A STUDY OF DOXORUBICIN TOXICITY

KULDIP GREWAL

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By

© Kuldip Grewal, B.Sc.(Hons.)

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Abstract

We examined myocardial injury following Doxorubicin use via analysis with the Automated Hitachi Analyzer of serum activity changes of enzymes specific for myocardial tissue. Sprague-Dawley rats were administered either saline, 3, 6, or 9 mg/kg doses of Doxorubicin by intraperitoneal injection. Blood samples were collected at times 0 and 48 hours posttreatment. There was an increase in serum creatine kinase (except the 9 mg/kg injected rats), lactate dehydrogenase (LD), including elevations of LD 1 and LD 2 isoenzymes and aspartate aminotransferase (AST) activities at 48 hours post Doxorubicin injection. Increasing drug dosage resulted in increasing myocardial tissue concentration of the drug as measured by high performance liquid chromatography. This suggests increasing Doxorubicin dosage leads to increased myocardial tissue concentration of drug and is associated with tissue damage and release of cardiac-specific enzymes.

Serum CK isoenzyme separation by electrophoresis revealed the presence of an additional band cathodic to CK-MM following administration of 6 and 9 mg/kg doses of the drug. Creatine kinase isoenzyme analysis of mitochondria also demonstrated this band in a position corresponding to that observed in the drug-treated animals. We suggest that

at these doses mitochondrial damage resulted in the release of mitochondrial CK.

At a drug dose of 9 mg/kg, increased serum alanine aminotransferase (ALT) and LD 4 and LD 5 isoenzymes was observed suggesting hepatotoxicity.

In rats, 24 hour urine specimens were analyzed for various biochemical parameters with the use of the Automated Hitachi Analyzer, the Astra-8 Automated Analyzer and the Chemstrip 9 Dipstick screen test, to detect evidence of renal damage. The only abnormality noted was a decreased urine volume with increased dose of the drug.

In the New Zealand white rabbit model, we demonstrated a serum CK-MB activity greater than five percent of total CK activity in all rabbits 48 hours subsequent to treatment with 10 mg/kg Doxorubicin. There was no such effect in the saline-treated controls. Myoglobin was detected in the serum 48 hours following a 10 mg/kg dose using the Rapitex Myoglobin kit. The origin of CK-MB was believed to be cardiac muscle; however the source of myoglobin could have been either skeletal muscle or myocardium.

