin fractions. After 14 days the adduct levels decreased to half in globin (33 vs 16) as well as in albumin (12 vs 6.7). As indicated earlier, the adduct loss can be attributed to the red cell/ albumin degradation faster than the normal half life of red cell or albumin. When DNB was given repeatedly (7 doses), the adduct levels were significantly higher



(131 p moles). Similarly when the dose was reduced to 1 mg daily for 7 days the adduct levels(8 p moles) were decreased accordingly. In albumin, the adduct levels were less, but the trend remained the same as in globin. When the rats were given 6 mg/kg/daily for seven or 14 days the adduct levels (for globin 77 or 74 p moles/mg protein and for albumin 21 or 22 p moles/mg protein) appear to have reached steady state levels(Table-2). In contrast, under the same treatment regimen DNB adducts in DNA (liver, kidney or spleen)did not reach the steady state level. For example, after seven daily doses (6mg/kg BW) the adduct levels in liver, kidney or spleen respectively were 9.7, 4, and 3.7 p moles/mg DNA. After 14 daily doses (6 mg/kg BW) the adduct levels increased to 23, 12, 11. p moles in liver kidney and spleen respectively (Table 6). Unlike the blood proteins, DNA adducts were stable and persisted much longer(Tables 6). It is clear from Table 3 that the DNA adduct levels were maximum at more than one time point. For example, in the liver, 24 h after exposure 16.8 p moles were present, followed by a decrease up to 4 days and then the adduct levels were increased to 24 p moles after 6 days. In the kidney and spleen, the adduct levels were low but the trend remained the same. The stability and persistence of DNB-DNA adducts (after single oral exposure) can be seen in Table 5. In

the liver the adduct levels were decreased significantly with time and detectable adduct levels (0.39 p moles) were present 70 days after signal oral exposure. In the kidney and spleen DNA the adduct level increased with time and significant amount of adducts were present in kidney (2.37 pmoles/mg DNA), and spleen (11 pmoles/mg DNA), 70 days after exposure.

Earlier it was recognized that 3-nitroaniline (NA) is the adducted metabolite of albumin and hemoglobin. Figure 13 represents the HPLC elution pattern of DNB treated rat liver DNA digest. Gradient table and chromatographic conditions are depicted in Figure 13. Figure 14 shows the radioactivity plot of the radioactive fractions (1 ml) collected from Figure 13 and plotted the retention times based on the radioactivity peaks. None of the retention times based on the radioactivity peaks. None of the retention time of the radioactive peaks correspond to NA. This suggests that DNB metabolites other than NA are present as DNA adducts in the liver. Fifty seven percent of the radioactivity collected (3 peaks, and one of the peak co-eluted with deoxyguanosine) could not be identified due to lack of appropriate standards. Significant amount of radioactivity (43%) did not elute from the column during 50 minutes run.

## CONCLUSIONS:

DNB forms adducts with blood proteins and DNA in a dose dependent manner. Hence, protein and DNA adducts appear to have promise as dose monitors for nitroaromatic munitions and their by-products. However, further studies are needed for the characterization and development of chemically based (non-radioactive methods) analytical methods for protein and DNA adducts of DNB may be useful as biological markers of exposure for nitroaromatic compounds.

**TETRYL:** The figures and Tables listed tetryl are shown in Appendix C.

Fischer rats treated with tetryl (single or multiple doses) formed stable adducts with blood proteins and tissue DNA (liver, kidney, and spleen) in a dose dependent manner (Figures 1A-8, 8A-11, and Tables 1-6). Blood proteins were fractionated into albumin flobulins, hemoglobin or globin according to the fractionation protocol described in Figure 1. Dose dependent formation of albumin adducts with tetryl is presented in Figure 1A. It is apparent from the figure that the adduct levels are not dose dependent during 8h (0.33 days) time point (746, 833, 749 p moles respectively for 300, 600 and 1200 mg tetryl dose groups. Maximum

albumin adducts are formed 1 day after treatment in a dose dependent manner. At 150, 300 and 600 mg dose groups the adduct levels were linear with dose (379, 831 and 1505 p moles). The binding sites or, loss of adducts due to break down of the albumin induced by excessive adduct load. In all dose groups the loss of adducts are much faster than the normal break down of albumin (half-life 2.66 days) (Skipper and Tannenbaum 1990), and by day four 75-80% by day seven 90% of the adducts were removed from albumin.

The formation and persistence of albumin-tetryl adducts are shown in Figure 2. Similar to TNB and DNB, the loss of adduct follow a bi-phasic pattern. The adduct loss was drastic between day 1 and day 2, followed by a gradual decline. The pattern of albumin-tetryl adducts loss over a period of 6 weeks is also presented in figure 2. During albumin precipitation from serum significant amount of radioactivity was recovered in the albumin soup. HPLC of the albumin soup revealed a major unknown radioactive peak that eluted at the solvent front and a minor peak identified as picric acid are presented in Figure 8A. The dose dependent formation and persistence of globulin-tetryl adducts are presented in figure 3 and 4. The adduct levels are relatively less and the pattern of adduct loss was very similar to albumin. Dose

dependent formation (150-600 mg dose groups) of hemoglobin tetryl adducts are shown in Figure 5. In the 1200 mg dose group, the adduct formation was not proportional to dose, perhaps for the reasons discussed earlier. Optimum adduct levels are formed by 24 hours. Similar to albumin and globulins a rapid loss of adducts are seen between day 1 and day 4. At the end of seven days 10-12% adducted radioactivity remained intact. The loss of radioactivity (adducts) in albumin, globulin or in globin is mainly due to the degradation of protein due to adduct loss (accelerated breakdown at an earlier time points) or due to normal metabolic processes (synthesis and breakdown) at later time points (day 4-7).

Formation and persistence of tetryl hemoglobin adducts are shown in Figure 6. It is clear from the table that the adduct levels are maximum with 600 mg dose group at 24 h (672 p moles) while in the 1200 mg dose the adduct levels are relatively less (547 p moles). The decrease may be due to the reasons explained before (instability due to adduct load followed by an accelerated decay or selected removal of adducted RBC in the spleen). The loss of radioactivity in hemoglobin, and in globin (Figure 8) is faster than the normal decay (loss) of red cells (half life 30-35 days) (Skipper and Tannebaum 1990). The dose dependent formation of globin adducts with tetryl are presented in Figure 7. The adducts

formed with hemoglobin/globin are stable (not acid or alkali labile) and no radioactivity released during globin precipitation. Maximum globin adducts are seen with 600 mg dose group. The adduct loss was bi-phasic and was very similar to that of albumin and globulins Figures (6&8).

Acid and alkali hydrolysis of albumin and hemoglobin adducts revealed that the adducts are very stable and no radioactivity was released due to hydrolysis and hence, no radioactivity (adducts) was extracted into organic phase (ether, water saturated ethyl acetate or butanol). Similar results were reported with orthotoluidine (DeBord et al., 1992) and 4,4-methylenebis(2-chloroaniline) (MOCA) (Sabbioni and Neumann 1990). Figure 8A represents of three different chromatograms that are overlaid. The three chromatograms are as follows: 1) HPLC of standard picric acid (UV at 254, retention time 25.03 min). 2) HPLC of tetryl treated rat serum albumin soup (UV and C-14 radioactivity monitoring channel), with a huge UV and radioactive peaks that are unknown and peaks at 25.18 min (UV) and 25.24 min (radioactive) that correspond to the picric acid peak retention time. 3) HPLC of serum albumin soups spiked with picric acid. It is apparent from the figure that the radioactive metabolite present in the albumin soup is picric acid (figure 8A). This suggests that the picric

acid released into the albumin soups may be circulating as free metabolite into the serum or bound to serum proteins other than albumin and globulins and was likely acid labile and released from serum and recovered in albumin soup.

An overview of the stability and persistence of tetryl blood protein adducts at different weeks of exposure (1 to 10) are presented in table 1. From week 1 to week 10 the loss of adducts are gradual and may be caused due to the normal metabolic breakdown of adducted hemoglobin/albumin. DeBord, et al. (1992), have reported the half life of o-toluidine adducted albumin and hemoglobin as 2.66 and 12.3 days respectively. Only trace amounts of tetryl albumin and globulin adducts were detected after 6 weeks. Tetryl adducts in hemoglobin/globin persisted up to 10 weeks (life span of rat RBC) Table 1). The effect of single vs. multiple divided multiple doses are presented in Table 2. Seven days after single administration of 300 mg tetryl/kg BW, 45-40 p moles of tetryl hemoglobin/globin, 87 p moles of tetryl albumin and 5 p moles of tetryl globulin adducts were formed and as expected, the adduct levels were significantly decreased to 24, 24, 9 and 1 p moles respectively for hemoglobin, globin, albumin and globulin by 2 weeks. When the same dose was given (300 mg/kg BW) daily for seven or fourteen days, the adduct levels were increased and seems

to have reached a steady state level. For example after seven and fourteen doses the hemoglobin adduct levels were 517 and 601 p moles of tetryl/mg globin. Similarly for albumin the adduct levels are 2354 and 2221 (Table 2). The adduct levels in single dose exposures (dose A) are significantly lower than the adduct levels seen in multiple exposures (dose B). The adduct levels detected in divided multiple exposure (dose C) are decreased significantly according to the administered dose (approximately 1/14 of the value seen with dose (B) (Table 2). At lower doses (divided multiple exposures, dose C) adduct levels did not reach a steady state level. In contrast, DNA adducts in liver, kidney and spleen, did not reach steady state level under these treatment conditions/ However, adduct levels were increased in a dose dependent manner, when multiple doses of tetryl was given (dose B or C) Table 7). Dose dependent formation of liver DNA-tetryl adducts are presented in Table 3. Unlike blood protein adducts (1 day), the DNA adducts reached their maximum by 2 days, in a dose dependent manner. The liver DNA adduct levels for 1200, 600, 300 and 150 mg/kg BW respectively are 147, 75, 41 and 18 p moles/mg DNA. The data also shows that the adduct formation is bi-phasic in all dose groups. In all these dose groups the adduct levels were maximum on day two followed by a decrease on day four with subsequent increase on day



7 and a gradual decrease up to 70 days (Table 3 and 6). For example, the adduct levels for 600 mg dose group for day 2, 4, 7, 14, 28, 42, 56 and 70 respectively are 75, 44, 70, 42, 20, 13, 10 and 7 p moles tetryl/mg DNA. About 10% of the adducts were intact 70 days after single exposure (Table 6 column 1). Similar trends were observed in kidney DNA-tetryl adducts (Table 4 and 6) with an exception that more than 60% of the adducts (as compared to the maximum adduct level seen after 2 weeks) were intact after 10 weeks (Table 6 column 2). In the spleen the DNA adduct levels reached maximum by day 1 (600, 300 and 150 mg dose groups) and decreased gradually with time. In spleen DNA about 14% of abducts were intact after 70 days (600 mg dose) Table 5 and 6 column 3). Figure 9 depicts the HPLC of liver DNA enzymatic digest and base line resolution of 4 deoxy nucleosides from liver DNA. Chromatographic gradient conditions are also presented. Figure 10 shows the radioactive counts plotted against the liver DNA digest (1 ml fractions from DNA digest chromatography (Fig. 9) and based on retention times where radioactivity was eluted). It is clear from the chart most of the radioactivity was not recovered from the column. About 22% of the radioactivity eluted could not be identified due to lack of standards. The radioactive peaks with retentimes similar to picric and picramic acids accounted for 4 and

8% respectively. Two of the unknown radioactive peaks were closely associated with deoxy guanosine and deoxy adenosine suggesting that these are the adducts of guanine and adenine. Picric and picramic acids are released during enzyme digestion. Figure 11 shows the radioactivity plot with kidney DNA digest. Similar to the liver, 63% of the radioactivity was not recovered and 19% of the radioactivity eluted could not be identified. Fifteen per cent of the radioactivity recovered was identified as picric acid. In the kidney DNA hydrolysate picramic acid was not detected. The unidentified radioactivity was associated with guanine and adenine and considered to be adducts of adenine and guanine.

#### **CONCLUSIONS:**

Tetryl forms adducts with blood proteins and tissue DNA in a dose dependent manner. Unlike the adducts formed with nitroaromatic compounds (TNB and DNB), the adducts formed with nitramine (tetryl) are very stable and not hydrolyzable with acid or alkali. Protein and DNA adducts of tetryl can be used as exposure biomarkers, with proper characterization and development of chemically based analytical methods (non-radioactive) for proteins and DNA. Further studies are needed for methods development.

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# APPENDIX A

**Figure 1**

**Blood Fractionation Protocol**

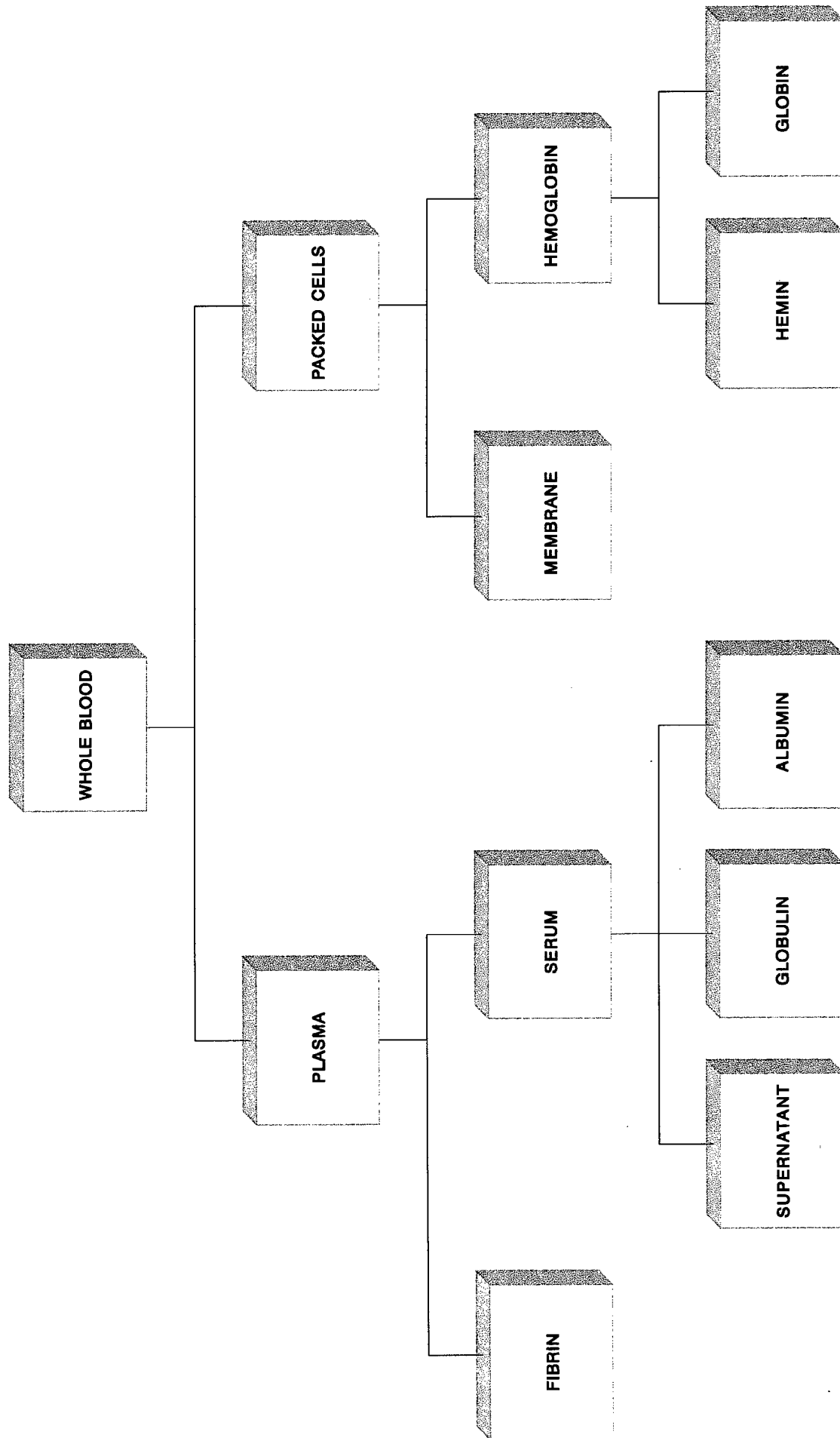




Figure 2

FORMATION AND PERSISTENCE OF TNB BLOOD PROTEIN ADDUCTS

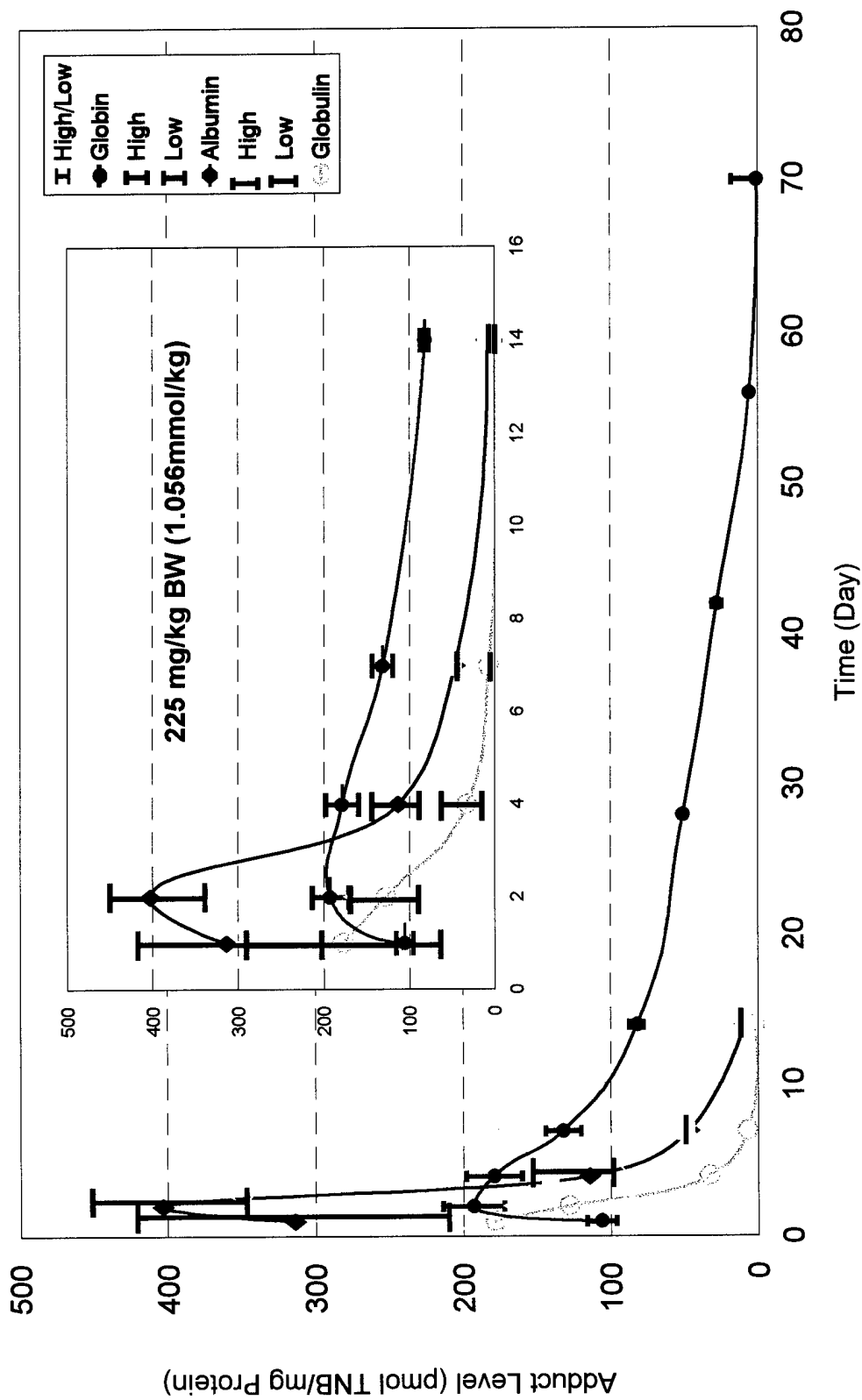


Figure 3

DOSE DEPENDENT FORMATION OF TNB ALBUMIN ADDUCTS

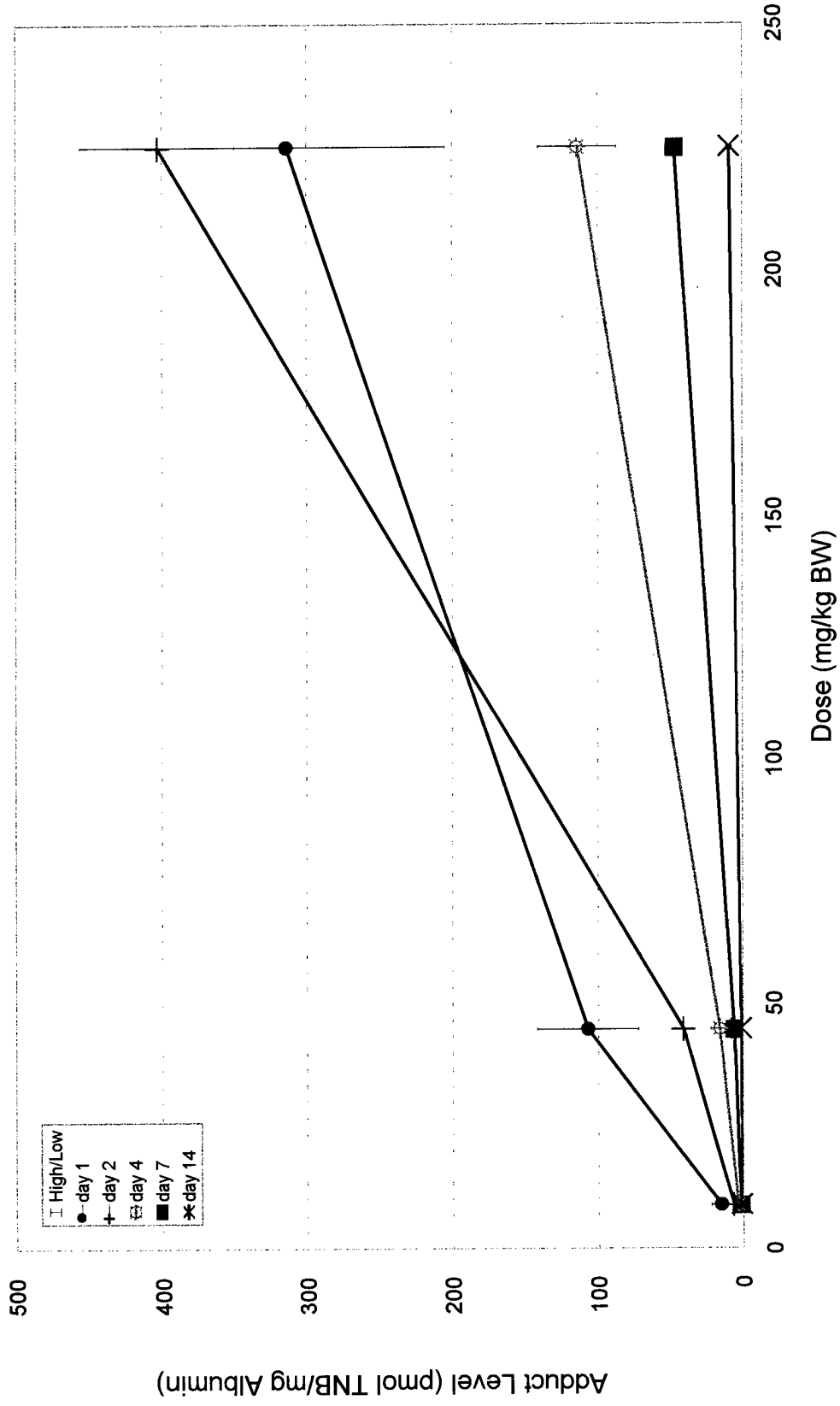


Figure 4

DOSE DEPENDENT FORMATION OF TNB GLOBIN ADDUCTS

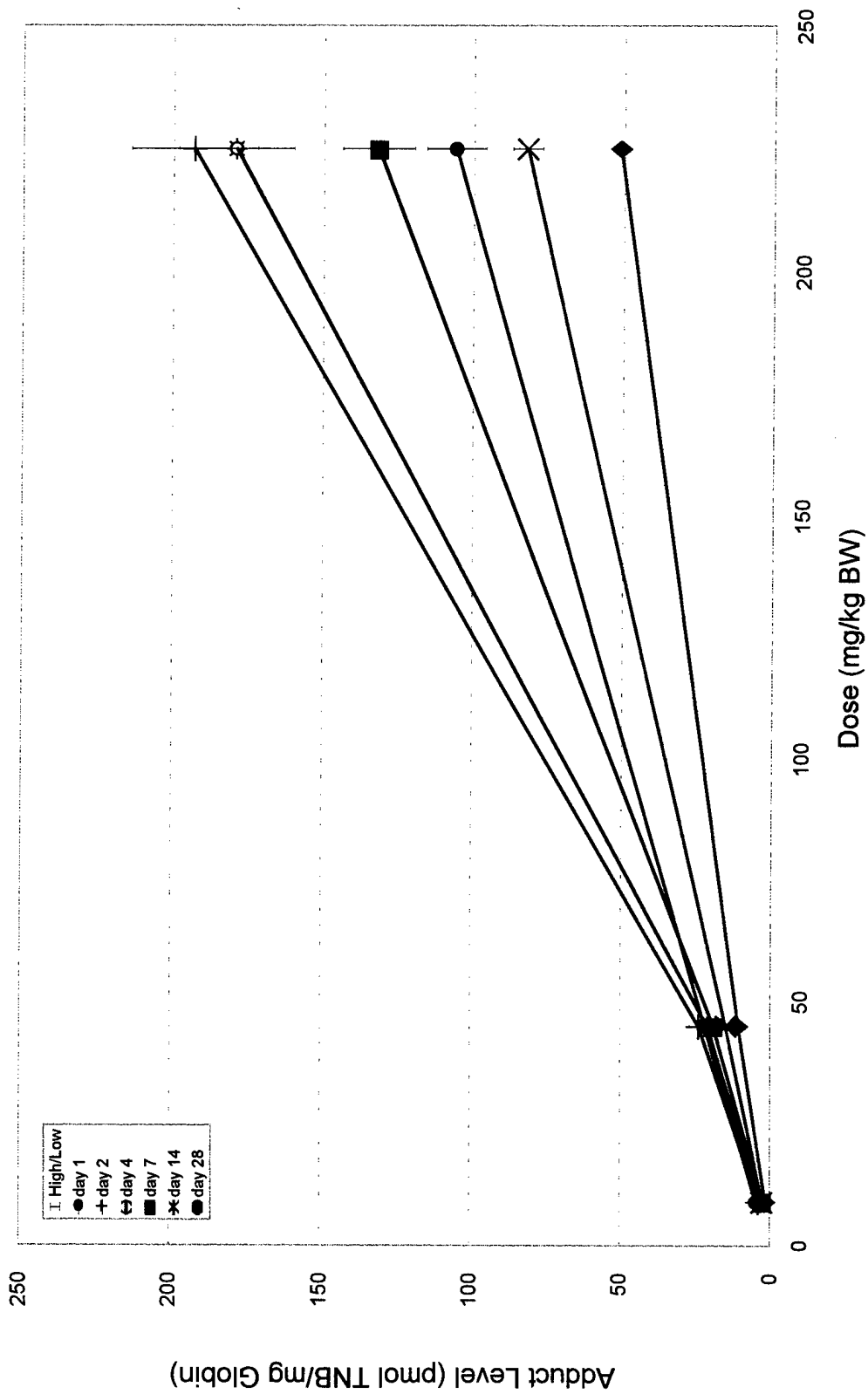


Figure 5

DOSE DEPENDENT FORMATION OF TNB DNA ADDUCTS IN THE LIVER

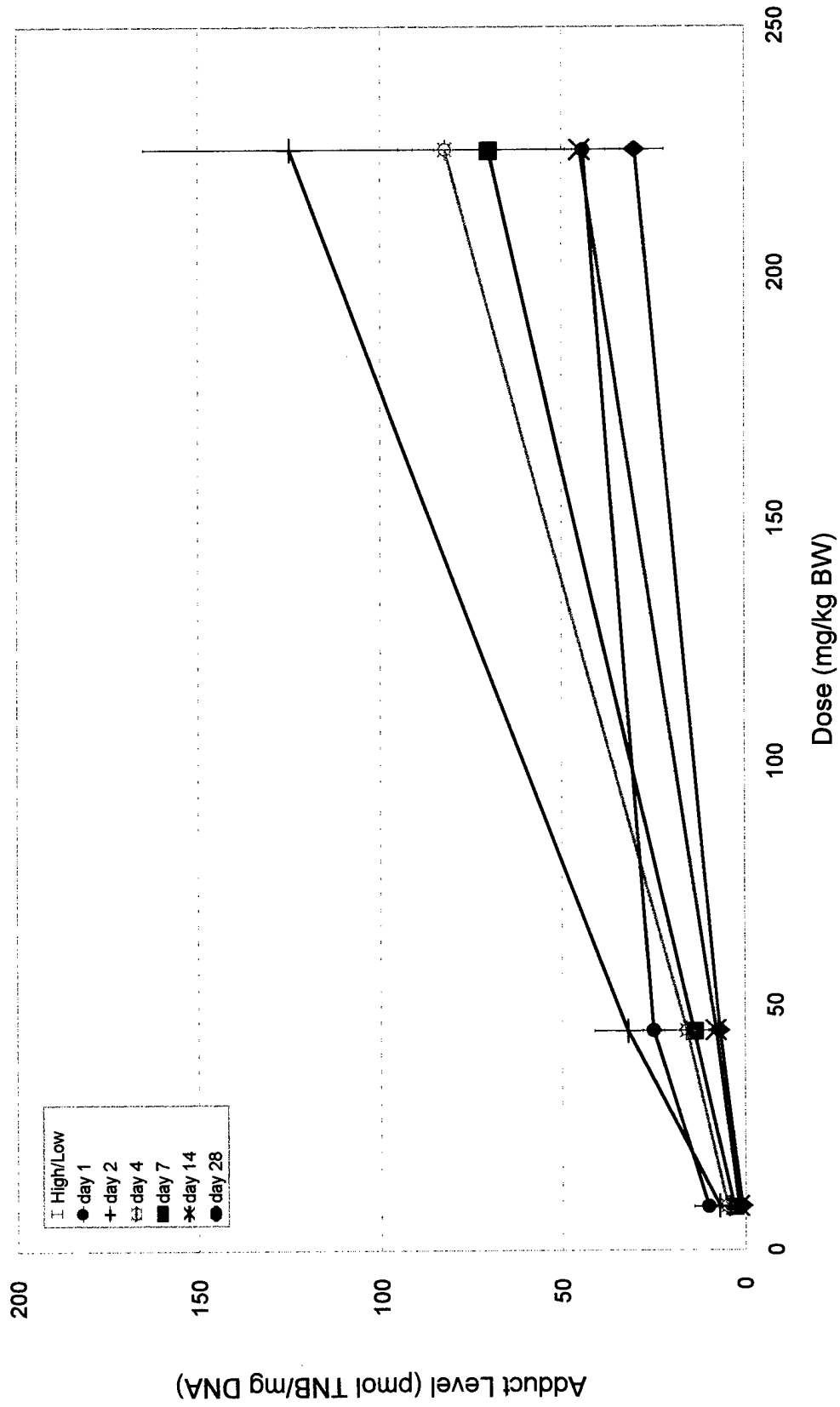


Figure 6  
 FORMATION AND PERSISTENCE OF TNB DNA ADDUCTS

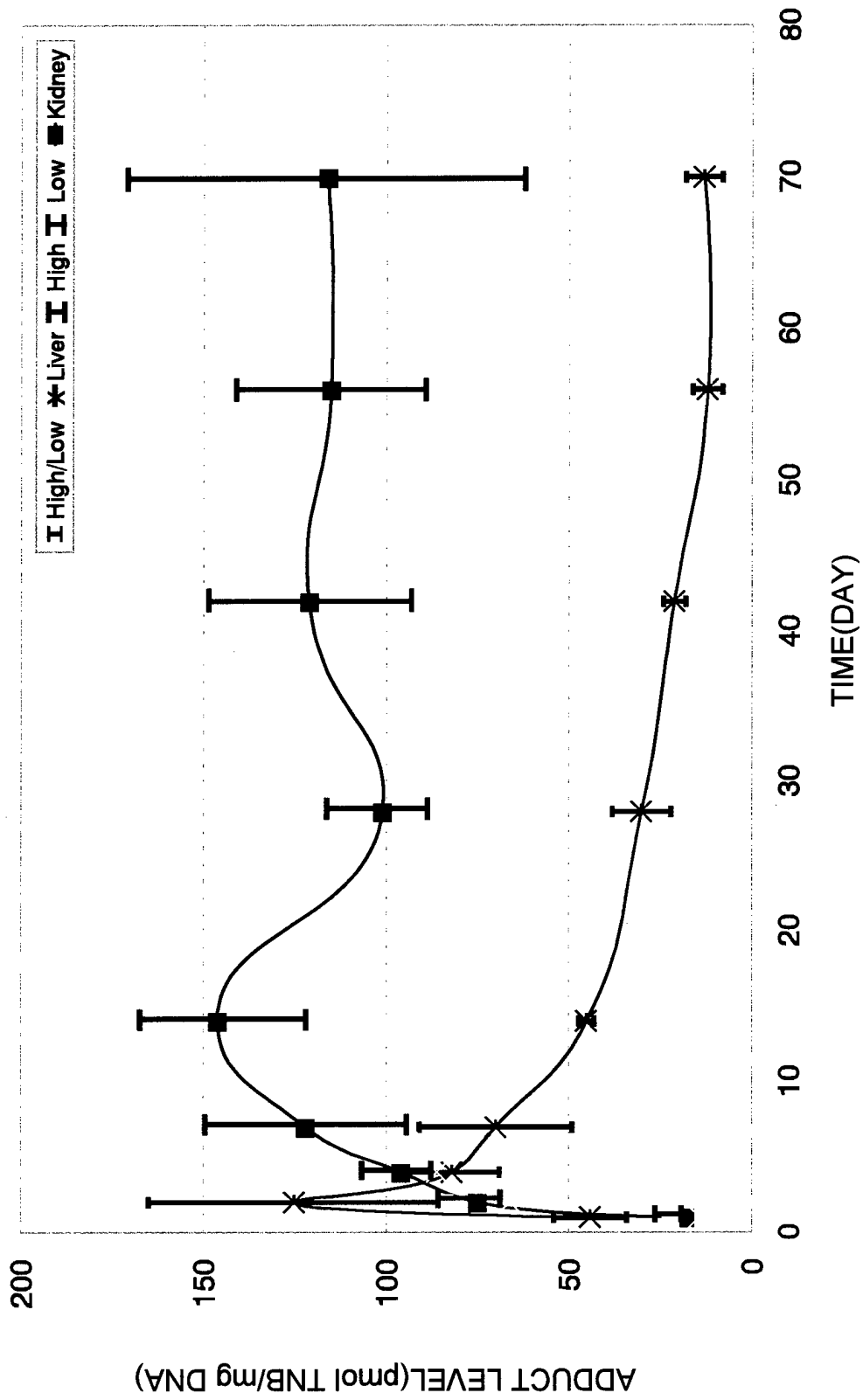


Figure 7

HPLC of Standard Nucleoside Mixture

W600 Gradient Table

#	Time (min)	Flow (ml)	%A (%)	%B (%)	%C (%)	%D (%)	Curve
1	0.00	3.00	95.0	5.0	0.0	0.0	0
2	10.00	3.00	85.0	15.0	0.0	0.0	6
3	20.00	3.00	50.0	50.0	0.0	0.0	6
4	25.00	3.00	0.0	100.0	0.0	0.0	6
5	50.00	3.00	0.0	100.0	0.0	0.0	6
6	55.00	3.00	95.0	5.0	0.0	0.0	6