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List of Abbreviations

AST/GOT: Aspartate aminotransferase/glutamic-oxaloacetic transaminase

ALT/GPT: Alanine aminotransferase/glutamic-pyruvic transaminase

CHF: congestive heart failure

CK: Creatine kinase

CK 1: creatine kinase-1

CK 2: creatine kinase-2

CK 3: creatine kinase-3

CK-MB: creatine kinase-MB

cu mm: cubic millimeter

ECG: electrocardiograph

G-C: guanine-cytosine

HPLC: high performance liquid chromatography

H_2O_2 : hydrogen peroxide

i.p.: intraperitoneal

i.v.: intravenous

Kp: tissue-to-plasma partition coefficient

LD: lactate dehydrogenase

LD 1: lactate dehydrogenase-1

LD 2: lactate dehydrogenase-2

LD 3: lactate dehydrogenase-3

LD 4: lactate dehydrogenase-4

LD 5: lactate dehydrogenase-5

mmol: millimole

ng: nanogram

nm: nanometers

$O_2^{\cdot}$: superoxide free radical

$OH^{\cdot}$: hydroxyl free radical

rpm: revolutions per minute

SOD: superoxide dismutase

sq m: square meter

µg: microgram

µl: microliter

U/L: units per liter

µm: micrometer

µM: micromole

U.V.: ultraviolet

V: volt

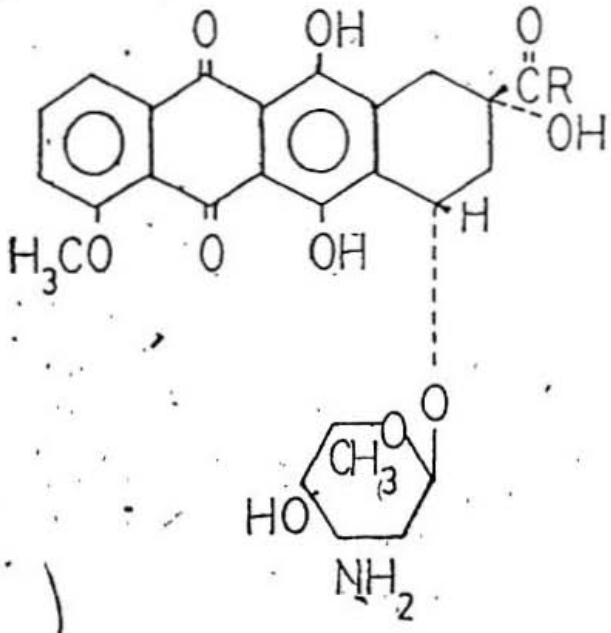
CHAPTER 1

INTRODUCTION

1.1. General Introduction

Doxorubicin (Adriamycin) is a cytotoxic anthracycline antibiotic isolated in 1967 by the Farmitalia Research Laboratory in Milan, Italy from the soil fungus Streptomyces peucetius var. caesius. It can also be chemically prepared from Daunorubicin (Vigevani and Williamson, 1980). The Doxorubicin molecule is an analog of Daunorubicin (Daunomycin) and differs from it only by an additional hydroxyl group at position 14 (Figure 1). Structurally the Doxorubicin molecule contains the aminosugar daunosamine linked via a glycosidic bond to the 4-ring anthracycline structure. Daunorubicin was discovered in 1957. Its antitumor activity was demonstrated in 1963 by DiMarco (Weiss et al., 1986). As a result of the structural similarity of the two, animal studies were initiated and in 1968 the antitumor effectiveness of Doxorubicin was demonstrated. The first clinical trial was carried out in 1969 by Bonadonna and coworkers who established its substantial antitumor action in the treatment of acute and chronic leukemias, lymphomas, Hodgkin's Disease and Ewing's sarcoma (Wang et al, 1971). Studies in the United States began shortly after and in 1974 the Food and Drug Administration approved its use. It is a broad spectrum antineoplastic agent, used successfully singly or in combination chemotherapy for a wide variety of

Figure 1. Structures of Doxorubicin and Daunorubicin



	R
Doxorubicin	CH_2OH
Daunorubicin	CH_3

malignancies. It is the most active single agent in the management of endometrial cancer in that it produces objective responses in 40% of patients. It is the most effective single agent in metastatic breast cancer. It produces regression in osteogenic and soft tissue sarcomas. Chemotherapy combinations utilizing Doxorubicin have been reported to produce complete responses in lymphomas and hematologic malignancies (Portlock and Groffinet, 1986).

The mechanism of antitumor activity of Doxorubicin is related to its ability to interact with specific sites on DNA, preferably G-C base pairs, via intercalation of the anthracycline ring between adjacent nucleotide bases. This results in steric obstruction and template disordering with consequent inhibition of the enzymes involved in DNA replication-DNA dependent DNA polymerase and transcription-DNA dependent RNA polymerase (Blum and Carter, 1974; Barranco, 1984). Doxorubicin also inhibits protein synthesis. The drug is not cell cycle specific but rather cell cycle active in that it acts at all stages of the cell cycle (Barranco, 1984).

Cell culture systems treated with Doxorubicin have demonstrated inhibition of viral, bacterial and mammalian cell DNA-dependent DNA polymerases and bacterial RNA polymerases. A 5 µg/ml dose of the drug inhibits

incorporation of tritiated thymidine into DNA and uridine into RNA (Momparler et al., 1976).

The interaction of Doxorubicin with DNA produces both single and double strand breaks which are not seen with purified DNA but only with cellular DNA indicating this may be the result of the action of intracellular enzymes such as repair endonucleases or topoisomerases in response to topological perturbations in DNA structure (Ross and Bradley, 1984).

The standard dosage schedule of Doxorubicin when used as a single agent is 60-75 mg/square meter by intravenous (I.V.) injection once every 3 to 4 weeks. There is substantial plasma protein binding, perhaps approaching fifty percent. Doxorubicin exhibits first order type kinetics in its elimination from the plasma. It has a triphasic plasma disappearance curve with approximate half-lives of 10-30 minutes, 10 hours and 24-48 hours (Young et al., 1981). Pharmacokinetic studies reveal fairly high concentrations of the metabolites soon after native drug administration.

When administered intravenously Doxorubicin is rapidly cleared from the blood and distributed to the tissues. There are clear differences in the apparent tissue-to-plasma partition coefficients (Kp) among tissues, but in

all cases this Kp value is unusually large. This drug binds to the DNA so it is postulated that the differences in tissue distribution depend on tissue DNA concentration (Terasaki et al., 1982; Terasaki et al., 1984). Terasaki suggests other mechanisms such as a lower capacity of the drug to bind to the DNA and/or the existence of a barrier to its entry in those tissues that exhibit a lower Kp value.

The metabolism of Doxorubicin is similar to that of Daunorubicin. The metabolic pathway is illustrated in Figure 2. The primary metabolite is Adriamycinol, the C-13 hydroxylation product of the action of NADPH-dependent aldo-keto reductase, a cytoplasmic enzyme ubiquitously distributed in man. The daunosamine moiety is then cleaved by microsomal glycosidases, present in most tissues, to produce the inactive aglycones Deoxyadriamycinol aglycone and Adriamycinol aglycone which are subsequently demethylated to Demethyldeoxyadriamycinol aglycone. The demethylated metabolites are conjugated to sulfate and glucuronide esters and excreted.

The predominant route of elimination of the drug and its metabolites is via the bile. Approximately 40-50% of the administered dose is excreted by this route over a period of 7 days. The majority (42%) of drug fluorescence is

accounted for by the parent drug. Adriamycinol accounts for 22% and 39% is attributed to the remaining metabolites. Only 7-10% of the dose is eliminated in the urine over 5 days; 40% as Adriamycin, 29% as Adriamycinol and 31% as other metabolites (Pratt and Ruddon, 1979).

Figure 2. Metabolic Pathway of Doxorubicin in Humans. Heavy arrows indicate preferred products.