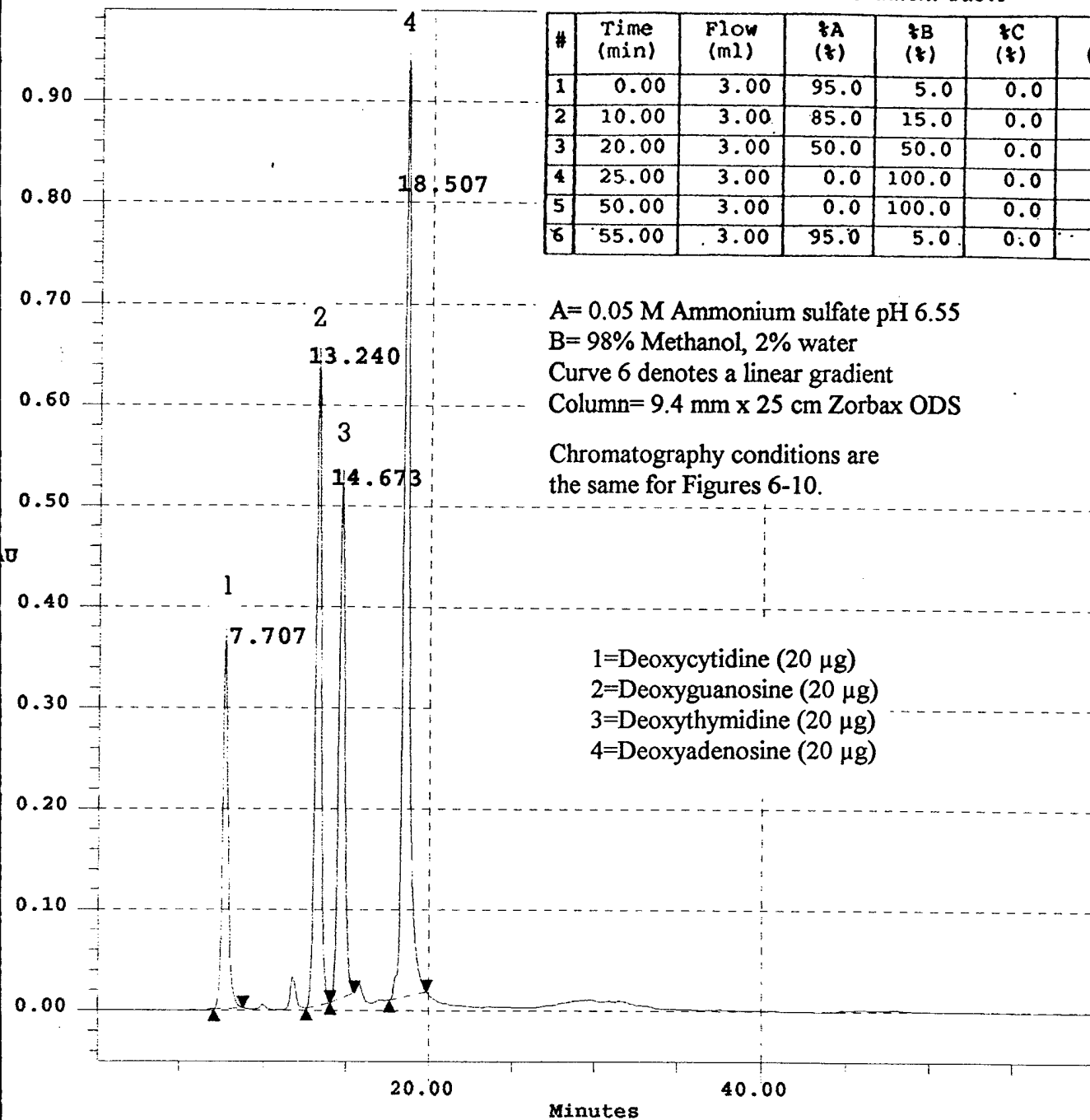


Figure 8

HPLC of TNB Metabolite Standards

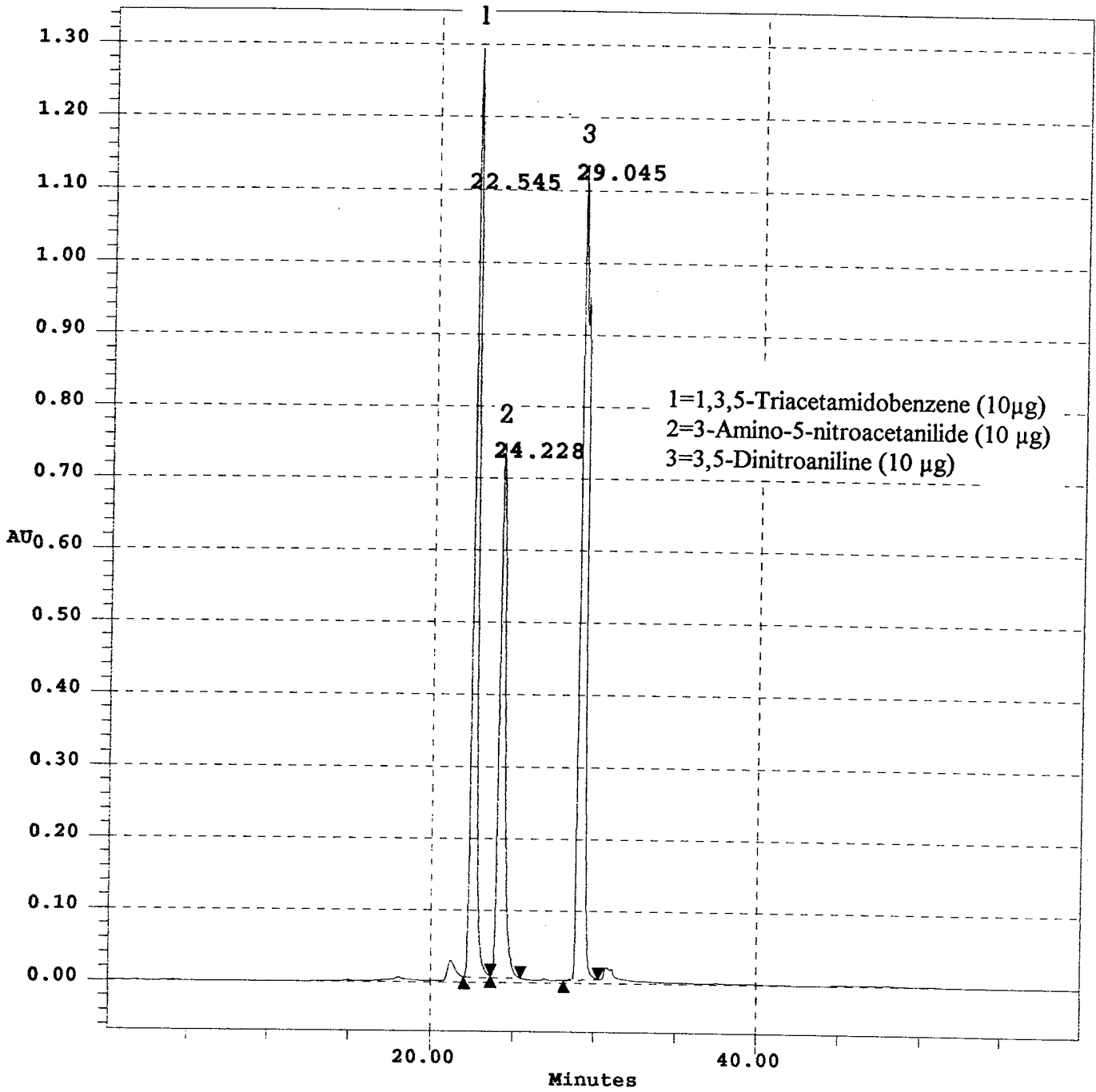


Figure 9

HPLC of DNA Nucleosides and TNB Metabolite Standards

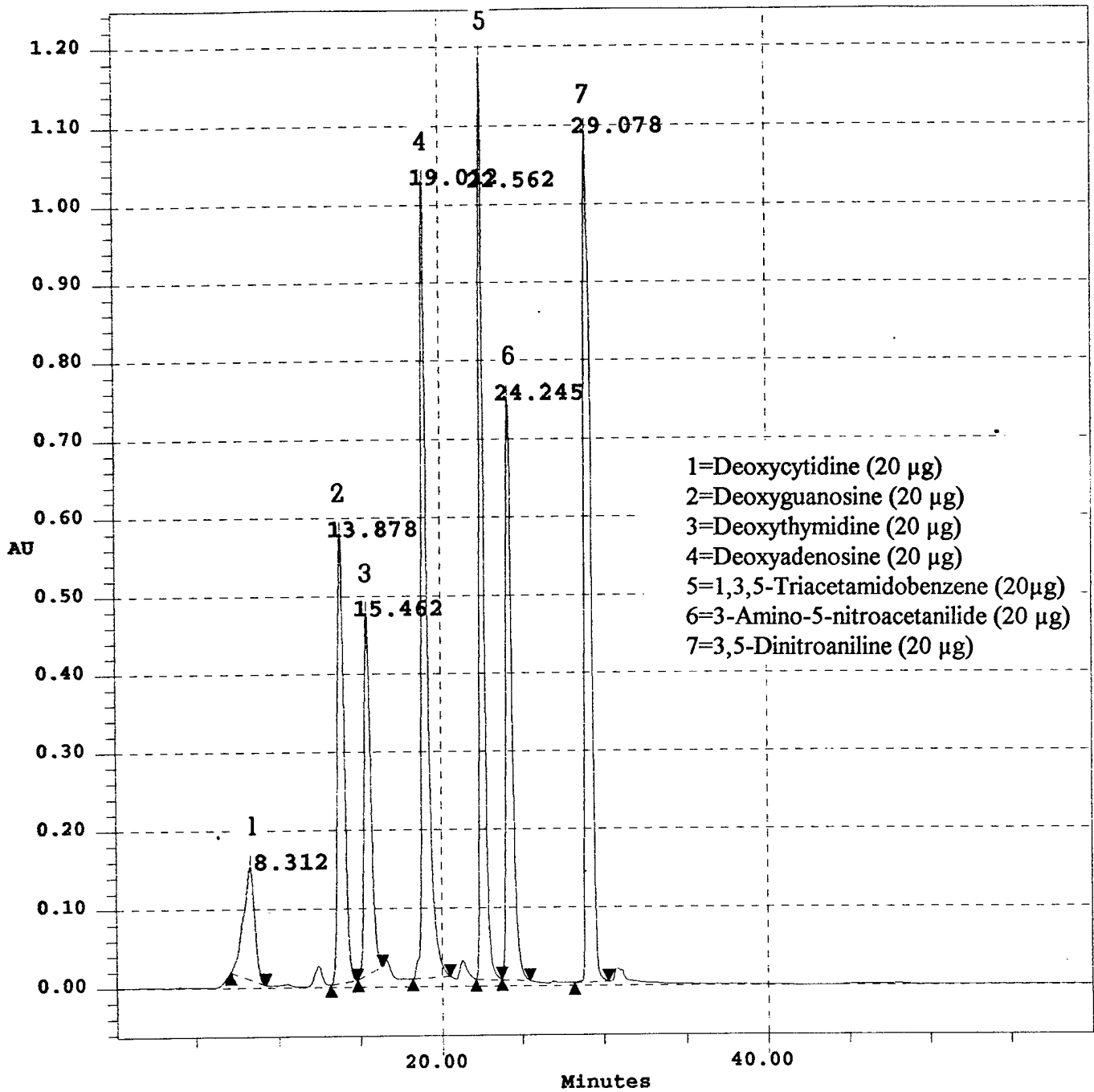
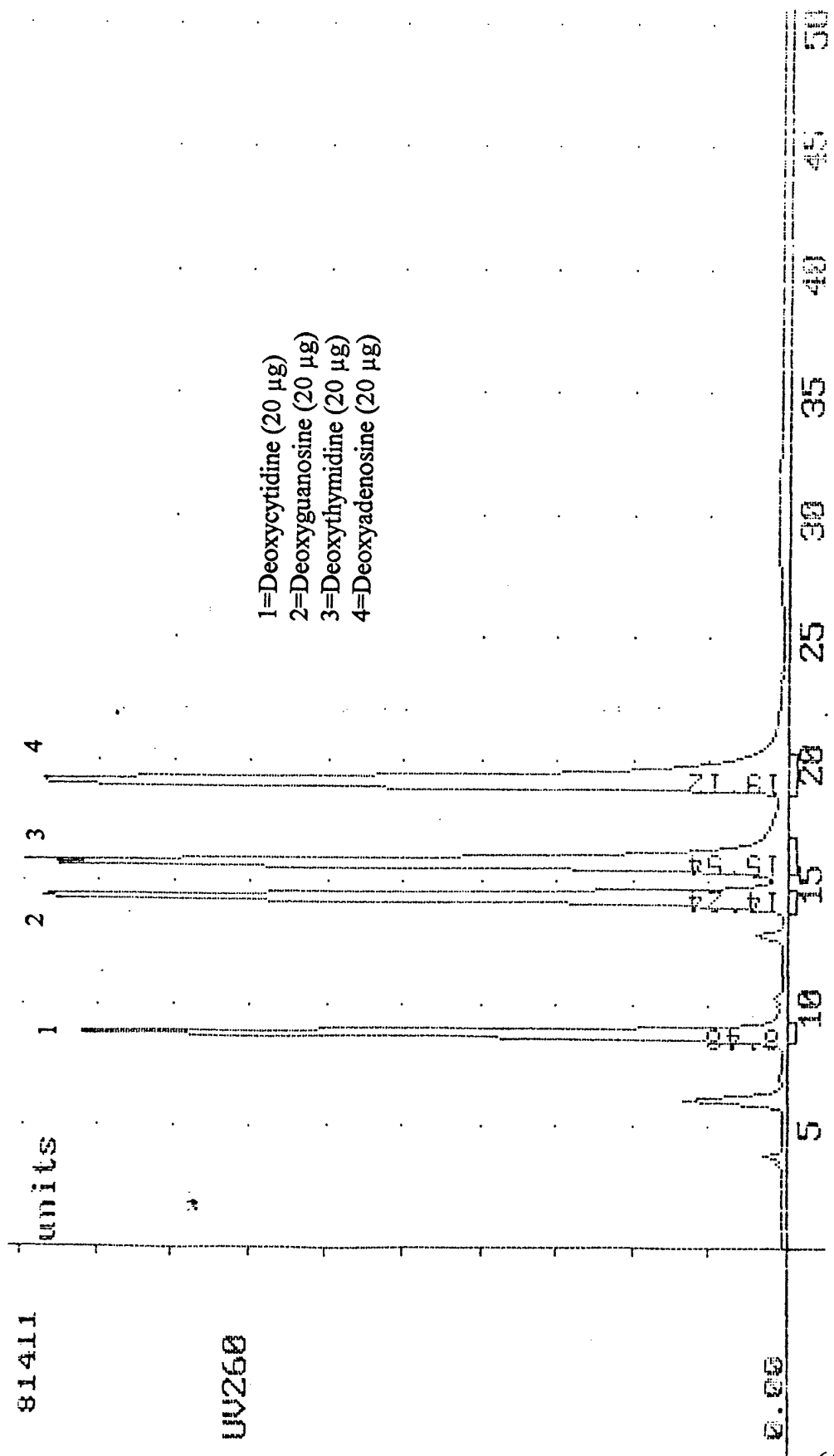




Figure 10  
HPLC of Liver DNA Digest from Rats Treated with TNB (225 mg/kg BW)



**TABLE 1**

**ACID LABILE RADIOACTIVITY % RECOVERERED IN ALBUMIN SUPENATANT AND HEME FRACTION DURING ALBUMIN AND GLOBIN PRECIPITATION**

Sacrifice Time (Days)	Dose 225 mg TNB/kg BW	
	% Radioactivity in Albumin supernatant	% Radioactivity in Globin supernatant
1	50±12	50± 7
2	45±8	37±6
4	31±9	24±6
7	5±1	12±2
14	ND	10±3
28	ND	10±2
42	ND	10±2

Mean± standard deviation(N = 5)

ND = Not detected

**TABLE 2**

**CLEARANCE OF <sup>14</sup>C] TNB AND/ OR METABOLITE(S) FROM RAT  
PLASMA AFTER SINGLE ORAL DOSE**

Sacrifice Time(Days)	<u>Picomole TNB/20 <math>\mu</math>l plasma</u> TNB Dose		
	225 mg/kg BW	45 mg /kg BW	9 mg /kg BW
1	1696 $\pm$ 355	389 $\pm$ 201	19.56 $\pm$ 5.6
2	1944 $\pm$ 822	44.5 $\pm$ 9.70	7.68 $\pm$ 1.17
4	92 $\pm$ 9	15.95 $\pm$ 6.03	3.67 $\pm$ 0.48
7	37 $\pm$ 1.4	6.11 $\pm$ 1.12	1.56 $\pm$ 0.34
14	7 $\pm$ 0.04	1.18 $\pm$ 0.19	0.23 $\pm$ 0.17
28	1 $\pm$ 0.08	0.18 $\pm$ 0.08	0.07 $\pm$ 0.03

Mean  $\pm$  standard deviation (N = 5)

**TABLE 3**

**FORMATION AND PERSISTENCE OF TNB-GLOBIN ADDUCTS  
IN RAT AFTER SINGLE ORAL EXPOSURE<sup>A</sup>**

<b>Sacrifice Time Weeks</b>	<b>Picomoles TNB/mg globin</b>
1	131.8±26.2
2	82.2±11.1
4	50.7±3.7
6	28.2±2.9
8	5.6±1.2
10	0.8±0.2

Mean ± standard deviation (N= 5)

A= 225 mg/kg BW

**TABLE 4**

**FORMATION AND PERSISTENCE OF TNB ADDUCTS WITH RAT LIVER DNA AFTER SINGLE ORAL EXPOSURE**

Sacrifice Time(Days)	Picomole TNB/mg DNA TNB Dose		
	225 mg/kg BW	45 mg/kg BW	9 mg /kg BW
1	44.4 ± 9.53	24.52± 2.52	9.94± 3.63
2	125.36±39.65	31.63± 8.76	7.15± 1.19
4	82.07±13.10	16.03± 5.94	4.86± 0.67
7	69.53±20.86	14.06± 3.72	3.22± 1.09
14	44.99± 2.17	7.95± 1.2	1.66± 0.65
28	30.43± 8.28	6.52± 2.24	0.82± 0.13
42	21.39± 3.43	ND	ND
56	11.63± 3.59	ND	ND
70	12.78± 4.59	ND	ND

Mean ± standard deviation (N= 5)

ND = Not detected

**TABLE 5**  
**FORMATION AND PERSISTENCE OF TNB ADDUCTS WITH RAT**  
**KIDNEY DNA AFTER SINGLE ORAL EXPOSURE**

Sacrifice Time/Days	<u>Picomole TNB/mg DNA</u> TNB Dose		
	225 mg/kg BW	45 mg/kg BW	9 mg/kg BW
1	67.73±20.97	24.78± 5.34	ND
2	79.23± 9.39	28.68±25.85	2.11± 0.47
4	57.47± 8.48	7.00± 1.62	2.43± 0.77
7	36.61± 6.48	7.04± 1.75	ND
14	26.44± 6.12	5.54± 1.9	1.98± 0.92
28	15.88± 7.09	4.25± 2.67	1.17± 0.42
42	11.55± 3.80	ND	
56	6.23± 1.23	ND	ND
70	6.73± 2.27	ND	ND

Mean ± standard deviation (N = 5)

ND = Not detected

TABLE 6

FORMATION AND PERSISTENCE OF TNB ADDUCTS WITH RAT  
SPLEEN DNA AFTER SINGLE ORAL EXPOSURE

Sacrifice Time/Days	Picomole TNB/mg DNA TNB Dose		
	225 mg/kg BW	45 mg/kg BW	9 mg/kg BW
1	7.45±3.05	6.01± 2.36	2.14± 0.6
2	14.30± 1.32	3.11±0.78	1.03± 0.24
4	26.74± 1.98	3.18± 0.97	1.18± 0.23
7	24.95± 6.62	2.51± 0.75	0.98±0.16
14	39.04±11.00	2.71± 0.97	0.61± 0.21
28	30.29±9.45	1.49±0.41	0.37± 0.24
42	21.12±10.11	ND	ND
56	11.94±3.36	ND	ND
70	11.44± 4.98	ND	ND

Mean ± standard deviation (N = 5)

ND = Not detected

# APPENDIX B



**Figure 1**  
**Blood Fractionation Protocol**

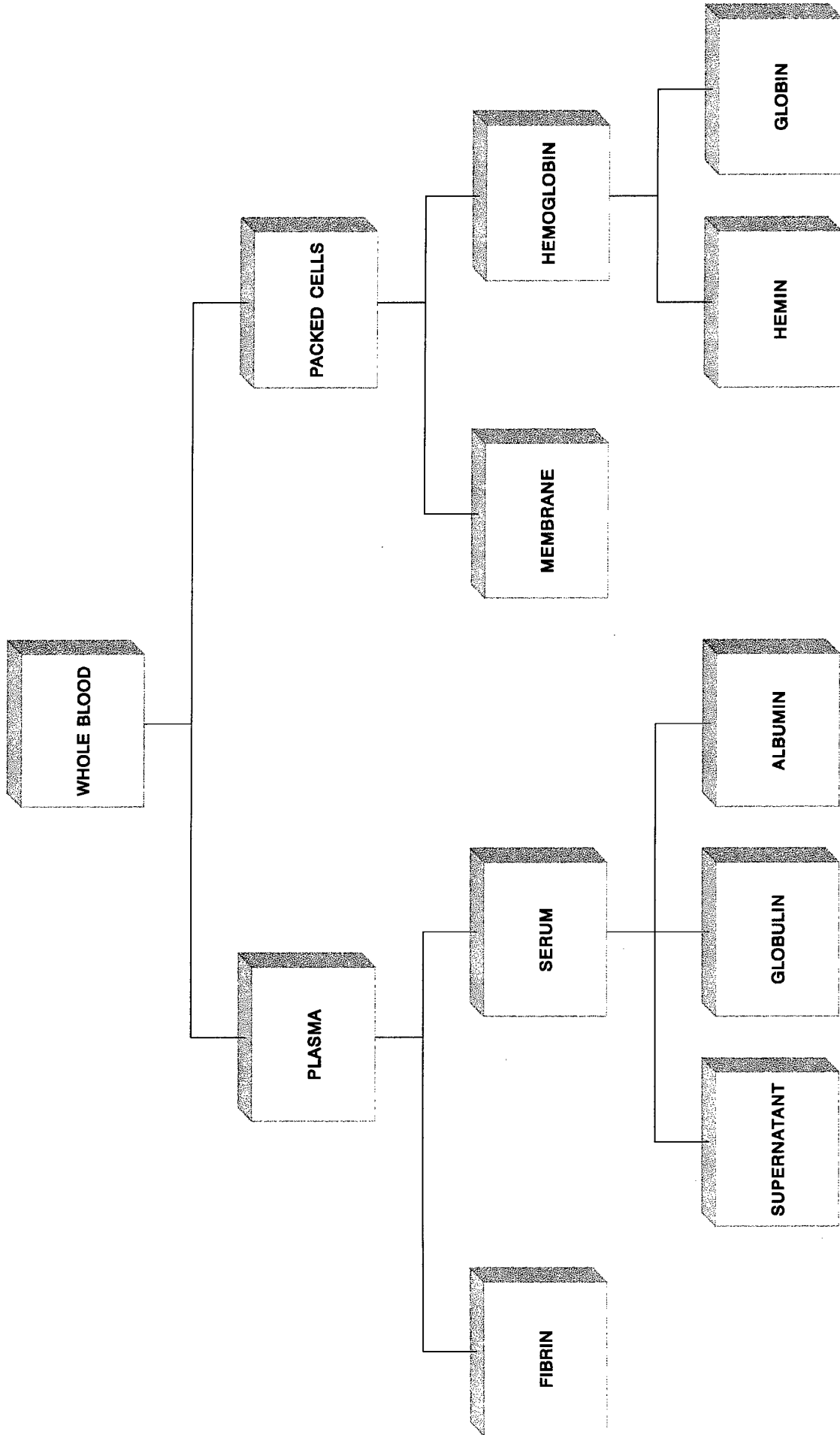


Figure 2

FORMATION AND PERSISTENCE OF SERUM ADDUCTS IN RATS TREATED WITH  
DNB (30mg/kg BW)

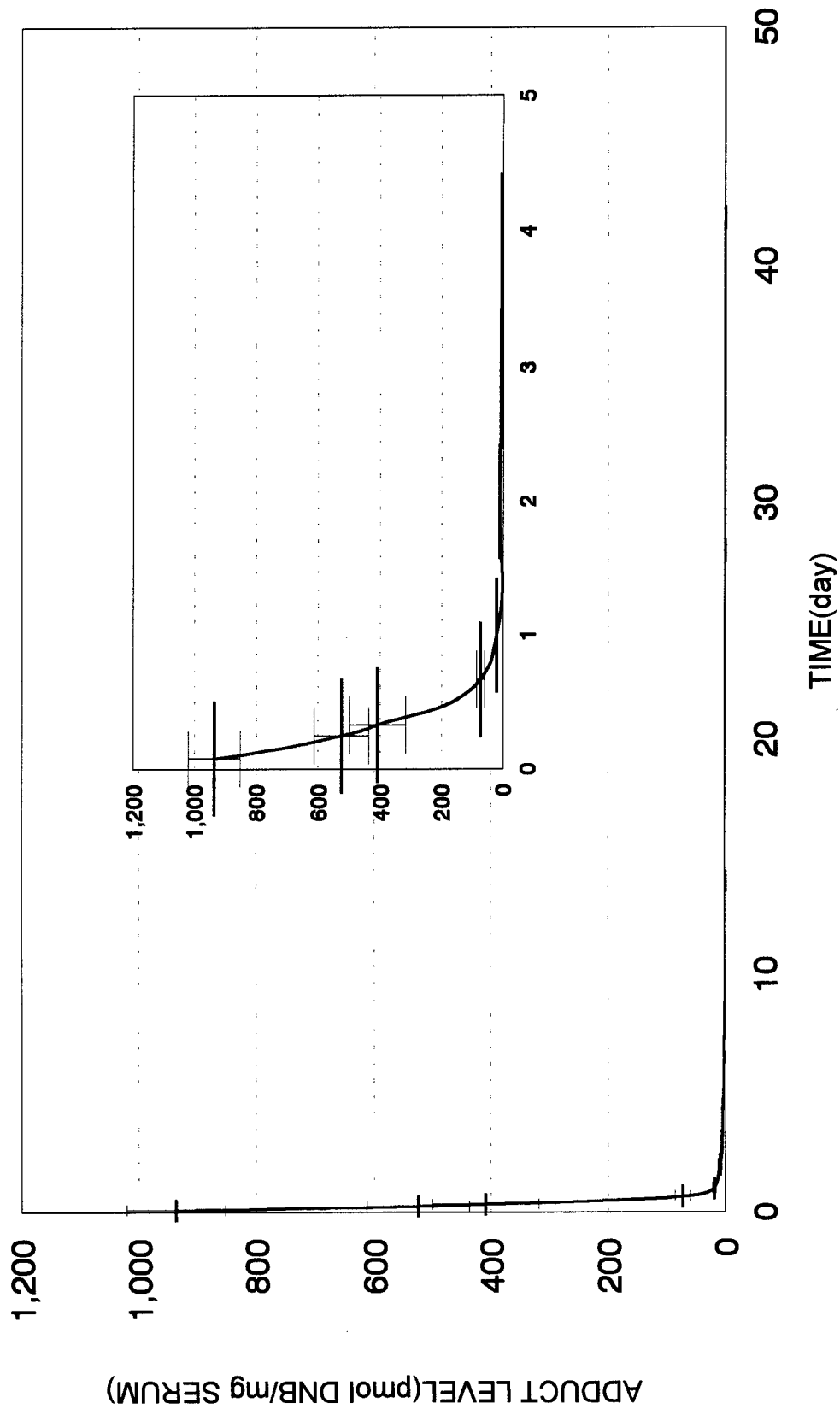


Figure 3

FORMATION AND PERSISTENCE OF ALBUMIN ADDUCTS IN RATS TREATED WITH DNB (30mg/kg BW)

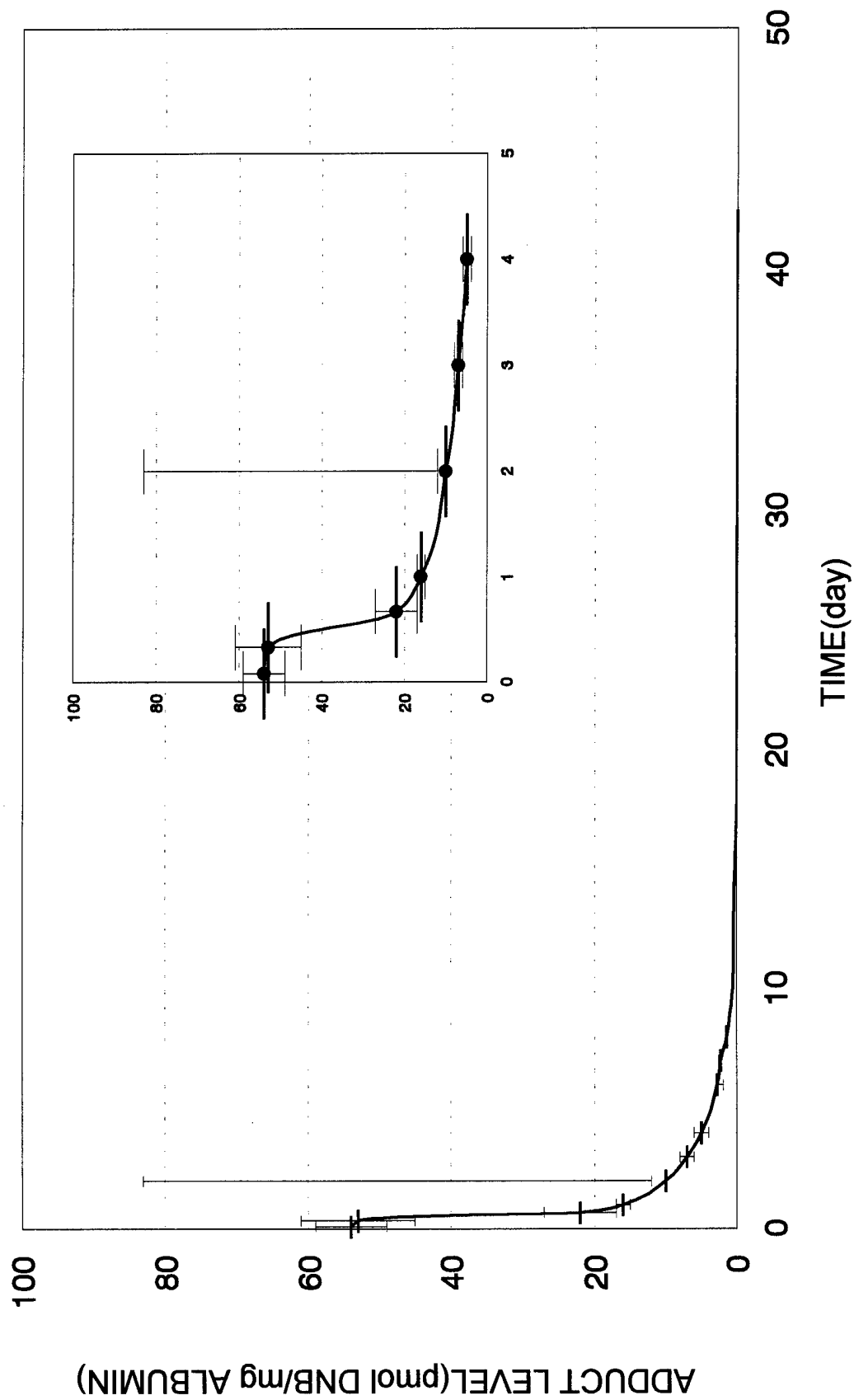


Figure 4

FORMATION AND PERSISTENCE OF GLOBULIN ADDUCTS IN RATS TREATED WITH DNB (30mg/kg BW)