I. Adriamycin (Doxorubicin)

II. Adriamycinol

III. Deoxyadriamycin

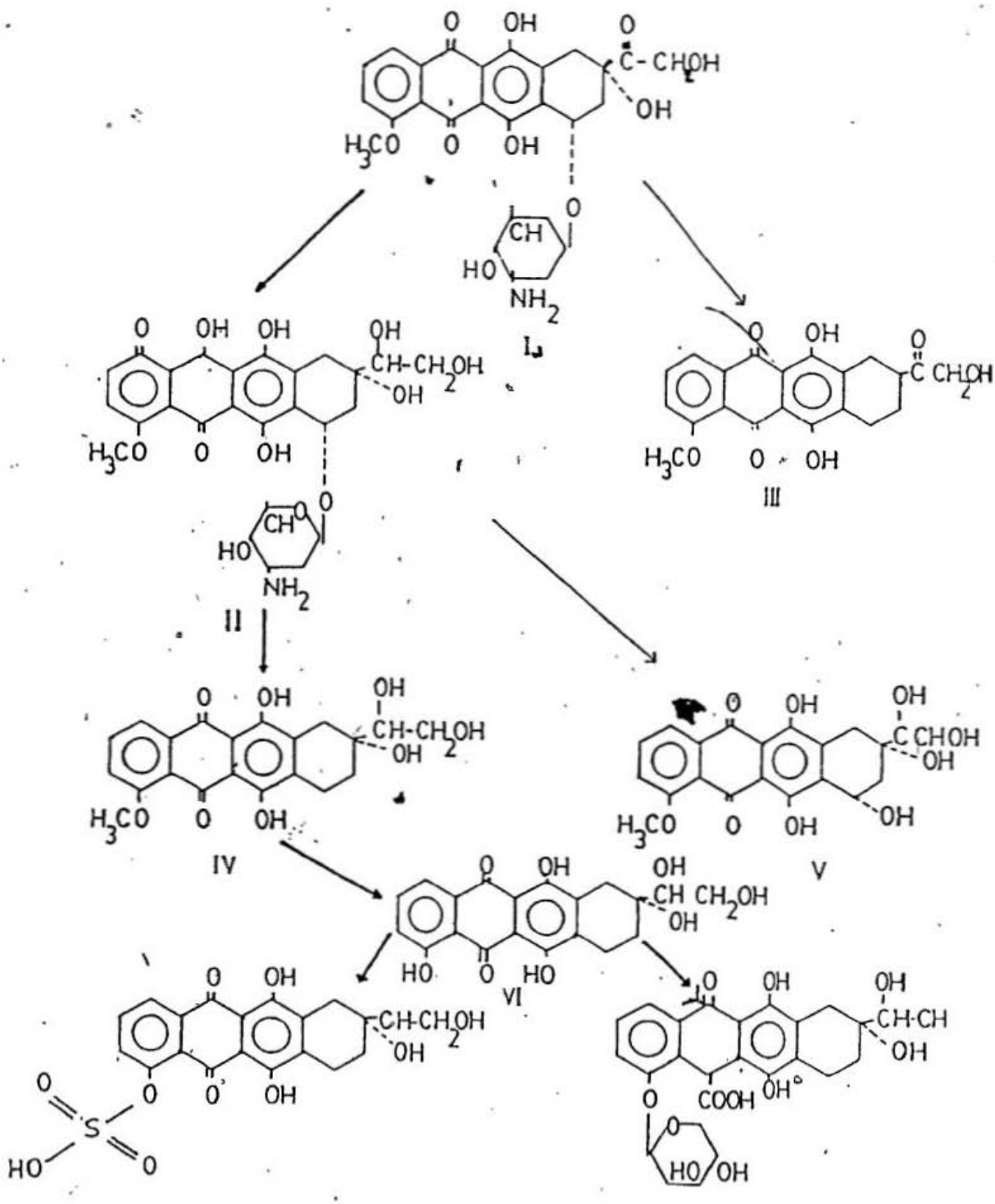
IV. Deoxyadriamycinol

V. Adriamycinol aglycone

VI. Demethyldeoxyadriamycinol

VII. Sulfate conjugate

VIII. Glucuronide conjugate


I
II
III
IV
V
VI
VII
VIII

Adriamycinol has the ability to bind to the DNA and therefore is thought to retain the toxic properties of Adriamycin. Although there is no direct evidence, there are observations which suggest that Adriamycinol can generate the production of cytotoxic superoxides (See section 1.5.1.). Also increased myocardial levels of this metabolite have been detected with the occurrence of functional and morphological cardiac alterations (Del Tacca et al., 1985).

1.2. Acute Toxicity

Myelosuppression is a dose-dependent, dose limiting complication in 60-80% of patients. It is manifest primarily as a neutropenia, reaching its nadir 10-14 days following treatment. White blood cell counts as low as 1000/cu mm should be expected. Thrombocytopenia and anemia may also occur but are less severe. Careful hematologic monitoring of WBC, RBC and platelets is necessary in the event that dose reduction or delay of therapy is required.

Complete reversible alopecia occurs in close to 100% of patients. Nausea and vomiting are frequent side effects. Stomatitis may occur 5 to 10 days following drug administration. Very rarely, fever and chills, urticaria, and anaphylaxis or hypertensive crisis may occur

immediately after injection. Other side effects include local tissue necrosis in the event of extravasation during injection, darkening of the veins used for administration and hyperuricemia (Becker, 1981).

Acute electrocardiographic (ECG) changes including tachycardia, ST-T wave changes, alterations in the QRS complex and extrasystolic contractions are frequent. There have been instances of sudden death following treatment which have been attributed to arrhythmias. However Freiss and coworkers question this. A study group of 30 patients who received Doxorubicin in combination with other chemotherapeutic agents were monitored by continuous ECG recording devices 24 hours prior to treatment and 24 hours during and following therapy. Nine of the 30 patients experienced pretreatment ventricular ectopy which did not increase in severity posttreatment. They suggested pretreatment monitoring to rule out drug therapy as its source (Freiss et al., 1985).

1.3. Nephrotoxicity

Doxorubicin-induced nephrotoxicity has been reported following only one dose of the drug in male Sprague-Dawley rats. Bertani described nephrotic alterations as early as 3 hours following treatment as a reduction in glomerular

membrane polyanions, which increased in severity with time (Bertani et al., 1982).

There is an apparent sequence of pathological changes with respect to the glomerulus. Firstly, there is the early loss of glomerular membrane polyanions, followed by fusion of the foot processes, microvilli formation and limited vacuolization. This latter change becomes more diffuse and encompasses disarrangement and sclerosis of the tufts (Giroux et al., 1980).

In addition to the alterations in renal morphology there are also changes in blood chemistries indicative of nephrotoxicity, such as elevation of serum cholesterol, triglycerides, total lipids, uric acid, creatinine and blood urea nitrogen (BUN).

The nephrotic syndrome produced with Doxorubicin use has been demonstrated in several animal species. However its occurrence in man has not been consistently reported. Doxorubicin therapy was thought to be responsible for renal damage in an elderly man who was treated for squamous cell carcinoma of the lung subsequent to treatment with 180 mg of the drug. Six weeks subsequent to the last dose renal biopsy revealed severe interstitial fibrosis and tubular atrophy with mild glomerular filtration, fusion and loss

of foot processes and abundant microvilli formation. There were increased serum levels of creatinine and BUN as well as proteinuria and hematuria (Burke et al., 1977).

1.4. Cardiotoxicity

The development of dose-dependent cardiotoxicity with chronic administration of Doxorubicin limits its use in oncologic practice. Gottlieb was the first to note that at cumulative doses above 550 mg/square meter the incidence of myocardial damage was unacceptably high and recommended cessation of drug therapy below this total dose level (Legha et al., 1982). However pathological changes in the myocardium have been described in patients who receive as little as 180 mg/sq m and have not been reported in others who receive in excess of 1000 mg/sq m. Therefore the clinician must decide whether to continue drug administration as a successful antitumor agent, concurrently risking the development of cardiotoxicity, or to halt therapy in a responding patient.

The cardiotoxicity is commonly categorized as either acute which is manifest primarily as electrocardiographic alterations or as chronic cardiomyopathy which may develop into congestive heart failure (CHF). The electrocardiographic abnormalities seen in acute toxicity

resolve within a few days to 2 months. The development of these changes is not an indication to halt therapy, however life threatening ventricular arrhythmias and death have been reported subsequent to Doxorubicin treatment.

An uncommon occurrence is the development of cardiomyopathy after only 2 courses of treatment but there is minimal functional deterioration until a patient-specific degree of damage is exceeded producing CHF. Clinically symptomatic biventricular CHF usually develops within a week to 2.5 months after the last dose of Doxorubicin, however it has been reported to occur as late as 7 years following therapy (Freter et al., 1986). If treated early it may respond to cardiac support.

The morphological features of Doxorubicin-induced cardiac toxicity have been well defined both in man and several animal models and characteristically include vacuolization and a decrease in the number of myocytes, interstitial edema, distention of the sarcoplasmic reticulum and swelling of cardiac mitochondria. All of these lesions are focal, for damaged tissue can be found adjacent to normal tissue, however with continuance of therapy they become disseminated. Many of these histologic changes are typical of cardiomyopathies of other origins (Von Hoff et al., 1982; Saltiel and McGuire 1986). Cohen reported a negative

correlation between the clinical and histologic signs of toxicity. In 23 of 69 patients who exhibited clinical signs of toxicity following treatment with Doxorubicin or Daunorubicin, only 10 had histologic findings in support of this, such as vacuolization and myofibrillar loss and interstitial fibrosis. In 21 of 46 patients who had no clinical signs there was histological confirmation of cardiotoxicity (Cohen et al. 1986).