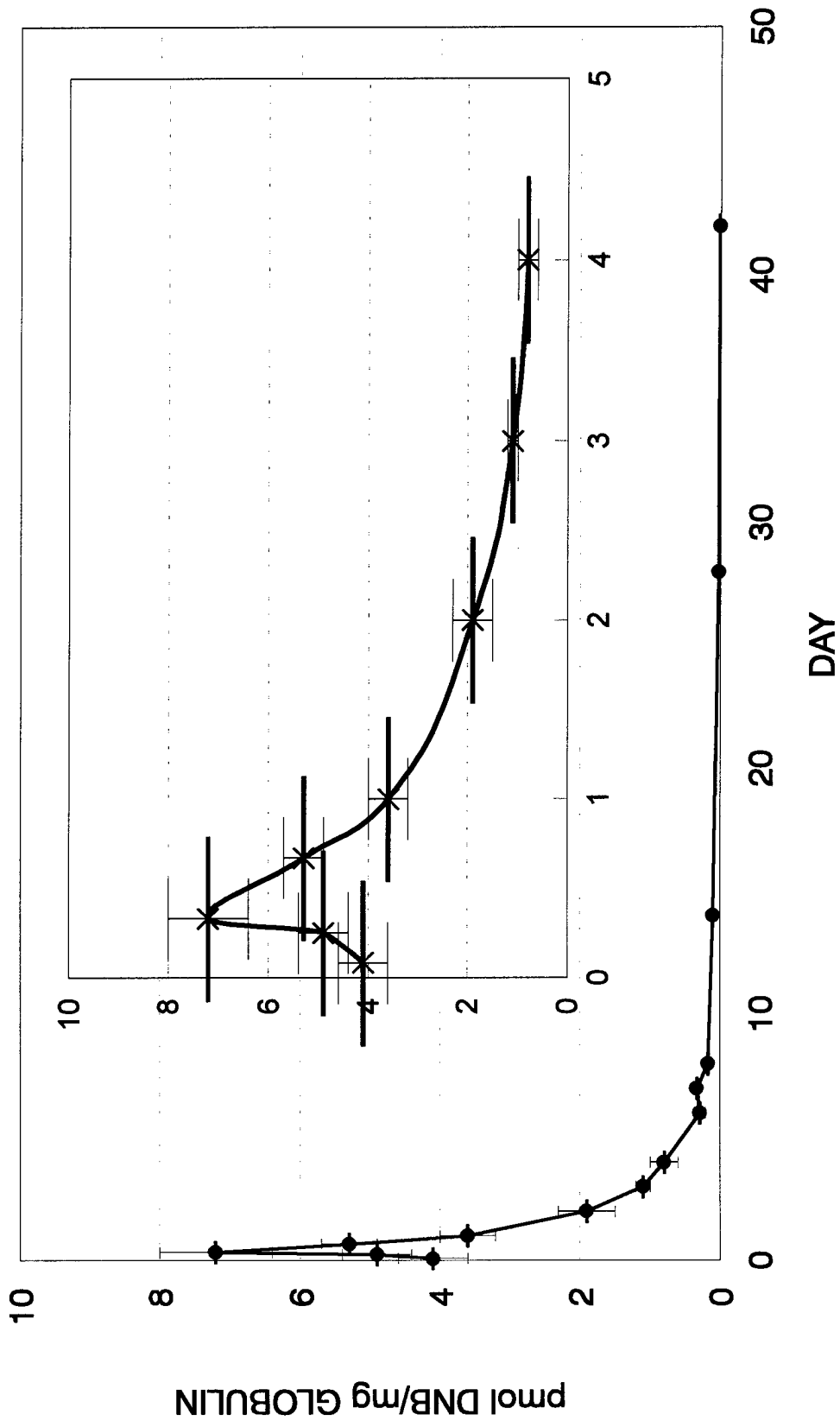


Figure 5

FORMATION AND PERSISTENCE OF HEMOGLOBIN ADDUCTS IN RATS TREATED WITH DNB (30mg/kg BW)

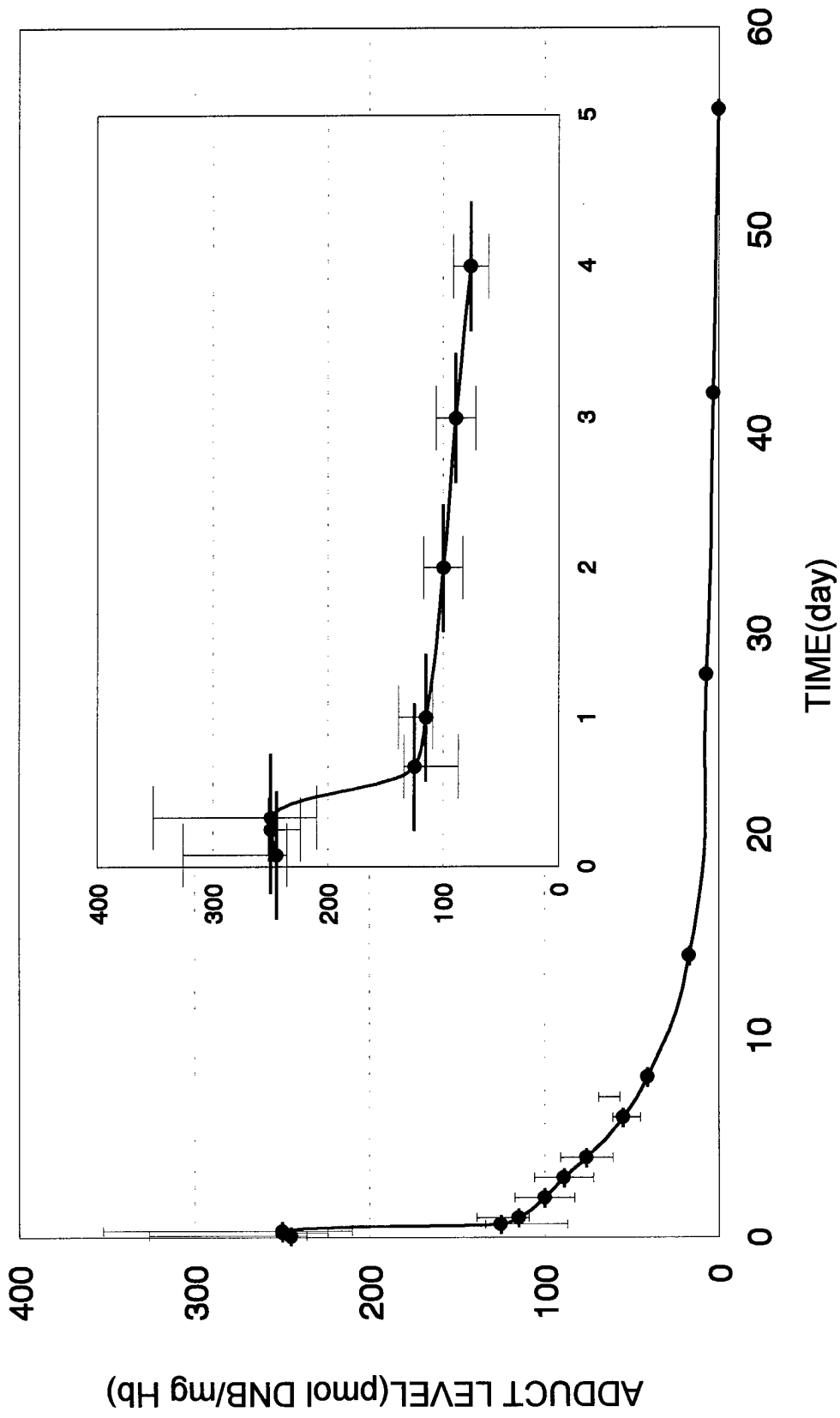


Figure 6

FORMATION AND PERSISTENCE OF GLOBIN ADDUCTS IN RATS TREATED WITH  
DNB (30mg/kg BW)