When a patient receiving Doxorubicin chemotherapy presents with a cardiac disorder one should suspect the drug as its cause however there may be other factors responsible such as tumor involvement of the pericardium, infectious complications of therapy, nonbacterial thrombotic endocarditis, nutritional deficiencies and complications of therapy (Tokaz and Von Hoff, 1984). The ECG alterations occur in anywhere up to 41% of patients. However many cancer patients are febrile, weak and malnourished which may be contributing factors. Several risk factors have been identified for the development of cardiomyopathy. They are: 1) AGE. Those 70 years of age or greater and the group more recently identified, young children, are thought to be at greater risk. 2) MEDIASTINAL IRRADIATION. Prior or concurrent mediastinal radiation particularly to the heart is associated with an increased risk of development of cardiomyopathy. 3) PREEXISTING CARDIAC DISEASE. An

association between pre-treatment cardiac disease and hypertension and a greater incidence of cardiomyopathy development has been documented by several authors. 4) DOSING SCHEDULE. The usual dosage regimen is once every 3 weeks but a weekly schedule is associated with less cardiotoxicity. 5) OTHER DRUGS. In studies of patients receiving multi-drug chemotherapy containing cyclophosphamide and Doxorubicin a more toxic response developed than in those receiving only Doxorubicin. 6) TOTAL DOSE. The most important risk factor as described earlier.

1.5. Mechanisms of Cardiotoxicity

1.5.1. Free Radical Theory

In spite of extensive investigations the mechanism of cardiotoxicity produced with Doxorubicin use is not known. Many theories have been forwarded which include excess calcium influx, disruption of the electron transport chain in myocardial mitochondria (Johnson et al., 1986), interaction with SH-group containing enzymes (Fabregat et al., 1984), alteration of the membrane-bound Na -K -ATPase pump (Gosalvez et al., 1979), direct effect on the cell membrane (Murphree et al., 1981), release of histamine and catecholamines (Jackson et al., 1984), and the generation of Doxorubicin-induced semiquinone free radical and

subsequent oxygen-derived species which can disrupt intracellular membrane systems, result in lipid peroxidation or react with DNA and/or other macromolecules (Berlin and Haseltine, 1981; Halliwell and Gutteridge, 1986).

It has been well documented that the one-electron reduction of Doxorubicin to the unstable yet active semiquinone free radical intermediate is catalyzed by microsomal and nuclear NADPH cytochrome P-450 reductase, by the transfer of electrons from NADPH to the quinone moiety of Doxorubicin. Bachur suggests that it is the semiquinone free radical which intercalates into the DNA to induce strand scission (Berlin and Haseltine, 1981), however there is more evidence in support of the theory that under aerobic conditions autoxidation of the semiquinone generates superoxide free radical ($O_2^{\cdot}$) formation. Dismutation of the superoxide radical species produces hydrogen peroxide (H_2O_2) which in turn can react with the Doxorubicin semiquinone intermediate to produce the hydroxyl free radical ($OH^{\cdot}$), the most reactive of all these species. The physiological consequences of the formation of these active oxygen species include DNA strand scission and lipid peroxidation (Sinha et al., 1984; Berlin and Haseltine, 1981).

Malondialdehyde is a toxic by-product of the degradation of lipid peroxides. An increase in its content in the hearts of animals treated with the drug would lend additional support to the free radical theory. However there are conflicting reports as to its production. In an acute model of cardiotoxicity by Jackson, male New Zealand white rabbits were administered an intravenous injection of 10 mg/kg Doxorubicin. In this study there was no difference in malondialdehyde production between the control and experimental groups (Jackson et al., 1984). On the other hand Myers reported its production following a 15 mg/kg dose by intraperitoneal (i.p.) injection (Myers et al., 1977b).

If free radical production is wholly or in part responsible for myocardial toxicity, free radical scavenger agents must confer some degree of cardioprotection. In mice treated with the free radical scavenger alpha tocopherol 24 hours prior to drug treatment cardiotoxicity was significantly reduced as determined by electron microscopic examination. A lethal dose for 85% of the animals was subsequently reduced to a lethal dose for 10% with alpha-tocopherol treatment (Myers et al., 1977).

The cell has various intracellular defenses to protect itself against the minute quantities of oxygen radical

species that are normally produced. Superoxide dismutase (SOD) removes $O_2^{\cdot}$, catalase removes H_2O_2 and glutathione peroxidase and glutathione-S-transferase remove hydrogen peroxide and lipid peroxides (Halliwell and Gutteridge, 1986). The reduction of Doxorubicin to the free radical metabolite is greater in the liver than in the heart but only the heart develops clinically significant morphological changes. Why is this? Doroshow demonstrated that catalase activity in mouse liver was 173.3 ± 10.5 U whilst cardiac catalase was 1.1 ± 0.4 U (< 0.6% of that in the liver). As well, the activity of SOD in the heart was 27.1% of that in the liver. An acute depression in glutathione peroxidase activity for 72 hours following a single dose of 15 mg/kg Doxorubicin was also observed. Therefore Doxorubicin may potentiate its own toxicity (Doroshow et al., 1980).