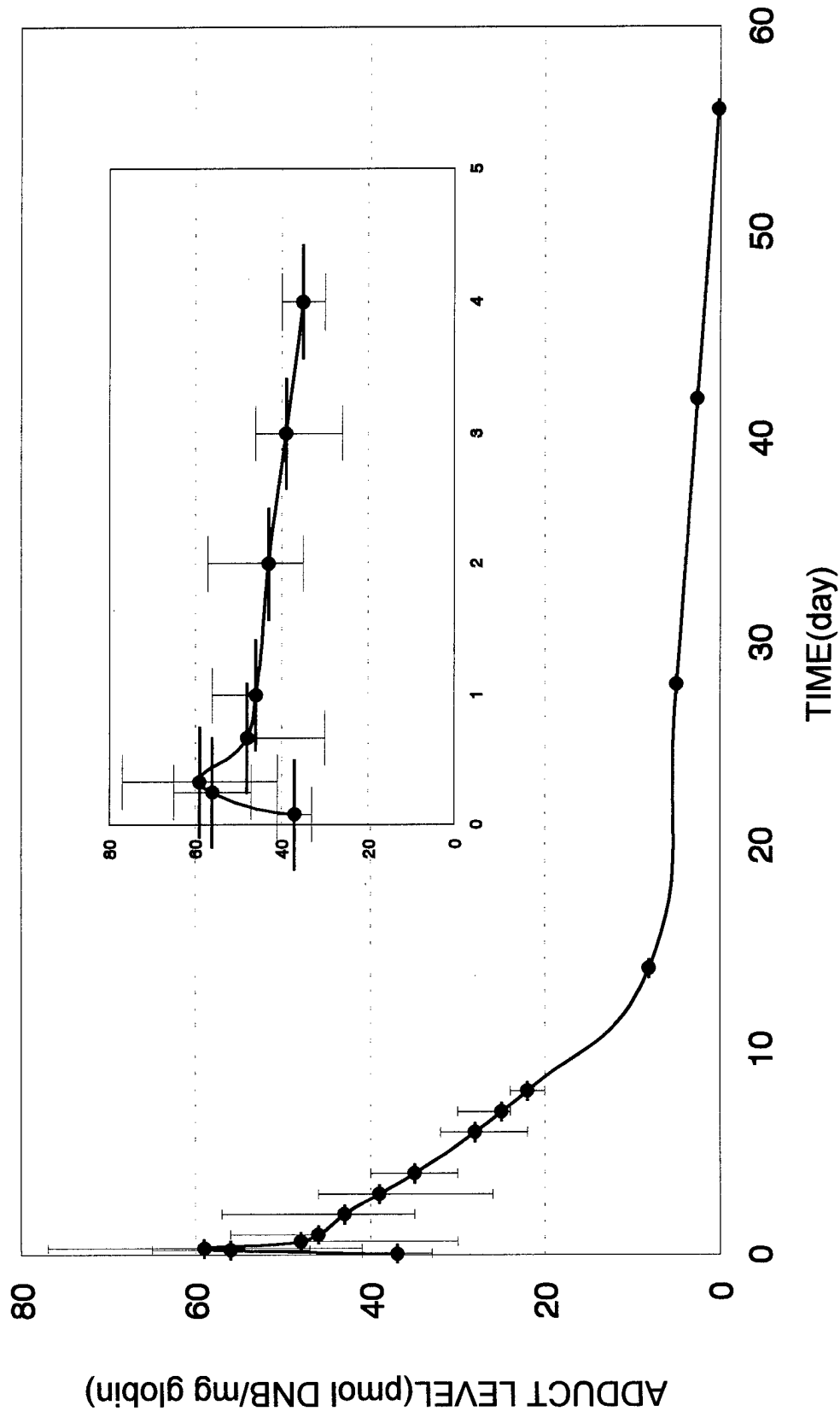


Figure 7

DOSE DEPENDENT FORMATION OF SERUM ADDUCTS IN RATS TREATED WITH DNB

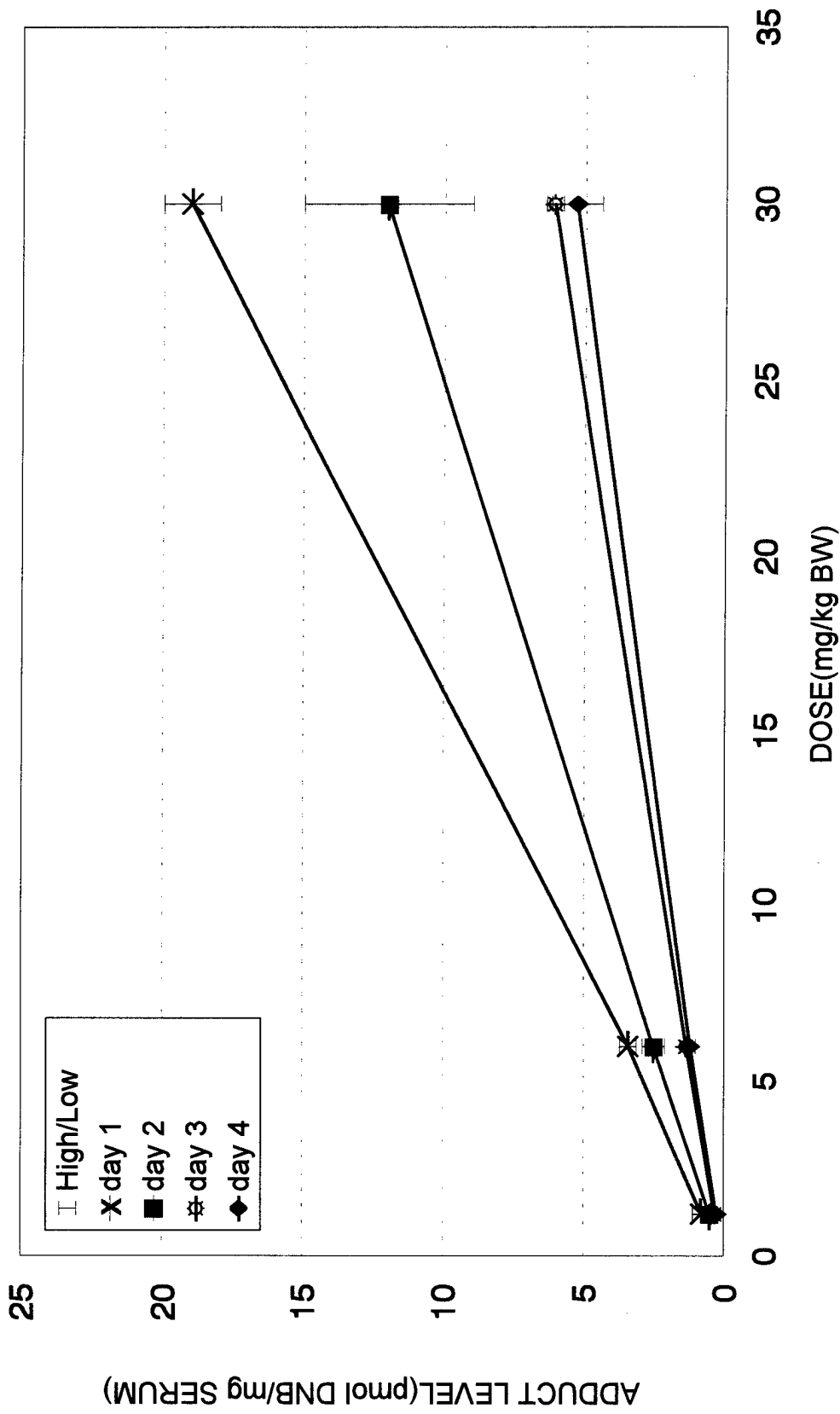


Figure 8

DOSE DEPENDENT FORMATION OF ALBUMIN ADDUCTS IN RATS TREATED WITH DNB

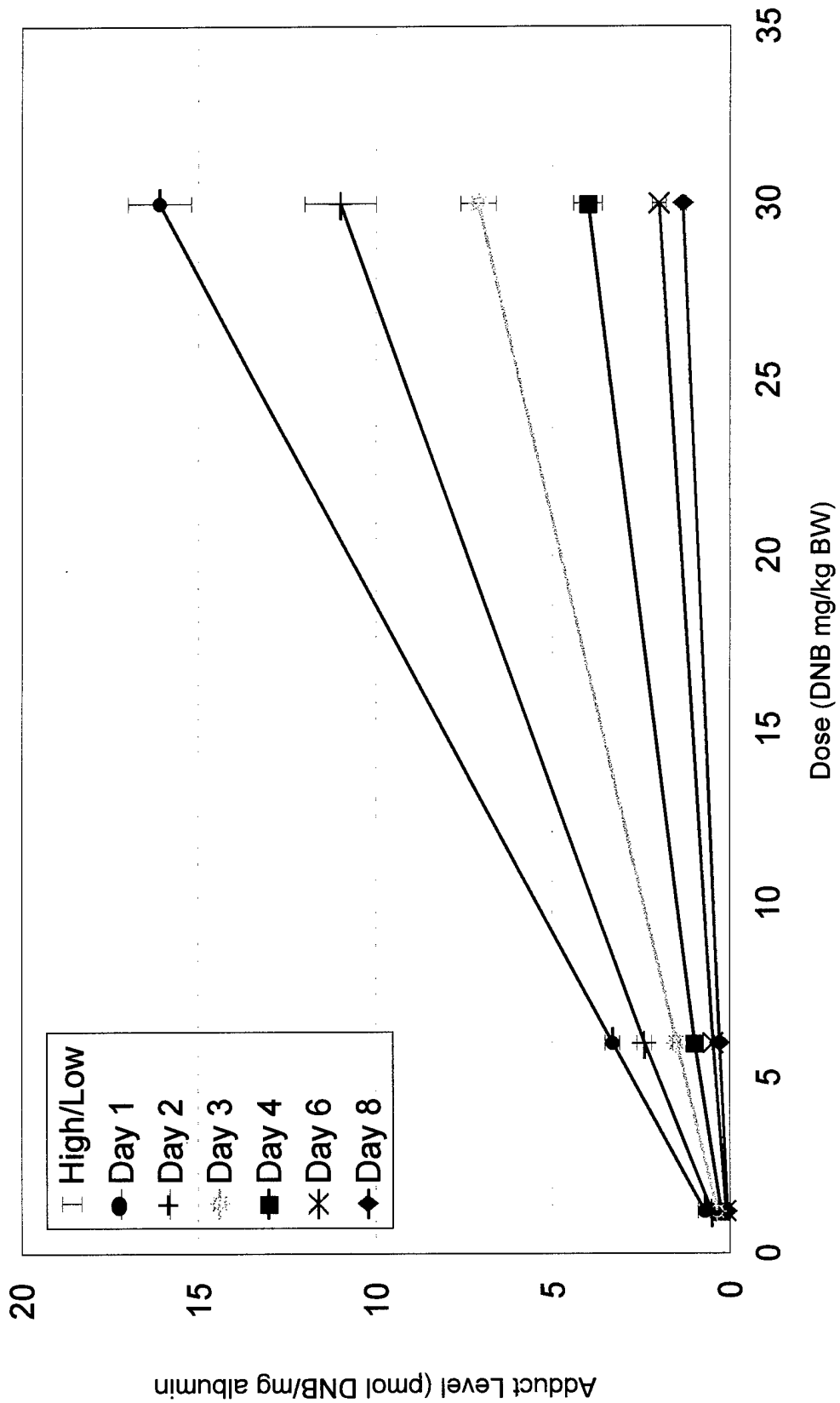




Figure 9

DOSE DEPENDENT FORMATION OF GLOBULIN ADDUCTS IN RATS TREATED WITH DNB

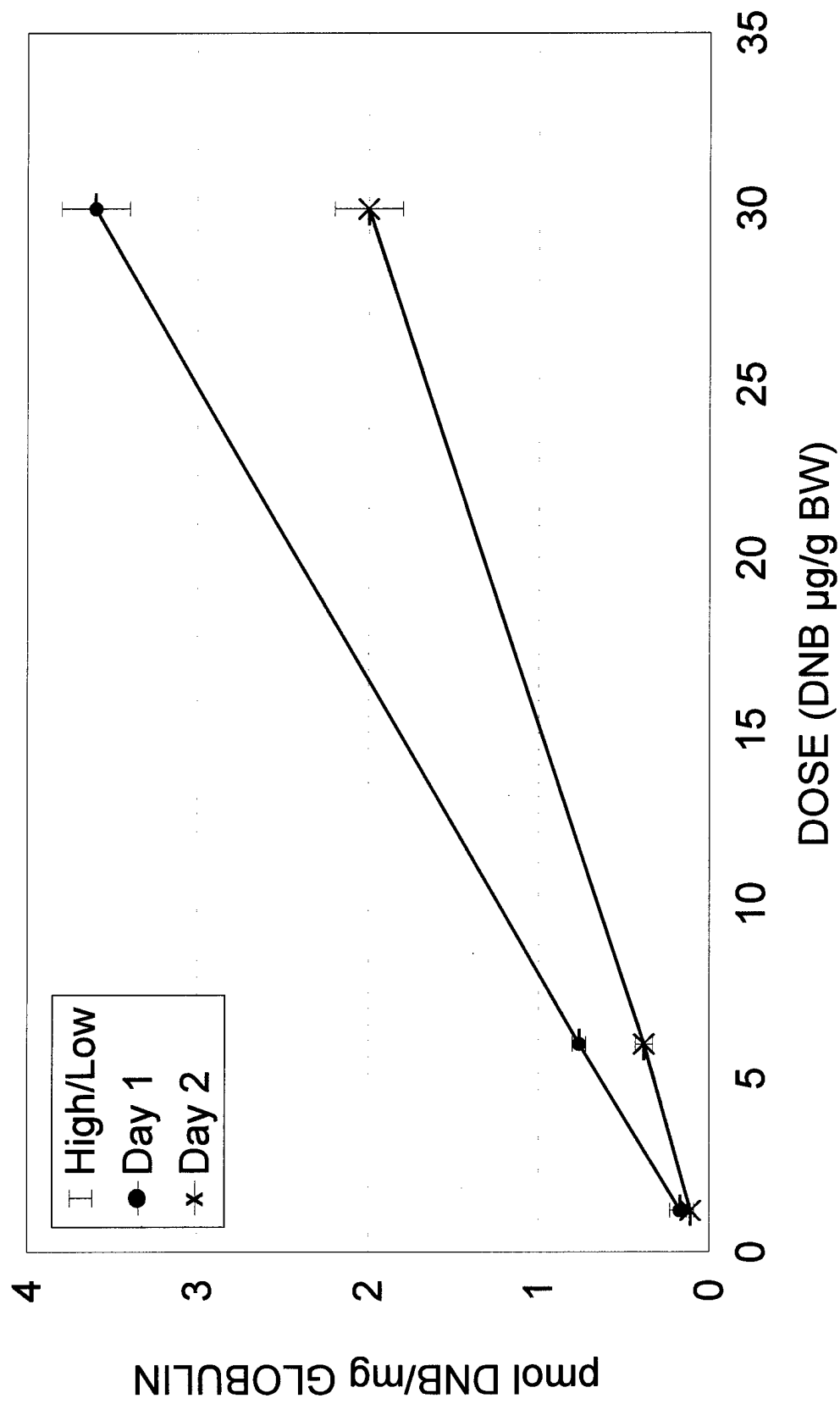


Figure 10

DOSE DEPENDENT FORMATION OF HEMOGLOBIN ADDUCTS IN RATS TREATED WITH DNB

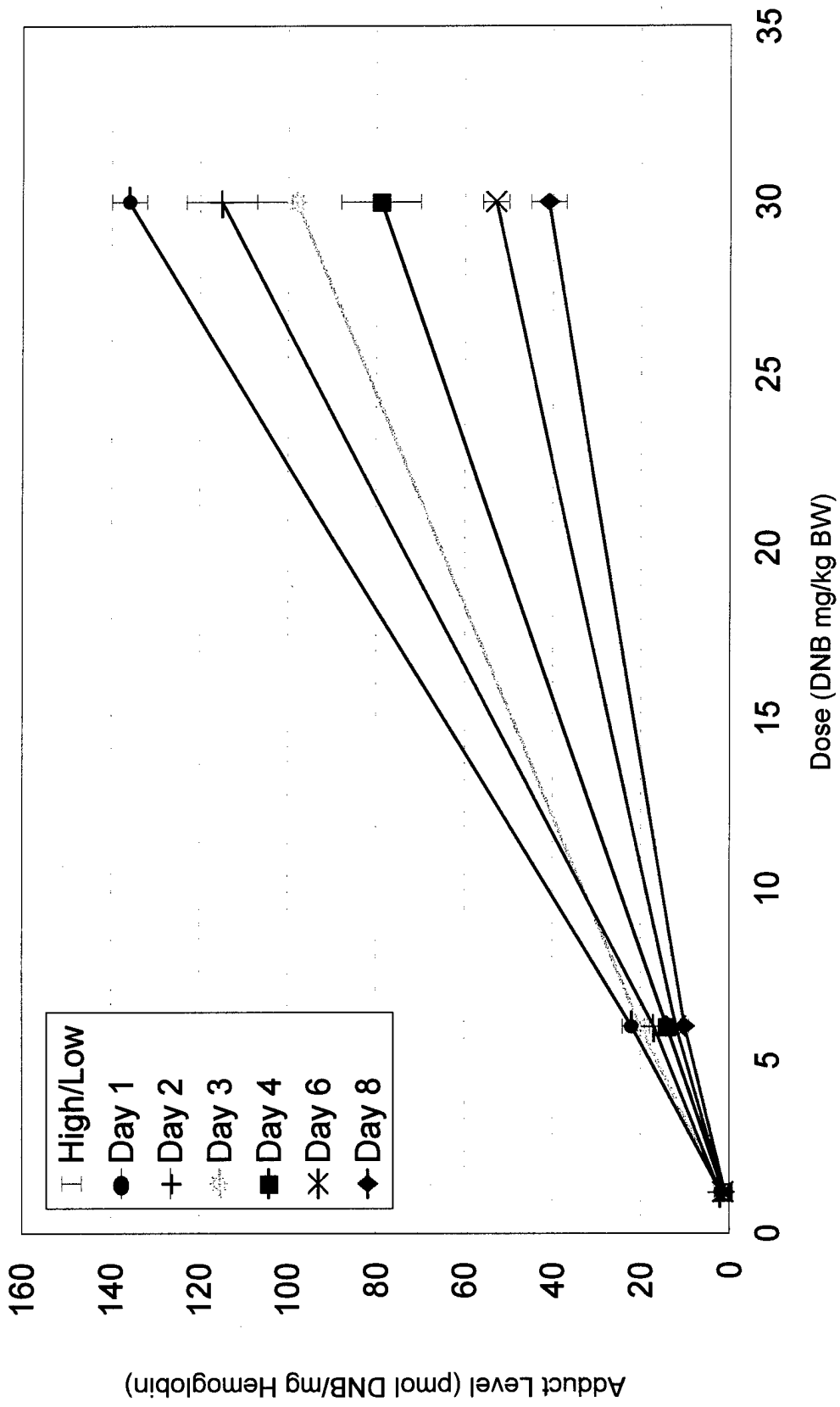


Figure 11

DOSE DEPENDENT FORMATION OF GLOBIN ADDUCTS IN RATS TREATED WITH DNB

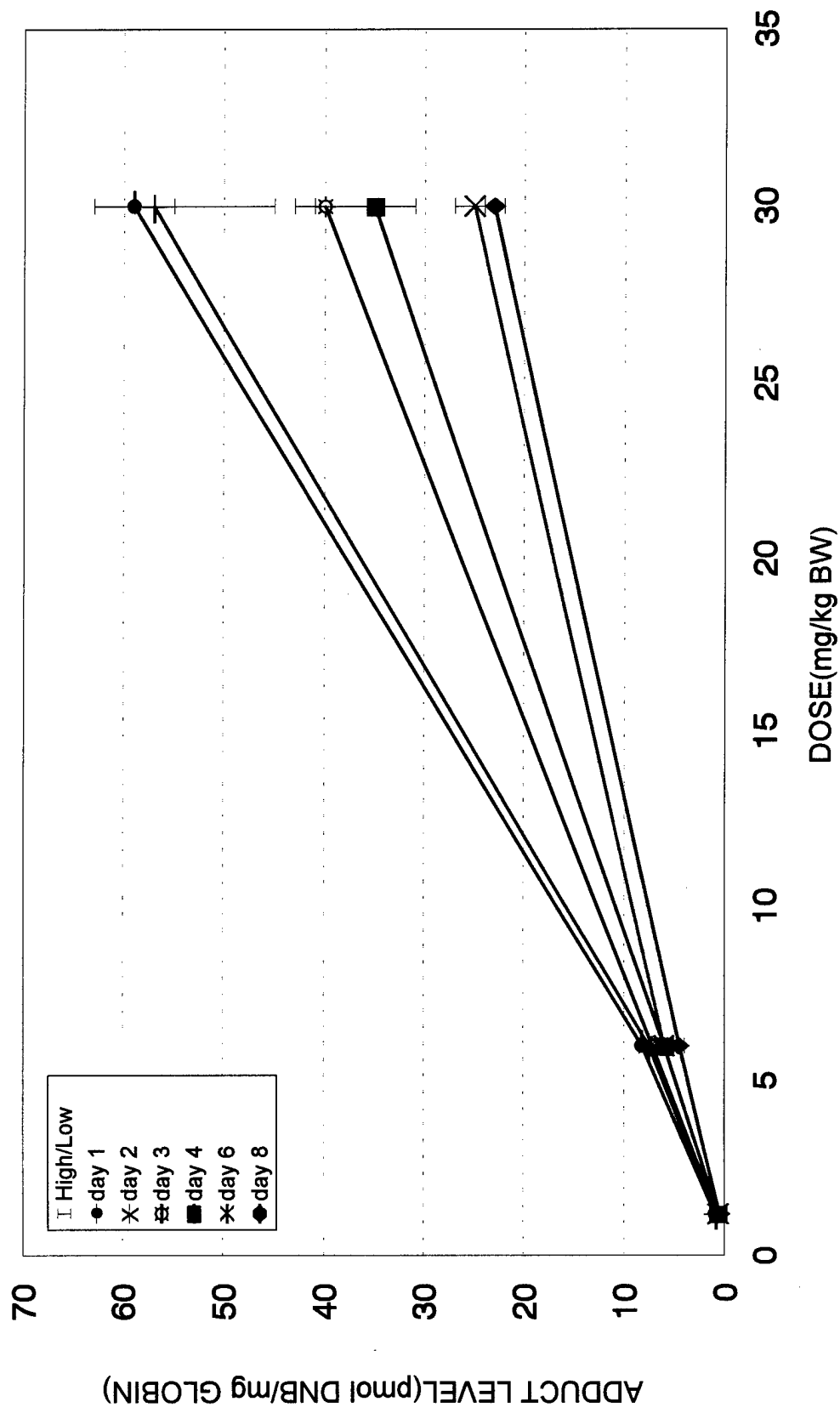
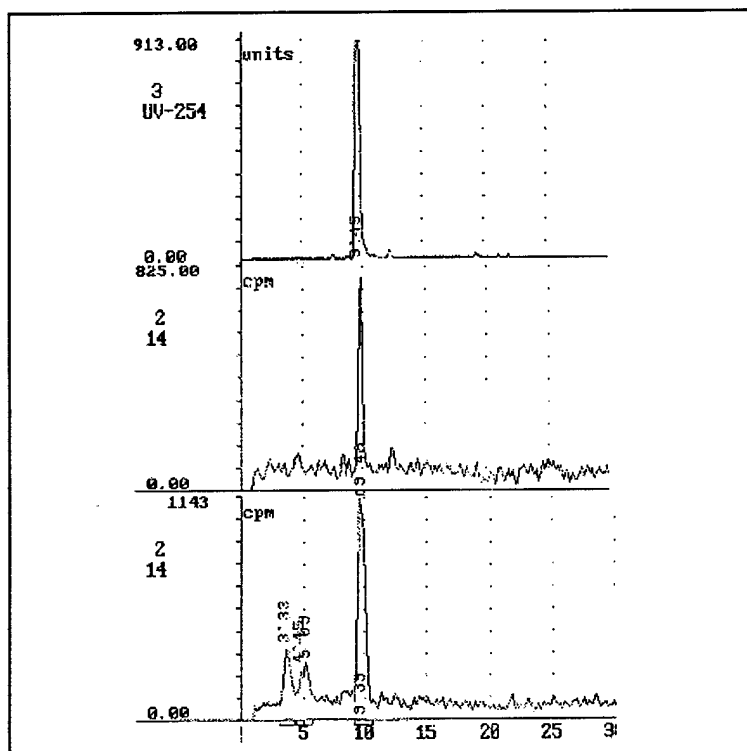


Figure 12  
HPLC of Hemoglobin Hydrolysate, Albumin Soup from rats treated with DNB and 3-Nitroaniline



Top: 3-nitroaniline(3NA) standard

Middle: extracts of Hb acid hydrolysis/Heme soup  
radioactive peak(3NA) accounted for 98% of the  
injected radioactivity

Bottom: albumin soup radioactive peak(3NA)  
accounted for 80% of the injected radioactivity

HPLC condition:

Column: Zorbax ODS C8 25cm x 9.4mm

Solvent: A:0.1%TFA in ddH<sub>2</sub>O pH 6~7

B: 0.1%TFA in acetonitrile pH 6~7

Gradient Table

Time(min)	Flow Rate(ml/min)	A%	B%	Curve
0.0	3.0	60	40	
30	3.0	0	100	6

Figure 13

HPLC of Liver DNA Digest  
from Rats Treated with DNB (30 mg/kg BW)

W600 Gradient Table

#	Time (min)	Flow (ml)	%A (%)	%B (%)	%C (%)	%D (%)	Curve
1	0.00	3.00	95.0	5.0	0.0	0.0	0
2	10.00	3.00	85.0	15.0	0.0	0.0	6
3	20.00	3.00	50.0	50.0	0.0	0.0	6
4	25.00	3.00	0.0	100.0	0.0	0.0	6
5	50.00	3.00	0.0	100.0	0.0	0.0	6
6	55.00	3.00	95.0	5.0	0.0	0.0	6

A= 0.05 M Ammonium sulfate pH 6.55  
B= 98% Methanol, 2% water  
Curve 6 denotes a linear gradient  
Column= 9.4 mm x 25 cm Zorbax ODS

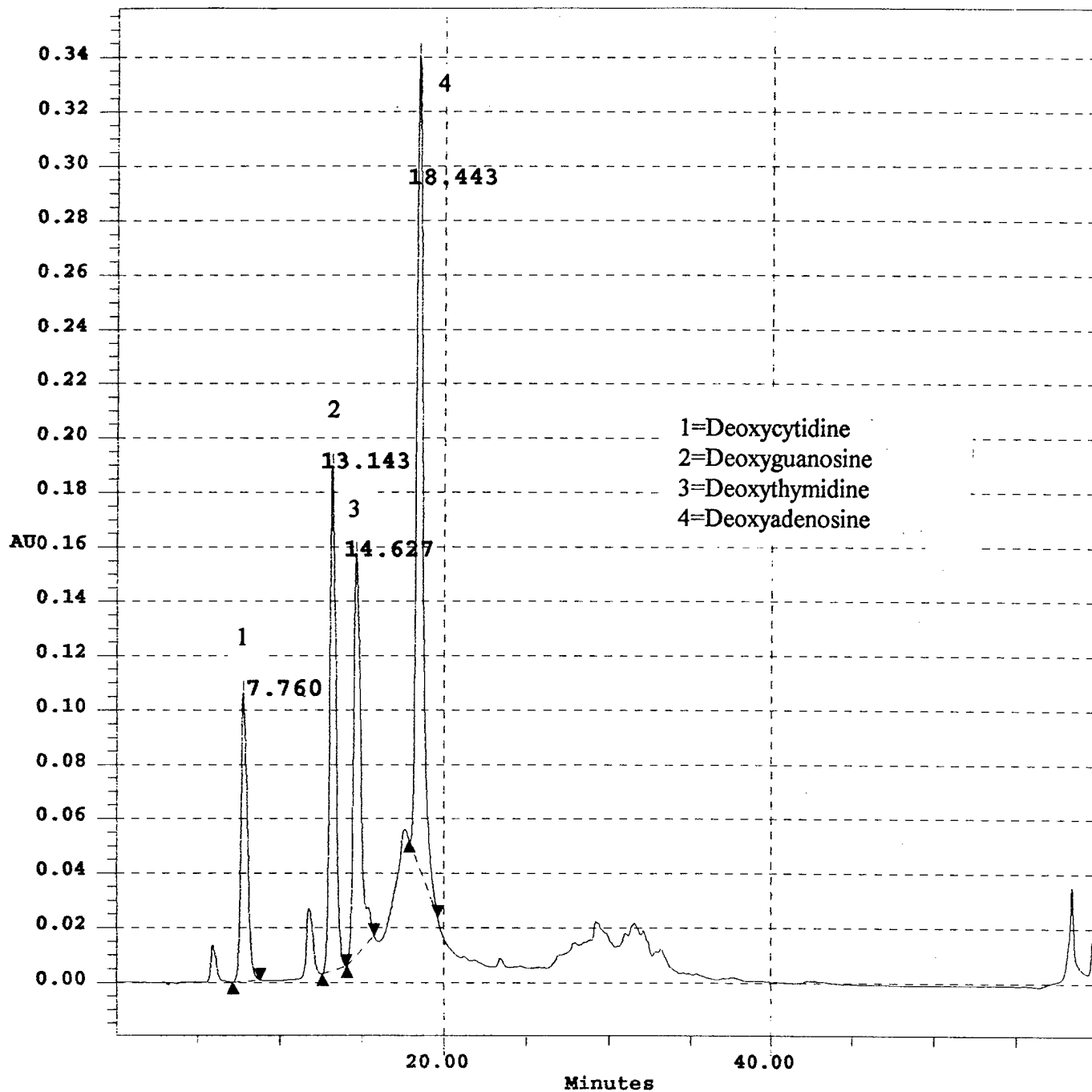


Figure 14

HPLC of Liver DNA Digest and Radioactivity Plot  
from Rats Treated with DNB (30 mg/kg BW)

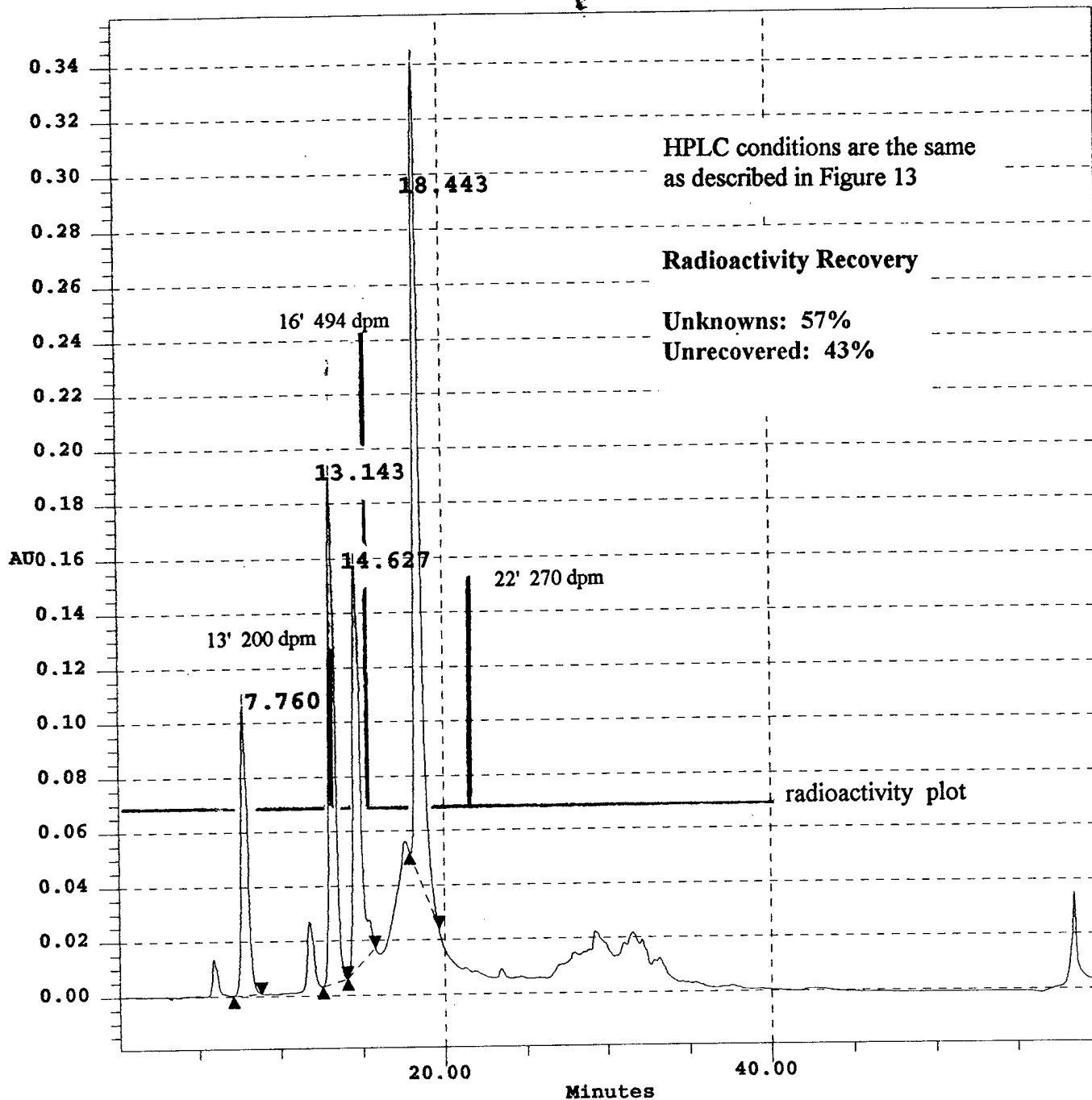


TABLE 1

DETERMINATION OF OPTIMUM TIME REQUIRED FOR MAXIMUM DNB<sup>A</sup>  
BLOOD PROTEIN ADDUCT FORMATION IN RATS TREATED WITH DNB

Sacrifice time hours	picomoles DNB / mg protein				
	serum	albumin	globulins	homoglobin	globin
8	409 ± 91	53 ± 8	7.2 ± 1	303 ± 84	59 ± 18
24	20 ± 3*	16 ± 1*	3.6 ± 0.4*	108 ± 7*	51 ± 5*
48	10 ± 3*	10 ± 2*	1.9 ± 0.4*	84 ± 8*	46 ± 11*
72	6 ± 1*	7 ± 1*	1.1 ± 0.01*	89 ± 4*	36 ± 10*
96	5 ± 1*	5 ± 1*	0.8 ± 0.2*	60 ± 6*	35 ± 5*
144	2 ± 0.3*	2 ± 0.6*	0.29 ± 0.08*	57 ± 5*	27 ± 5*
192	1 ± 0.1*	1 ± 0.1*	0.17 ± 0.04*	40 ± 28*	22 ± 2*

A. Dose: 30 mg DNB/kg BW (single oral dose)

Mean ± standard deviation (N=5)

\*Significantly different from 8 h. dose group, (P<0.05)

TABLE 2

EFFECT OF SINGLE VS MULTIPLE AND DIVIDED MULTIPLE DOSES OF DNB IN  
RAT BLOOD PROTEIN ADDUCTS FORMATION

Sacrifice Time (Days)	Dose	<u>picomol DNB/mg protein</u>	
		Globin	Albumin
7	A	33.0 ± 5.0	12.2 ± 1.3
14	A	16.0 ± 2.0	6.7 ± 0.6
7	B	131.0 ± 7.0*	44.0 ± 2.0*
7	C	8.3 ± 0.9 <sup>+</sup>	2.5 ± 0.2
7	D	77.0 ± 5.0	21.0 ± 0.9
14	D	74.0 ± 16.0	22.0 ± 3.0

Mean ± standard deviation (N=5).

A = Single dose of 15 mg DNB/kg BW

B = Daily doses of 15 mg DNB/kg BW

C = Daily doses of 15/14 mg DNB/kg BW

D = Single dose of 6 mg DNB/kg BW

\* Significantly different from dose A & D ( $p \leq 0.05$ ), dose or time comparison.



TABLE 3.

OPTIMUM TIME REQUIRED FOR MAXIMUM DNA ADDUCT FORMATION IN RATS TREATED WITH DNB<sup>A</sup>

Sacrifice Time (hours)	Average pmol DNB/mg DNA		
	Liver	Kidney	Spleen
2	1.42 ± 0.51*	4.73 ± 0.51	0.44 ± 0.11**
8	4.64 ± 1.24*	5.59 ± 1.67	1.10 ± 0.29
24	16.77 ± 3.86	7.87 ± 4.59	0.54 ± 0.36
48	9.69 ± 1.14*	4.10 ± 2.54	1.59 ± 0.39
72	7.25 ± 1.08*	4.09 ± 1.49	0.59 ± 0.22
96	12.89 ± 1.06	3.75 ± 1.25	0.77 ± 0.51
144	23.90 ± 11.14	4.35 ± 1.11	0.78 ± 0.24
192	10.16 ± 4.59	4.36 ± 1.46	1.93 ± 0.83

A. Dose=30 mg DNB/kg BW (Single oral dose).

Mean ± standard deviation (N=5)

\* Significantly different from adduct levels at 24 h, (P<0.05).

\*\* Significantly different from adduct levels at 48 h, (P<0.05).

TABLE 4

DOSE DEPENDENT FORMATION OF LIVER DNA-DNB ADDUCTS IN RATS TREATED WITH DNB<sup>a</sup>

Sacrifice Time (Days)	Dose		
	30 mg/kg BW	6 mg/kg BW	1.2 mg/kg BW
1	2.13 ± 0.50*	1.10 ± 0.10 <sup>+</sup>	0.71 ± 0.06
2	5.28 ± 0.58*	2.24 ± 0.57 <sup>+</sup>	0.37 ± 0.14* <sup>+</sup>
3	7.19 ± 2.56*	1.44 ± 0.36 <sup>+</sup>	0.32 ± 0.09* <sup>+</sup>
4	8.16 ± 0.85	0.51 ± 0.18* <sup>+</sup>	0.17 ± 0.04* <sup>+</sup>
6	14.37 ± 2.65	0.80 ± 0.46* <sup>+</sup>	0.24 ± 0.07* <sup>+</sup>
8	6.55 ± 1.04*	0.37 ± 0.09* <sup>+</sup>	0.16 ± 0.04* <sup>+</sup>

<sup>a</sup> Average pmol DNB/mg DNA (Single oral dose)

Mean ± standard deviation (N=5)

\* Significantly different from the maximum (p&lt;0.05), time comparison.

<sup>+</sup> Significantly different from the maximum (p<0.05), dose comparison.

TABLE 5

## STABILITY AND PERSISTENCE OF TISSUE DNB-DNA ADDUCTS IN RATS TREATED WITH DNB

Sacrifice Time (Days)	picomol DNB/mgDNA		
	Liver	Kidney	Spleen
0.33	1.00 ± 1.74*	0.70 ± 0.15*	1.08 ± 0.33*
1	4.70 ± 1.96	1.01 ± 0.24*	1.32 ± 0.43*
2	3.74 ± 1.86	1.31 ± 0.30*	0.86 ± 0.27*
3	1.00 ± 1.91*	2.83 ± 1.67*	2.03 ± 1.82*
4	3.05 ± 1.51	4.25 ± 1.28	1.87 ± 0.36*
7	5.36 ± 1.85	4.65 ± 0.59	4.94 ± 3.01*
14	1.77 ± 0.59*	5.10 ± 0.70	3.20 ± 1.03*
28	0.99 ± 0.30*	5.47 ± 0.55	6.30 ± 2.33*
42	1.00 ± 0.11*	2.28 ± 0.47*	6.10 ± 2.06*
56	0.45 ± 0.09*	2.05 ± 0.44*	13.52 ± 4.77
70	0.39 ± 0.03*	2.37 ± 0.51*	10.96 ± 5.76

Mean ± standard deviation (N=5), Dose: 30 mg DNB/kg BW (Single oral dose)

\* Significantly different from the maximum value ( $p \leq 0.05$ ).

TABLE 6

EFFECT OF SINGLE VS MULTIPLE AND DIVIDED MULTIPLE DOSES ON DNB-DNA  
ADDUCT FORMATION IN RATS TREATED WITH DNB

Sacrifice Time (Days)	Dose	picomol DNB/mg DNA		
		Liver	Kidney	Spleen
7	A	1.39 ± 0.56* <sup>+</sup>	1.62 ± 0.24* <sup>+</sup>	1.10 ± 0.15*
14	A	1.27 ± 0.26*	1.80 ± 0.64*	1.60 ± 0.46*
7	B	20.90 ± 7.22	5.70 ± 0.78	15.95 ± 7.88
7	C	2.20 ± 0.68* <sup>+</sup>	0.66 ± 0.15* <sup>+</sup>	0.95 ± 0.28
7	D	9.68 ± 2.96	3.92 ± 0.81	3.65 ± 1.40
14	D	23.26 ± 3.98	12.05 ± 1.31	11.52 ± 6.71

Mean ± standard deviation (N=5).

A = Single dose of 15 mg DNB/kg BW

B = Daily dose of 15 mg DNB/kg BW

C = Daily doses of 15/14 mg DNB/kg BW

D = Single dose of 6 mg DNB/kg BW

\* Significantly different from dose B ( $p \leq 0.05$ ), time comparison.

<sup>+</sup> Significantly different, dose A vs. dose C ( $p \leq 0.05$ ), time comparison.

**APPENDIX C**

**Figure 1**

**Blood Fractionation Protocol**

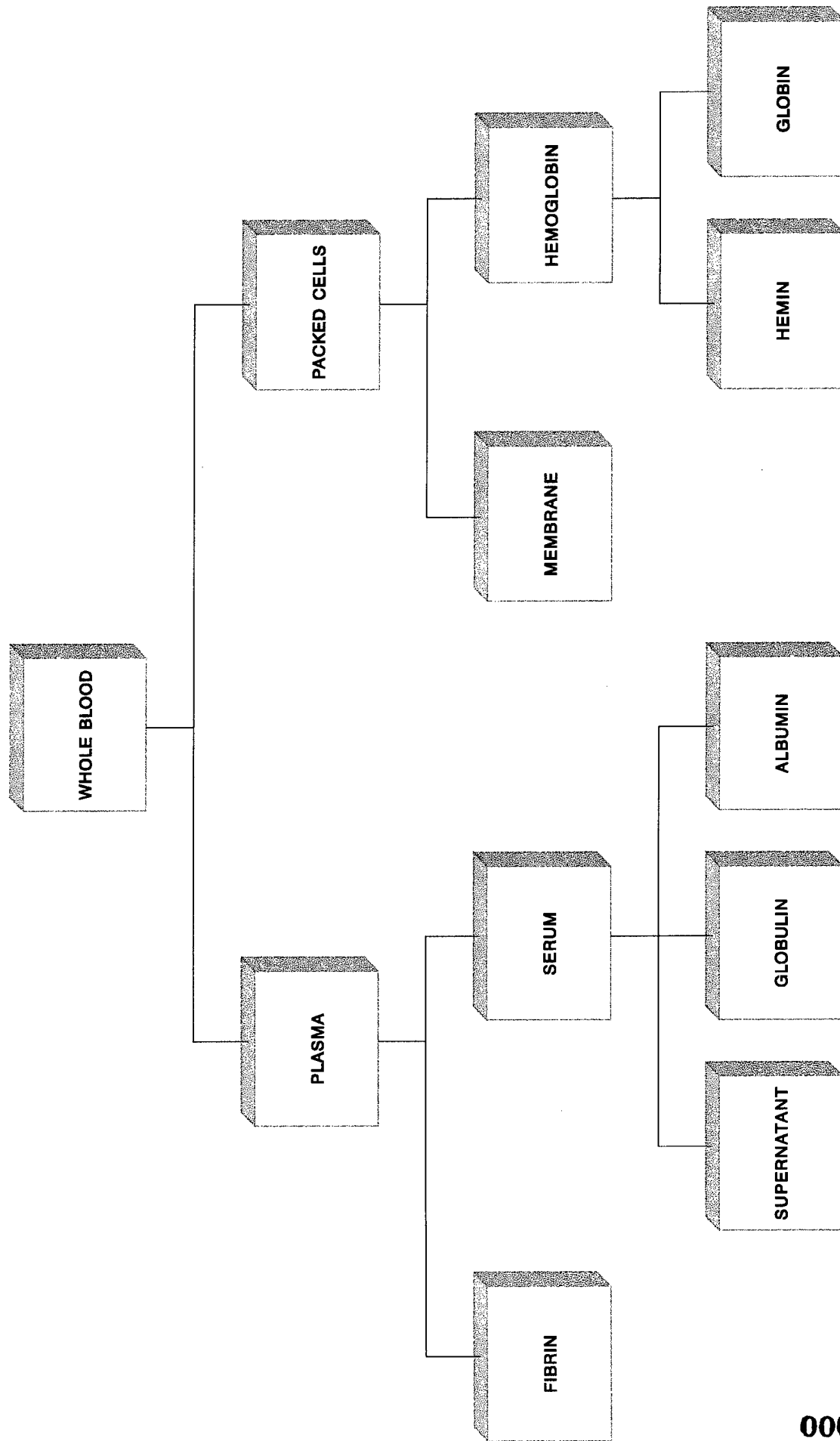


Figure 1A

DOSE DEPENDENT FORMATION OF ALBUMIN ADDUCTS WITH TETRYL

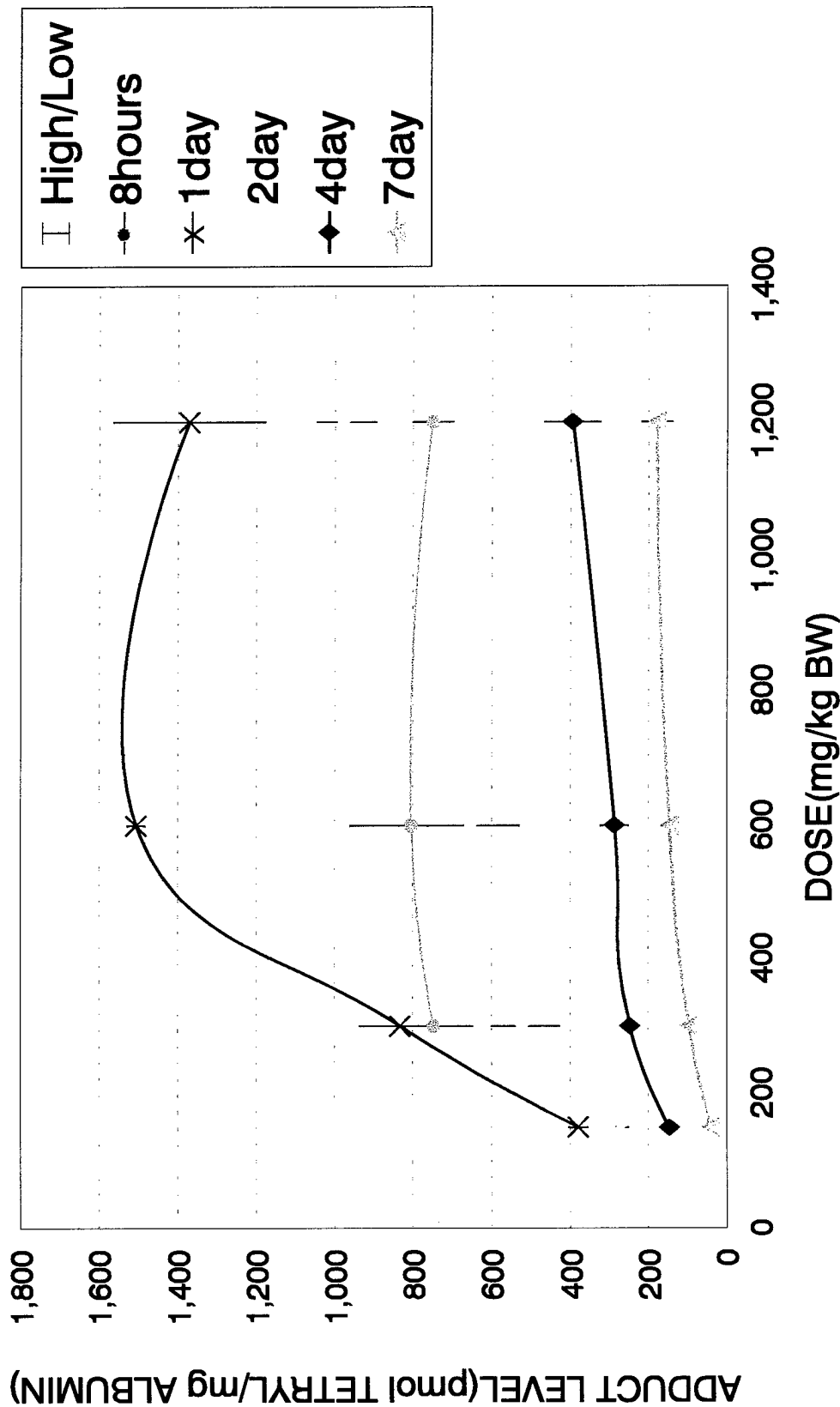


Figure 2

FORMATION AND PERSISTENCE OF ALBUMIN ADDUCTS IN RATS TREATED WITH TETRYL

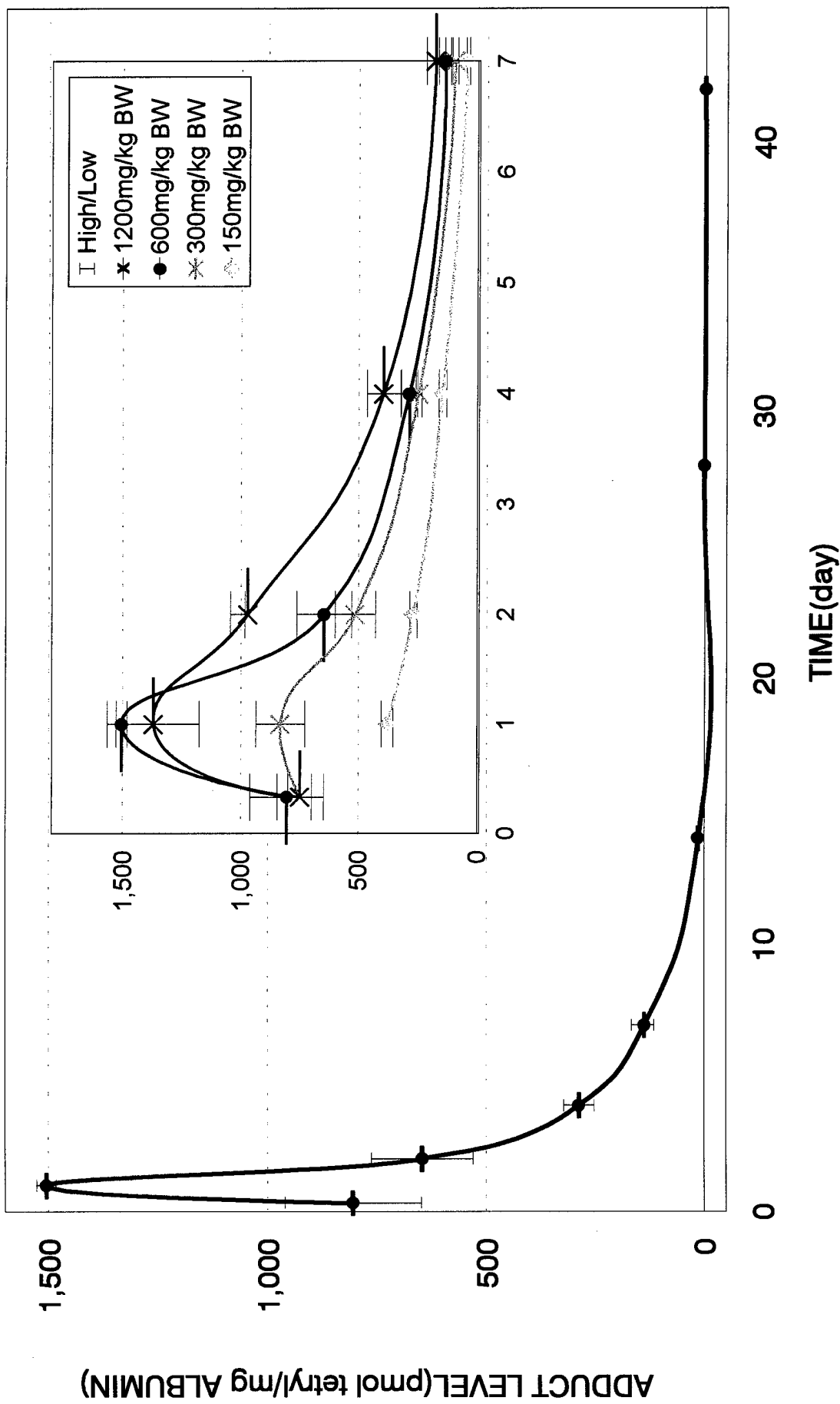




Figure 3

DOSE DEPENDENT FORMATION OF GLOBIN ADDUCTS IN RATS TREATED WITH TETRYL

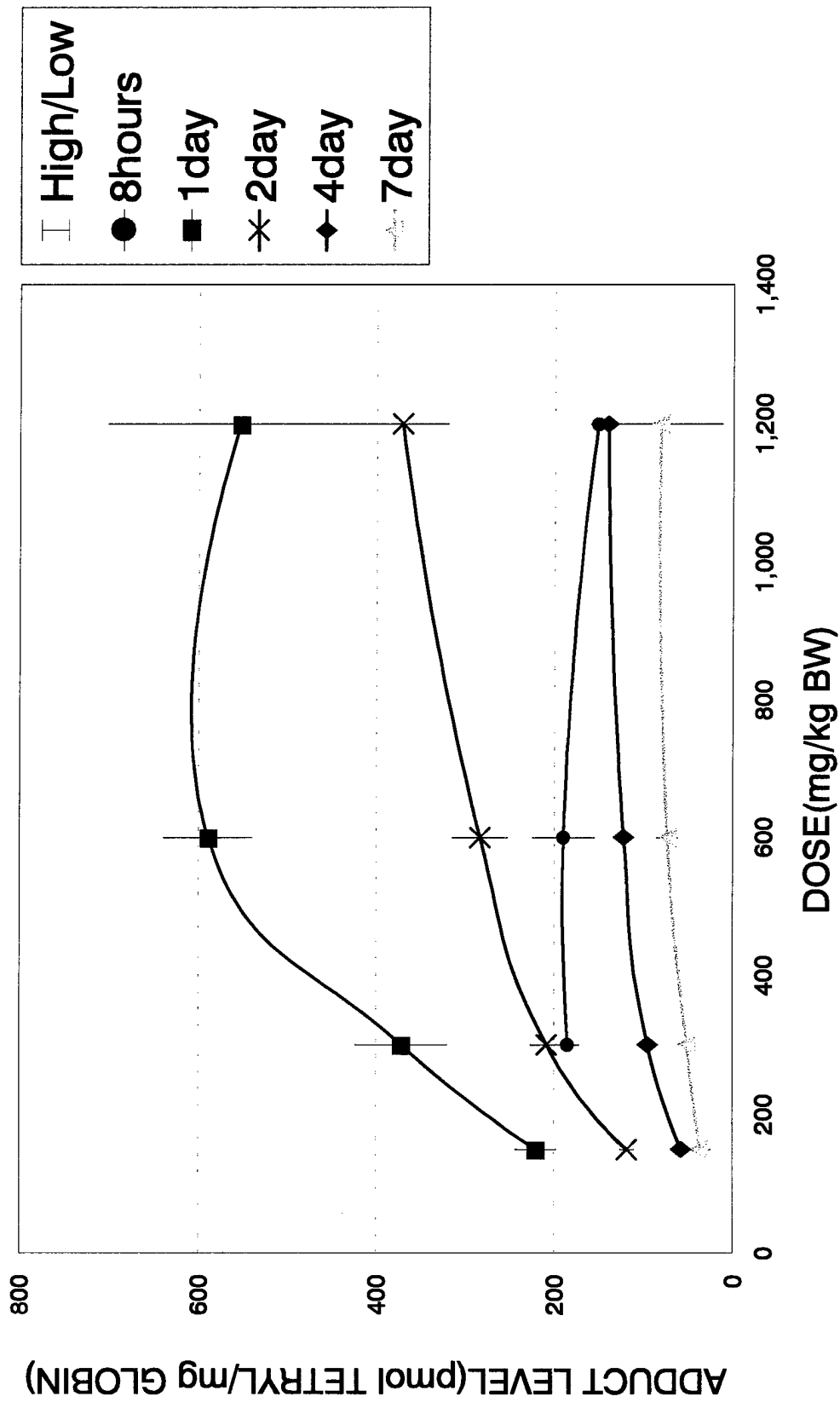


Figure 4

FORMATION AND PERSISTENCE OF GLOBULIN ADDUCTS IN RATS TREATED WITH TETRYL

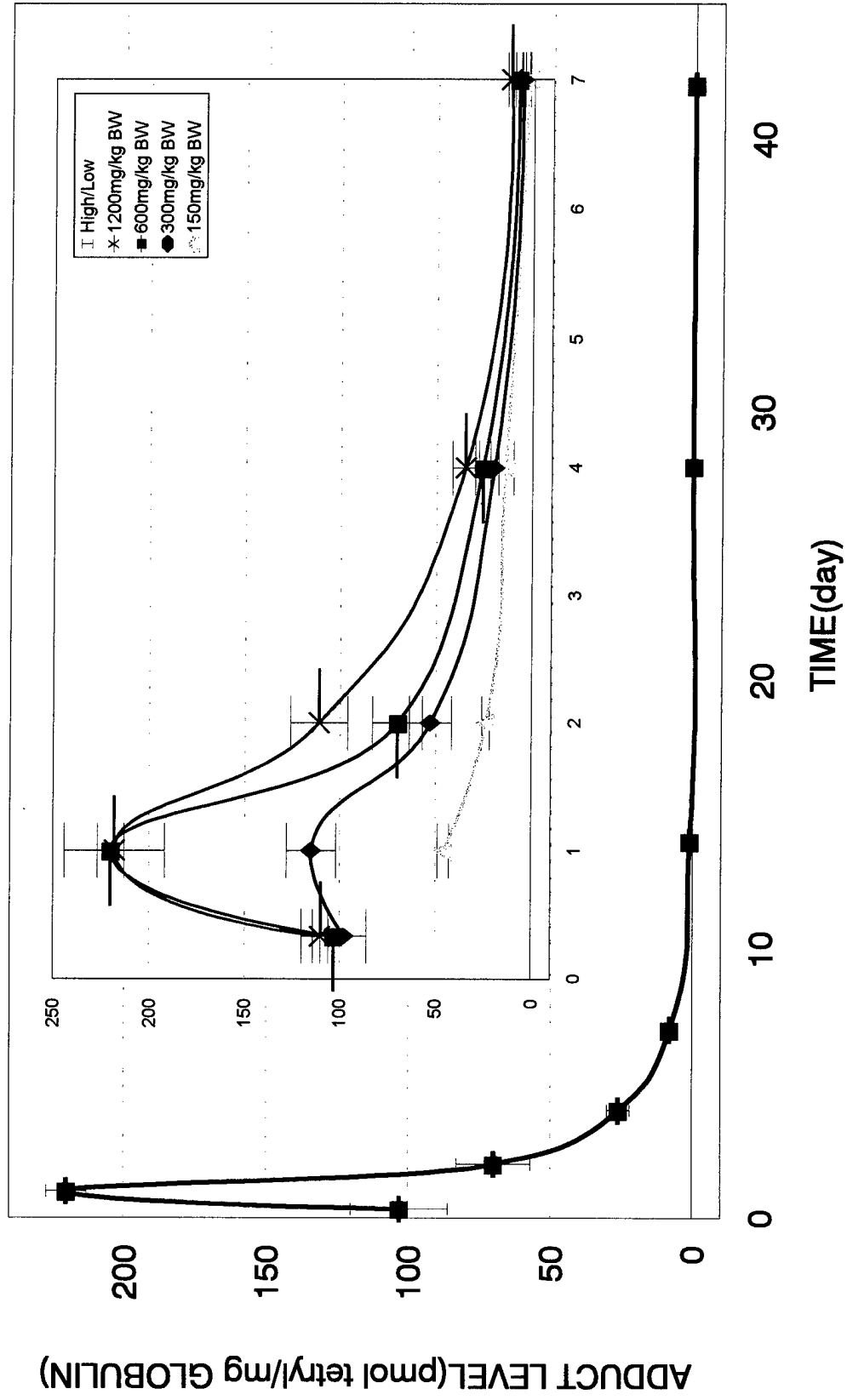


Figure 5

DOSE DEPENDENT FORMATION OF HEMOGLOBIN ADDUCTS IN RATS TREATED WITH TETRYL

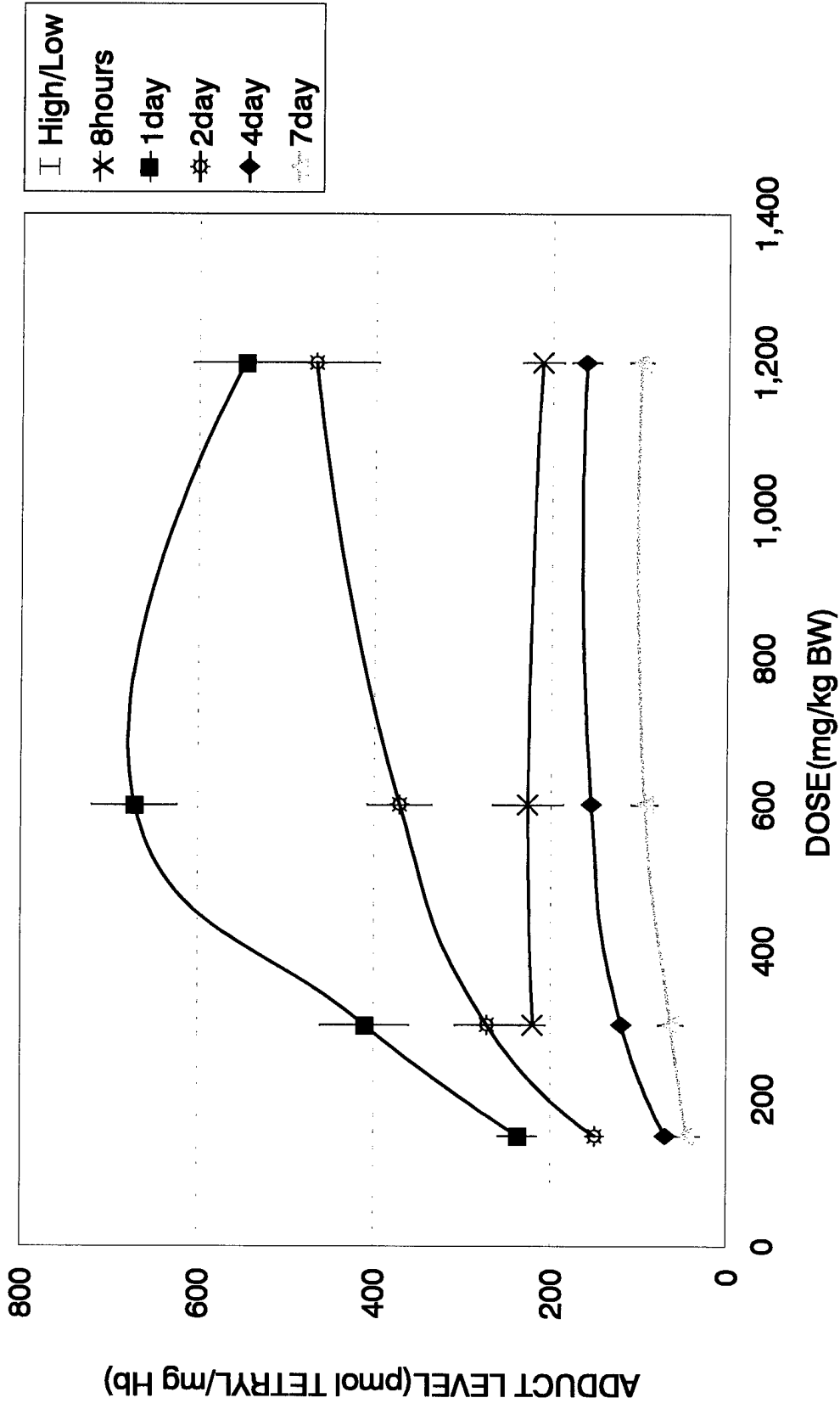


Figure 6

FORMATION AND PERSISTENCE OF HEMOGLOBIN ADDUCTS IN RATS TREATED WITH TETRYL

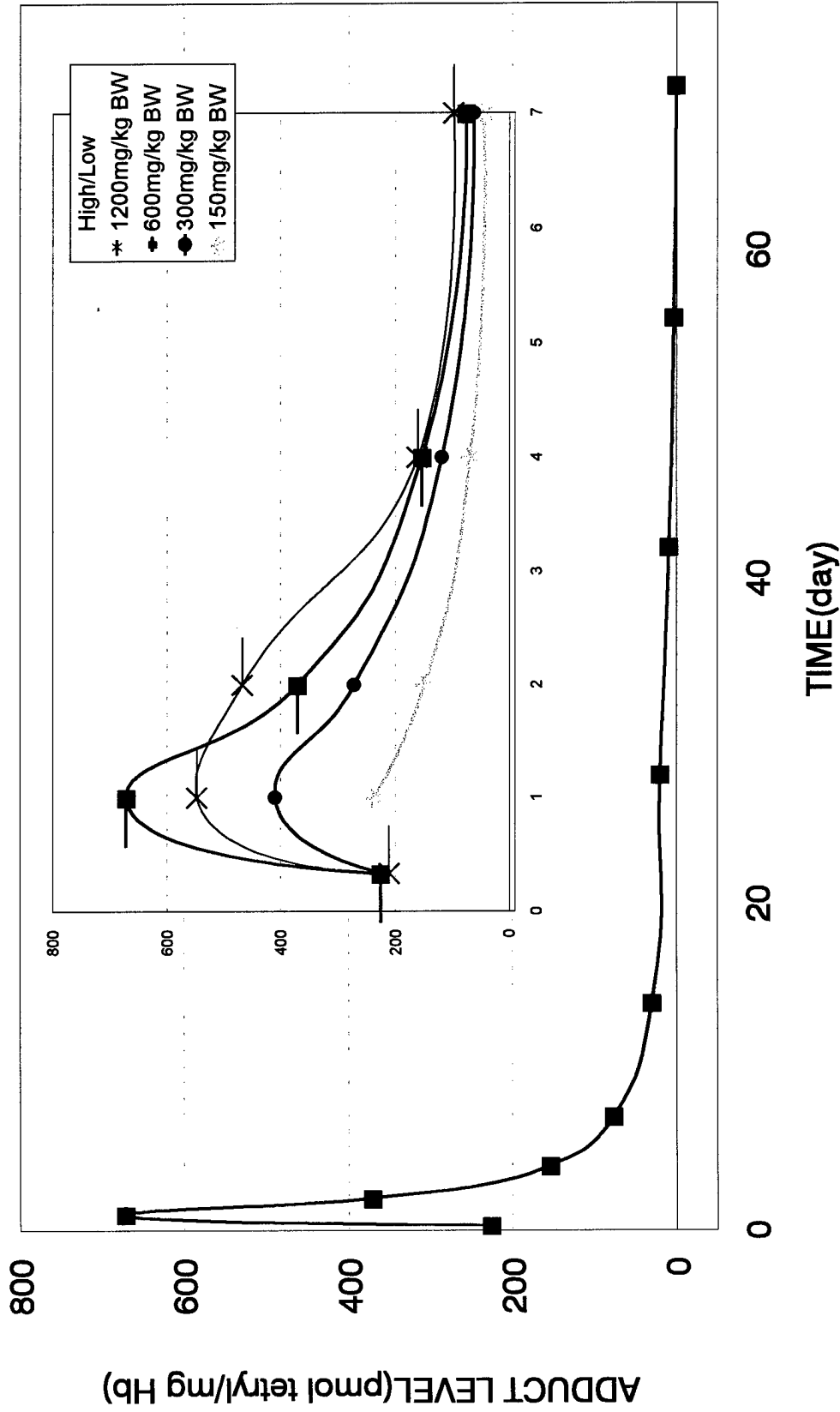


Figure 7

DOSE DEPENDENT FORMATION OF GLOBIN ADDUCTS IN RATS TREATED WITH TETRIL

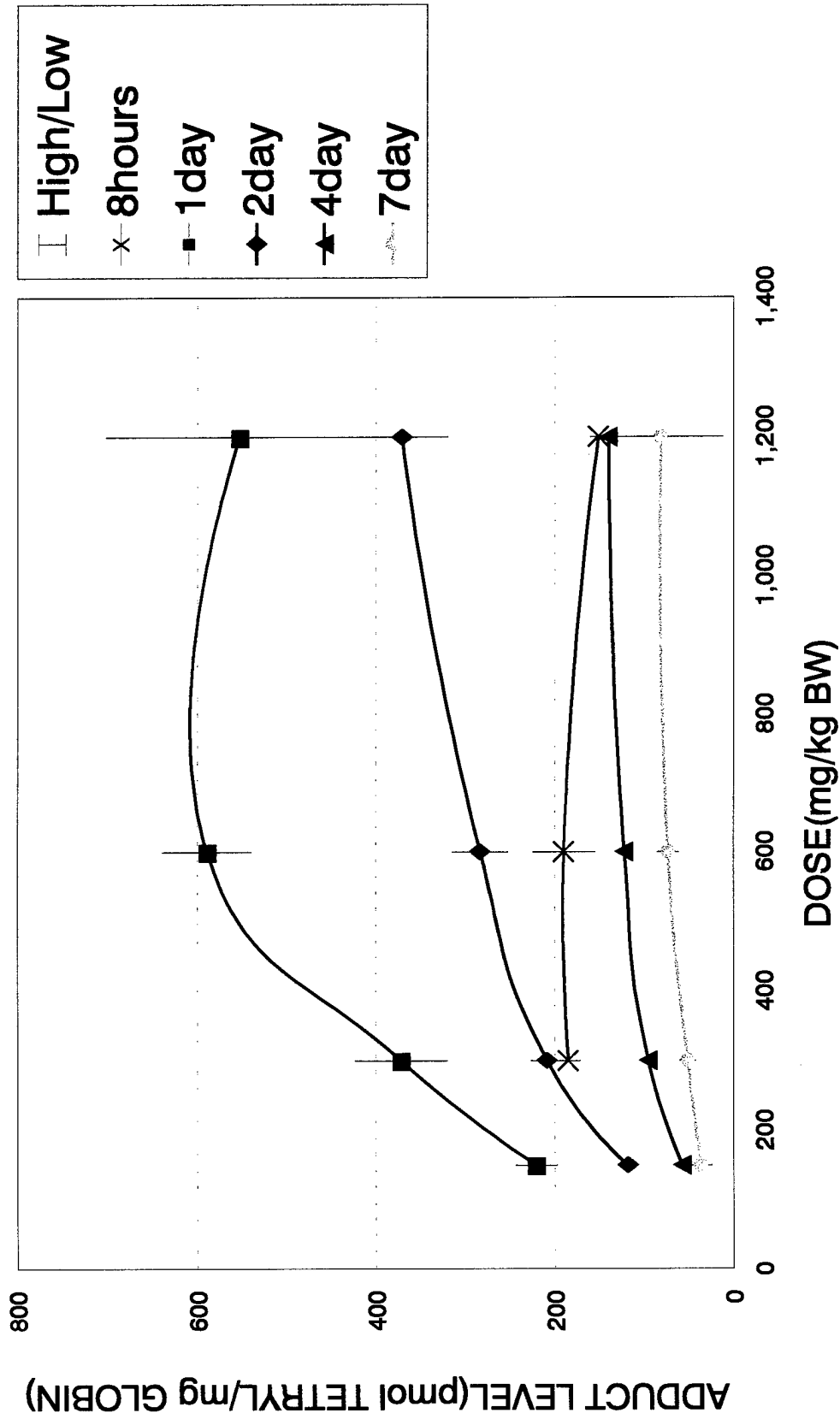


Figure 8

FORMATION AND PERSISTENCE OF GLOBIN ADDUCTS IN RATS TREATED WITH TETRYL

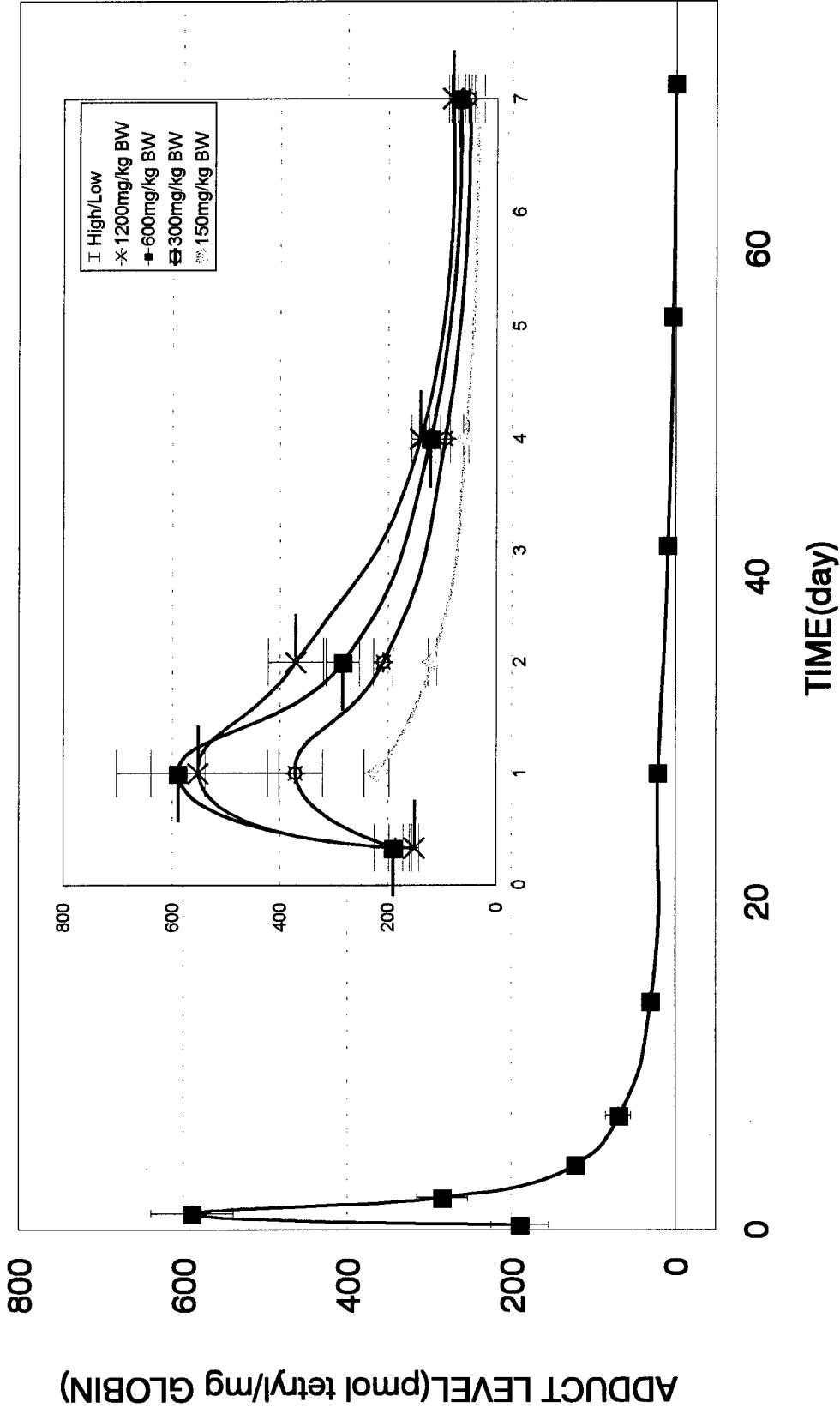
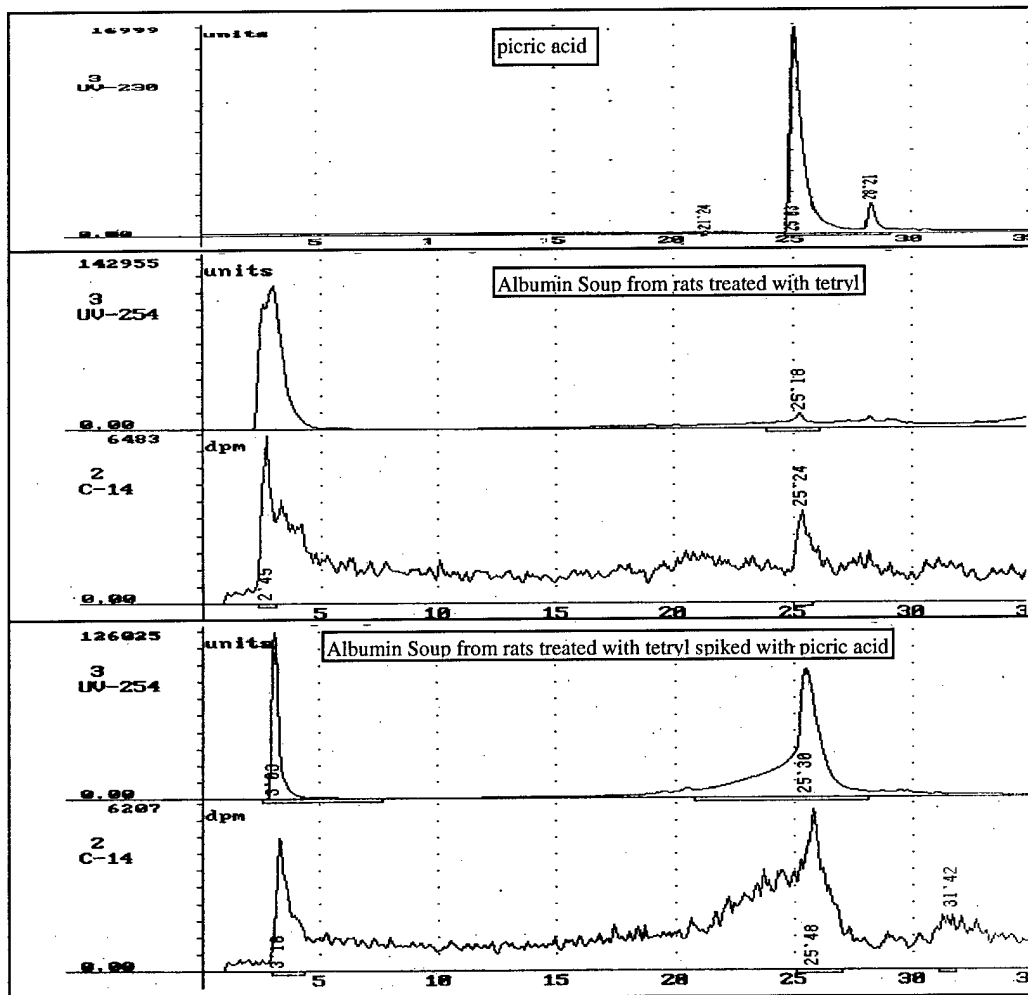


Figure 8A

HPLC of Albumin Soup from rats treated with tetryl spiked with and without picric acid and picric acid



HPLC condition:

Column: Zorbax ODS C18 25cm x 4.6mm

Solvent: A: 0.1%TFA in ddH<sub>2</sub>O

B: 0.1%TFA in acetonitrile

Gradient Table

Time(min)	Flow Rate(min)	A%	B%	Curve
0.0	1.0	100	0	
50	1.0	0	100	6

#	Time (min)	Flow (ml)	%A (%)	%B (%)	%C (%)	%D (%)	Curve
1	0.00	3.00	95.0	5.0	0.0	0.0	0
2	10.00	3.00	85.0	15.0	0.0	0.0	6
3	20.00	3.00	50.0	50.0	0.0	0.0	6
4	25.00	3.00	0.0	100.0	0.0	0.0	6
5	50.00	3.00	0.0	100.0	0.0	0.0	6
6	55.00	3.00	95.0	5.0	0.0	0.0	6

A= 0.05 M Ammonium sulfate pH 6.55

B= 98% Methanol, 2% water

Curve 6 denotes a linear gradient

Column= 9.4 mm x 25 cm Zorbax ODS

Chromatography conditions are the same for Figures 10 and 11.

- 1=Deoxycytidine
- 2=Deoxyguanosine
- 3=Deoxythymidine
- 4=Deoxyadenosine

Figure 9

HPLC of Liver DNA Digest from Rats Treated with Tetryl (600 mg/kg BW)

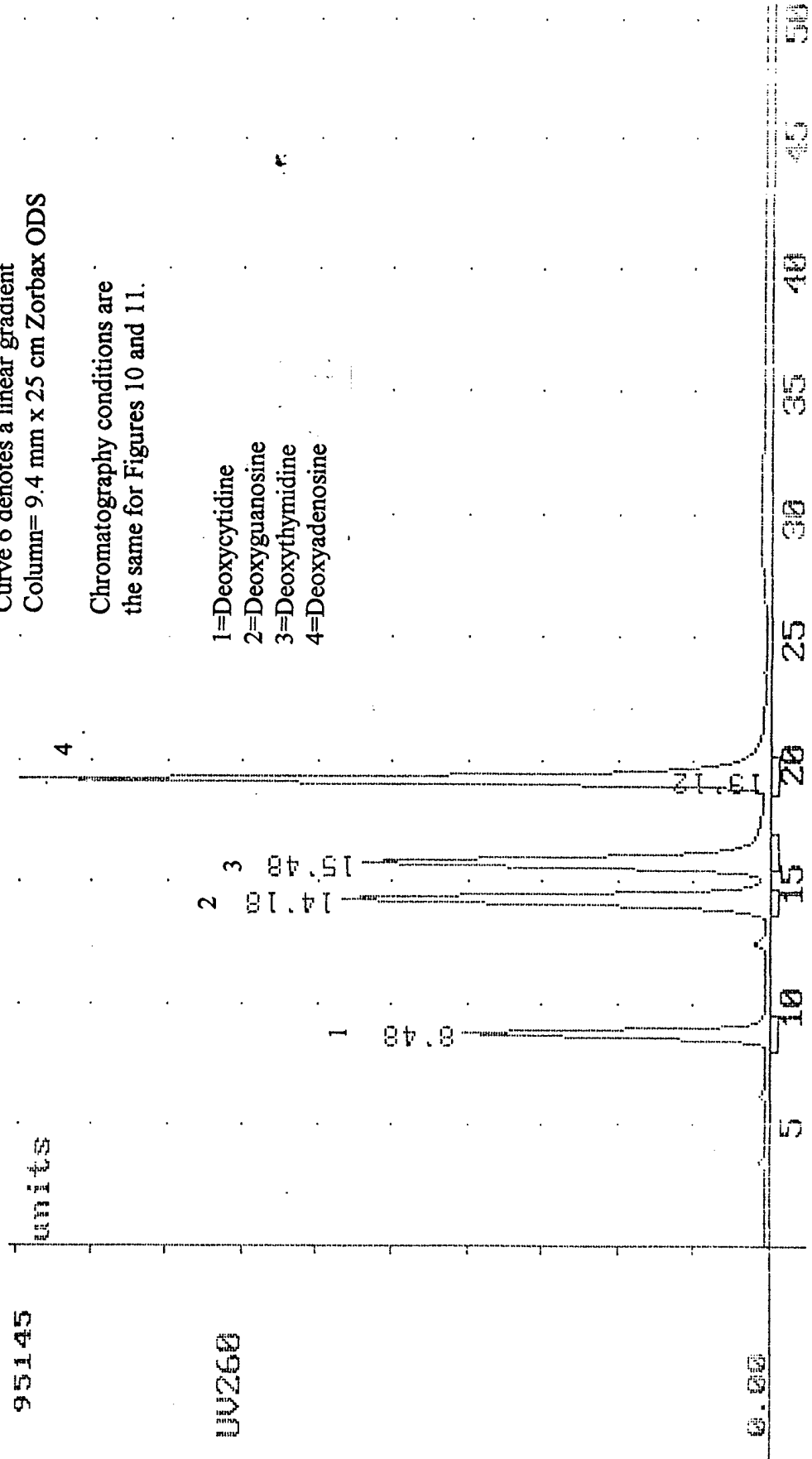




Figure 10

HPLC of Liver DNA Digest with Radioactivity Plot  
from Rats Treated with Tetryl (600 mg/kg BW)

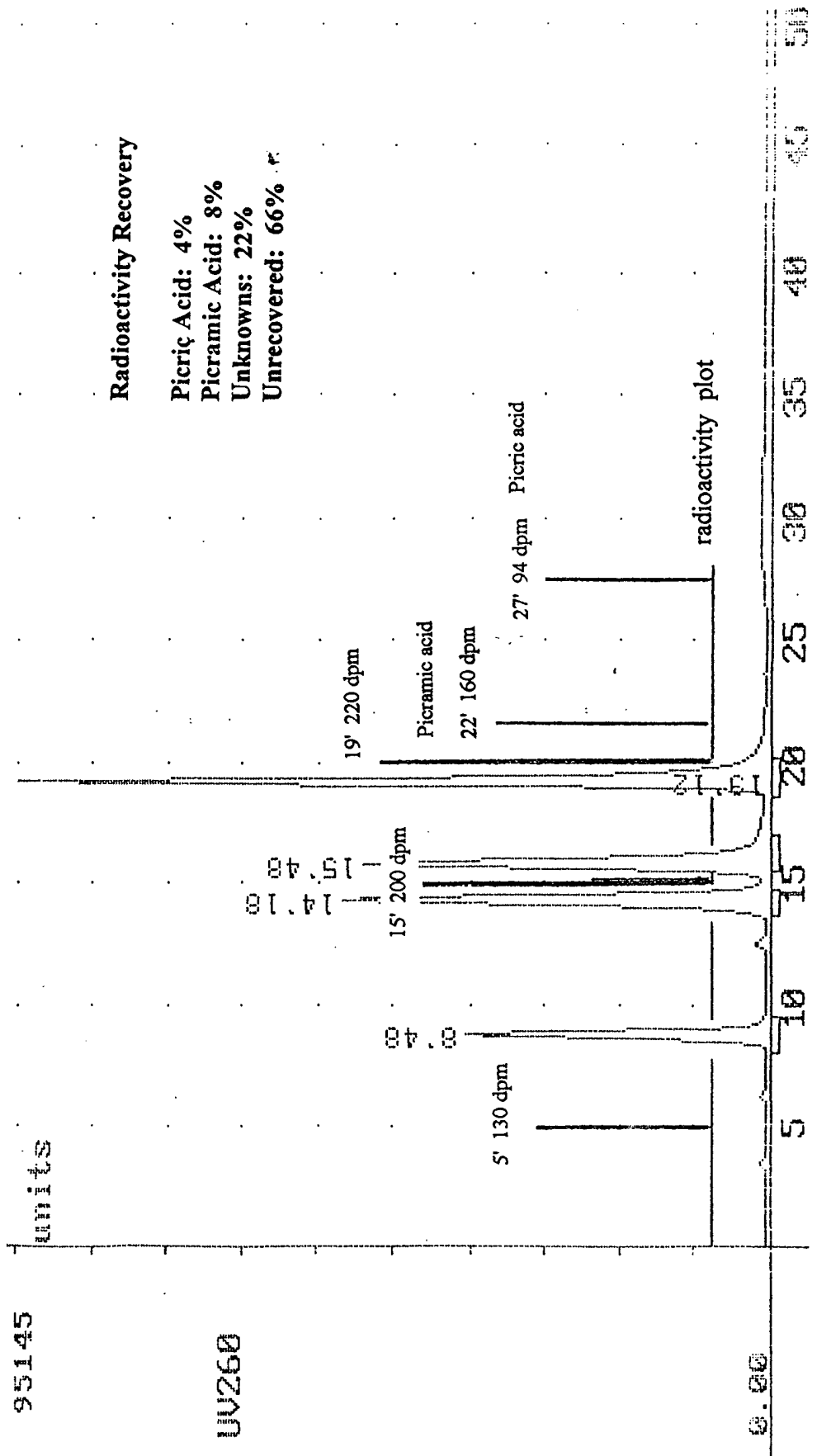
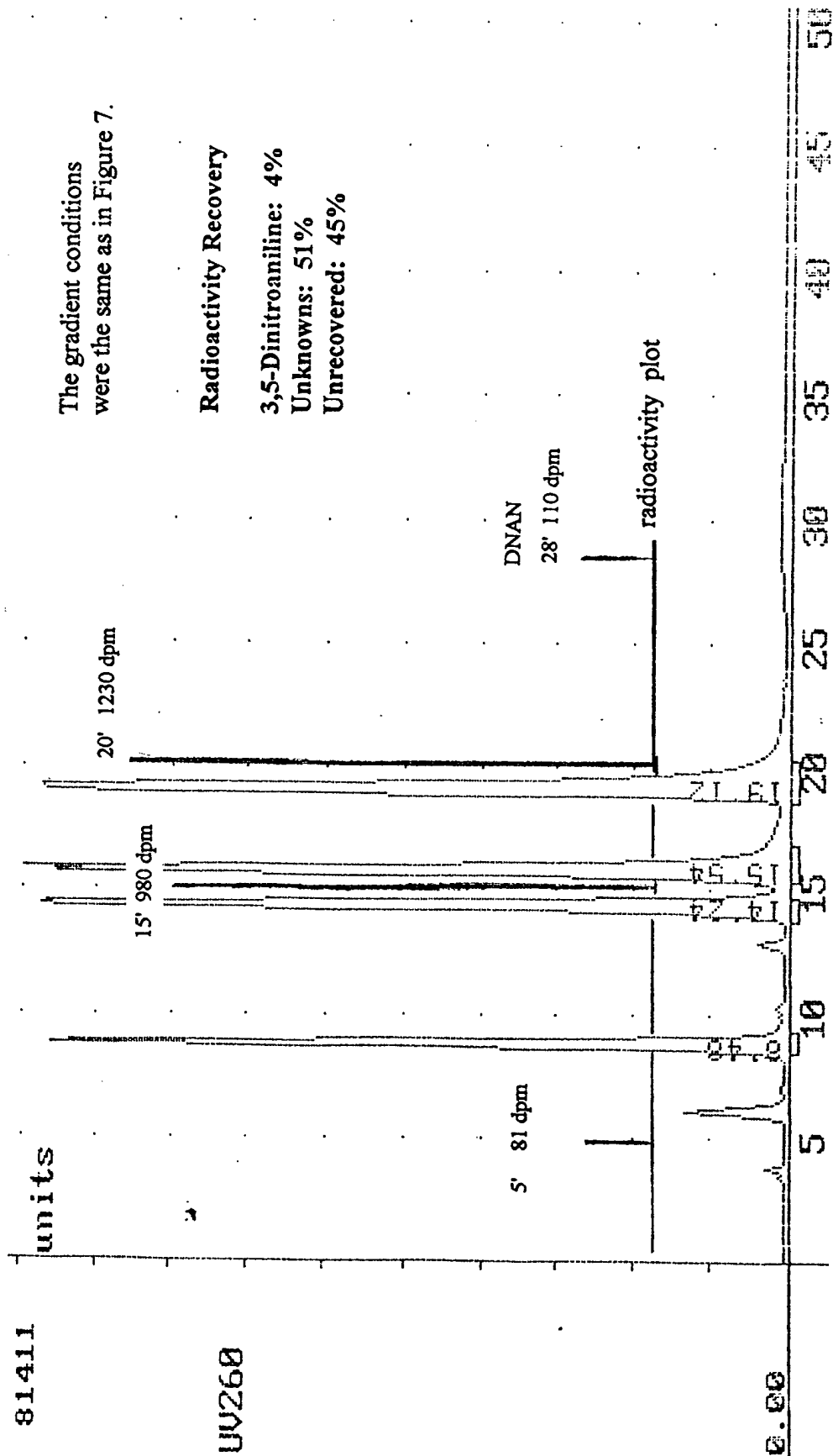


Figure 11

### HPLC of Kidney DNA Digest with Radioactivity Plot from Rats Treated with Tetryl (600 mg/kg BW)



The gradient conditions were the same as in Figure 7.

Radioactivity Recovery

3,5-Dinitroaniline: 4%

Unknowns: 51%

Unrecovered: 45%

TABLE 1

## FORMATION AND PERSISTENCE OF TETRYL- PROTEIN ADDUCTS\* IN RATS

Time (Weeks)	Hemoglobin	Globin	Albumin	Globulin
1	58.37 ± 9.04	63.28 ± 9.61	128.26 ± 15.05	7.04 ± 0.93
2	28.76 ± 3.26**	28.49 ± 4.05**	14.12 ± 2.57**	1.05 ± 0.09**
4	19.80 ± 3.78**	20.63 ± 3.71**	0.72 ± 0.21**	0.25 ± 0.07**
6	9.40 ± 1.39**	9.15 ± 1.25**	0.25 ± 0.07**	0.12 ± 0.06**
8	3.40 ± 0.81**	2.92 ± 0.68**	ND	ND
10	0.51 ± 0.08**	0.29 ± 0.04**	ND	ND

\* Average pmol tetryl/mg protein

Mean ± standard deviation (N=5)

ND: Not detected

Dose: 600 mg/kg BW (single oral dose)

\*\* Significantly different than the maximum value ( $p \leq 0.05$ ).

TABLE 2

## EFFECT OF SINGLE VS. MULTIPLE AND DIVIDED MULTIPLE DOSES ON TETRYL-PROTEIN ADDUCT FORMATION\* IN RATS

Time (Days)	Dose	Hemoglobin	Globin	Albumin	Globulin
7	A	44.96 ± 6.37** <sup>†</sup>	40.25 ± 6.62**	87.55 ± 8.14** <sup>†</sup>	4.64 ± 0.72** <sup>†</sup>
14	A	24.39 ± 3.22** <sup>†</sup>	24.08 ± 3.39** <sup>†</sup>	9.25 ± 1.20** <sup>†</sup>	0.77 ± 0.17** <sup>†</sup>
7	B	517.43 ± 84.7	478.35 ± 52.64	2354.12 ± 107.39	293.20 ± 61.63
14	B	601.27 ± 26.24	567.89 ± 19.03	2221.08 ± 217.95	207.25 ± 26.51
7	C	59.66 ± 8.87** <sup>†</sup>	51.55 ± 7.56**	221.28 ± 23.32** <sup>†</sup>	13.17 ± 3.76** <sup>†</sup>
14	C	80.22 ± 13.59** <sup>†</sup>	79.25 ± 13.67** <sup>†</sup>	283.74 ± 60.03** <sup>†</sup>	14.33 ± 2.65** <sup>†</sup>

\* Average pmol tetryl/mg protein

Mean ± standard deviation (n=5)

Dosages: A= Single dose of 300 mg/kg BW;

B= Daily doses of 300 mg/kg BW;

C=Daily doses of 300/14 mg/kg BW.

\*\* Significantly different from dose B (p≤0.05), time comparison.

<sup>†</sup>Significantly different (p≤0.05), A vs. C, time comparison.

TABLE 3

## DOSE DEPENDENT FORMATION OF LIVER DNA-TETRYL ADDUCTS\* IN RATS

Time (Days)	Dose			
	1200 mg/kg BW	600 mg/kg BW	300 mg/kg BW	150 mg/kg BW
0.33	21.52 ± 5.53**	11.90 ± 3.16**	23.01 ± 11.28**	ND
1	49.40 ± 4.07**	53.99 ± 26.67	30.65 ± 3.85 <sup>+</sup>	17.15 ± 3.25 <sup>+</sup>
2	147.47 ± 12.46	75.00 ± 12.73 <sup>+</sup>	41.43 ± 7.04 <sup>+</sup>	18.28 ± 1.85 <sup>+</sup>
4	65.90 ± 9.66**	44.46 ± 9.75**	36.86 ± 21.35	12.47 ± 3.96 <sup>+</sup>
7	104.27 ± 10.79	70.17 ± 10.59	43.25 ± 11.09	24.80 ± 5.66 <sup>+</sup>

\*Average pmol tetryl/mg DNA

Mean ± standard deviation (N=5)

ND: Not done

\*\* Significantly different from maximum value ( $p \leq 0.05$ ), time comparison.<sup>+</sup> Significantly different from maximum value ( $p \leq 0.05$ ), dose comparison.

TABLE 4

## DOSE DEPENDENT FORMATION OF KIDNEY DNA-TETRYL ADDUCTS\* IN RATS

Time (Days)	Dose		
	1200 mg/kg BW	600 mg/kg BW	300 mg/kg BW
0.33	10.16 ± 4.35**	15.70 ± 18.88**	6.97 ± 4.22**
1	33.02 ± 28.34**	24.00 ± 5.89	14.95 ± 3.15** <sup>+</sup>
2	65.93 ± 9.19	33.02 ± 3.55 <sup>+</sup>	14.93 ± 1.62 <sup>+</sup>
4	36.87 ± 19.84	33.29 ± 16.87	26.23 ± 7.79
7	43.33 ± 14.23	34.11 ± 7.57	15.57 ± 7.26** <sup>+</sup>
			18.94 ± 9.79
			10.52 ± 3.46 <sup>+</sup>
			9.45 ± 1.98** <sup>+</sup>
			6.68 ± 1.58** <sup>+</sup>
			ND

\* Average pmol tetryl/mg DNA

Mean ± standard deviation (N=5)

ND: Not done

\*\* Significantly different from maximum value (p ≤ 0.05), time comparison.

<sup>+</sup> Significantly different from maximum value (p ≤ 0.05), dose comparison.

TABLE 5  
DOSE DEPENDENT FORMATION OF SPLEEN DNA-TETRYL ADDUCTS\* IN RATS

Time (Days)	Dose			
	1200 mg/kg BW	600 mg/kg BW	300 mg/kg BW	150 mg/kg BW
0.33	6.03 ± 3.51**	8.16 ± 6.01	3.60 ± 3.21**	ND
1	19.83 ± 3.56	24.50 ± 8.31	11.44 ± 3.37	4.87 ± 1.29
2	24.58 ± 4.61	16.51 ± 5.06 <sup>+</sup>	7.05 ± 1.18** <sup>+</sup>	3.77 ± 1.23 <sup>+</sup>
4	17.01 ± 2.52	17.01 ± 6.94	12.82 ± 7.60	4.21 ± 2.57
7	15.00 ± 1.31**	11.01 ± 6.18	8.09 ± 3.73** <sup>+</sup>	2.25 ± 1.55 <sup>+</sup>

\* Average pmol tetryl/mg DNA

Mean ± standard deviation (N=5)

ND: Not done

\*\* Significantly different from maximum value ( $p \leq 0.05$ ), time comparison.

<sup>+</sup> Significantly different from maximum value ( $p \leq 0.05$ ), dose comparison.

TABLE 6  
FORMATION AND PERSISTENCE OF TETRYL- DNA ADDUCTS\* IN RATS

Time (Weeks)	Liver	Kidney	Spleen
1	70.17 ± 10.59	34.60 ± 7.57	11.01 ± 6.18
2	42.33 ± 2.14	53.00 ± 14.05	10.38 ± 2.85
4	20.38 ± 4.21**	40.72 ± 10.27	5.61 ± 2.51
6	13.30 ± 2.08**	20.58 ± 5.48**	4.44 ± 0.81**
8	10.30 ± 1.13**	30.44 ± 3.40**	4.00 ± 1.06**
10	7.11 ± 1.98**	31.51 ± 6.02**	3.47 ± 0.62**

\* Average pmol tetryl/mg DNA

Mean ± Standard deviation (N=5)

Dose: 600 mg/kg BW (Single oral dose)

\*\* Significantly different from maximum value ( $p \leq 0.05$ ).



TABLE 7  
EFFECT OF SINGLE VS. MULTIPLE AND DIVIDED MULTIPLE DOSES  
ON TETRYL-DNA ADDUCT FORMATION\* IN RATS

Time (Days)	Dose	Liver	Kidney	Spleen
7	A	12.99 ± 2.81** <sup>+</sup>	20.11 ± 10.35** <sup>+</sup>	3.15 ± 1.41**
14	A	10.36 ± 0.49**	10.73 ± 2.04**	5.61 ± 1.93** <sup>+</sup>
7	B	54.54 ± 18.89	100.50 ± 18.91	44.14 ± 2.97
14	B	181.29 ± 39.18	397.17 ± 177.80	87.21 ± 10.75
7	C	7.49 ± 1.59** <sup>+</sup>	6.53 ± 1.82** <sup>+</sup>	2.82 ± 0.28**
14	C	10.56 ± 3.08**	15.24 ± 5.43**	4.43 ± 0.74** <sup>+</sup>

\* Average pmol tetryl/mg DNA

Mean ± Standard deviation (N=5)

Dosages: A= Single dose of 300 mg/kg BW; B= Daily doses of 300 mg/kg BW; C=Daily doses of 300/14 mg/kg BW.

\*\* Significantly different from dose B (p ≤ 0.05), time comparison.

<sup>+</sup>Significantly different (p ≤ 0.05), A vs. C, time comparison.

# APPENDIX D

- 449 FORMATION & PERSISTENCE OF 1,3,5-TRINITROBENZENE ADDUCTS WITH BLOOD PROTEINS AND TISSUE DNA. T V Reddy, L Wan, E L C Lin, F B Daniel and G Reddy, USEPA, HERL, Cincinnati, OH and US Army, BMRD, Ft. Detrick, MD

Blood protein adducts of chemicals have been used as biological markers of exposure. We investigated the ability of 1,3,5-trinitrobenzene (TNB) to form adducts with blood proteins and tissue DNA. Forty male rats were gavaged once with  $^{14}\text{C}$ -TNB (1.06 mmol/kg, 9.47 mCi/mmol) and divided equally into eight groups and were sacrificed after day 1, 2, 3, 4, 7, 14, 21 and 28, respectively. Adducted TNB with blood proteins, albumin (ALB), globulin (GLBU) and globin (GLB) was determined. ALB and GLBU adducts reached maximum (218 pmol/mg, 100%); and (61 pmol/mg, 100%) by day 1 and by day 7 about 90-95% of the adducts were lost. In contrast, GLB adducts reached maximum by day 2 (200 pmol/mg, 100%) and after 28 days 20% of the GLB adducts remained. TNB also formed stable adducts with DNA by day 1 in the spleen (16 pmol/mg, 100%) and by day 3 in both the stomach (112 pmol/mg, 100%) and the liver (75 pmol/mg, 100%). After 28 days the residual adduct level in the liver and stomach was 25% and of spleen was 100%. Hence, protein and DNA adducts of TNB may be useful as markers for exposure assessment. (Abstract does not necessarily reflect EPA policy) Supported by US Army I.A. Project Order No. 90PP0812.

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FORMATION & PERSISTENCE OF 1,3-DINITRO BENZENE (DNB) ADDUCTS WITH RAT BLOOD PROTEINS AND TISSUE DNA. T V Reddy<sup>1</sup>, F B Daniel<sup>1</sup>, H Wang<sup>2</sup>, B Wiechman<sup>2</sup>, and G Reddy<sup>3</sup>, <sup>1</sup>USEPA, Cincinnati, Ohio, <sup>2</sup>PAI, West Chester, Ohio, <sup>3</sup>U.S. Army CHPPM, Fort Detrick, Frederick, MD

Macromolecular adducts of chemicals have been used as biological markers of exposure. We investigated the ability of 1,3-DNB, an environmental contaminant detected at Army installations and superfund sites, to form adducts with blood proteins and tissue DNA. Sixty male Fischer rats were gavaged once with <sup>14</sup>C-DNB (0.178 m mol/kg, 65.4 mCi/m mol), divided equally into twelve groups and sacrificed after day 0.33, 1, 2, 3, 4, 7, 14, 21, 28, 42, 56 and 70, respectively. DNB adducted with blood proteins, albumin (ALB), globulins (GLBU), and hemoglobin (HB) was determined. Formation of ALB, GLBU and HB adducts was maximum at 8 hours (53, 7.2, 281 pico moles/mg protein, respectively), and decreased with time. About 90% of the ALB and GLBU adducts were cleared within a week while HB adducts persisted (e.g., 2.5% HB adducts were intact after 6 weeks). DNB also formed adducts with DNA from liver, kidney, and spleen and persisted throughout. Blood protein and DNA adduct levels were increased several fold when DNB was given daily (0.178 m mol/kg) for 7 days. Thus, protein and DNA adducts of DNB may be useful as markers for exposure assessment. (This abstract does not necessarily reflect USEPA/US Army policy)

10.7 cm X 14.5 cm

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**000111**

This abstract was accepted and will be presented at the 18th Annual meeting of the Society of The Environmental Toxicology and Chemistry, NoV. 1997

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**Exposure Assessment of Tetryl Using Blood Protein(s) and DNA Adducts as Biomarkers** T.Reddy, and B. Daniel, EERD, NERL, USEPA, Cincinnati, OH, B. Wiechman, and H. Wang, PAI, Westchester, OH, G. Reddy, US Army, CHPPM, APG, MD. Tetryl (N-methy-N, 2, 4, 6-tetranitroaniline) has been widely used as an ammunition chemical and it has been detected as an environmental contaminant in the soil, surface and groundwater near Army ammunition plants and at certain Army installations. Quantitation of actual exposures is a critical element in performing meaningful ecological health risk assessment. In this study we investigated the utility of macromolecular adducts (e.g., hemoglobin (HG), albumin (ALB) and DNA adducts) of Tetryl to function as biological markers of exposure. Experimentally, sixty male Fisher rats were orally intubated with [<sup>14</sup>C]-Tetryl (2.1 mmol/kg BW, 3 mCi/mmol), and groups of five rats were sacrificed after 0.33, 1, 2, 4, 7, 14, 28, 42, 56 and 70 days. The amount of Tetryl bound to HG, ALB as well as the DNA of selected tissues (liver, kidney, spleen) was quantified. HG and ALB adducts were formed in a dose-dependent manner and were maximal at 24 h after administration (672 and 1505 picomoles/mg protein), and decreased with time thereafter. Two weeks after a single exposure, approximately 5% of HG and less than 1% of ALB adducts of tetryl were intact. Detectable levels of radioactivity associated with HG were present even after eight weeks. Blood protein adduct levels were increased several-fold when Tetryl was given daily 1.05 mmol for 7 or 14 days. Tetryl also formed stable adducts with liver, kidney, and spleen DNA. Hence, blood protein and DNA adducts may be useful as markers for exposure assessment. (This abstract does not reflect USEPA/US Army policy).

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