1.5.2. Cell Membrane Theory

The conventional theory that Doxorubicin had to enter the cell in order to produce its toxic effect is now being challenged. Cytofluorescence studies demonstrate that the drug is localized in the nucleus and that it intercalates into the DNA. However the current thesis is that the drug may produce its cytotoxic effects as a result of interaction with membrane systems. There are several pieces

of evidence in support of this. Firstly, active synthetic anthracyclines such as N-trifluoroacetyladriamycin-14 valerate, an analog of Doxorubicin, has no demonstrable ability to bind to DNA. For many anthracycline derivatives synthesized in the laboratory there is no correlation between inhibition of nucleic acid synthesis and cytotoxicity. Also detailed studies have shown that Doxorubicin can effect alterations in such properties of the cell membrane as the expression of hormone receptors, transport of small ions and molecules, phospholipid structure and organization, fusion properties and fluidity (Siegfried et al., 1983; Tritton and Yee, 1982).

Tritton and Yee produced a model which demonstrated that the drug does not have to enter the cell to be active. They exposed murine cancer cell lines to large insoluble polymeric agarose beads to which Doxorubicin was covalently attached. The cells were exposed to the free drug, the drug attached to the support (beads) and the agarose beads themselves. The free and immobilized drug reduced cell survival while the beads had no effect. The intracellular contents of the cell were examined by high performance liquid chromatography (HPLC) to show that Doxorubicin was not being released from the support polymer.

Doxorubicin induces changes in the morphology of red blood

cells without producing cell lysis. With concentrations below 100 µM it can alter the discocyte to echinocyte morphology probably as a result of ATP depletion or calcium loading (Hickman et al., 1985).

Doxorubicin has a high affinity for cardiolipin, a negatively charged phospholipid specific to the inner mitochondrial membrane. Several of the enzymes of the respiratory chain require cardiolipin for full activity. Cell culture studies with myocardial cells demonstrate that the rhythmic contractions of the cells cease with drug treatment and that this is due to the lack of ATP synthesis (Goormaghtigh and Ruysschaert, 1984).

1.6. Cardiac Enzymes

1.6.1. Creatine Kinase

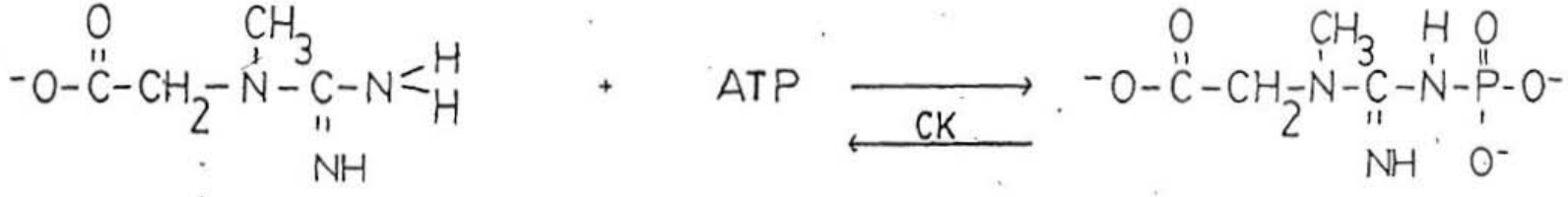
Creatine kinase (ATP:creatine N-phosphotransferase; CK; EC 2.7.3.2) is a dimer composed of 2 subunits with a molecular weight of 50,000 daltons each. It is present both in the cytoplasm and mitochondria of primarily metabolically active tissues such as the heart, brain and skeletal muscle. As shown in Figure 3 the enzyme is involved in the regeneration of ATP. An elevation in total CK activity is not characteristic of a specific disease state for it is observed in such varied conditions as myocardial

infarction, acute psychotic reactions and a severe fall. There are 3 isoenzymes of CK all of which are composed of combinations of the M and B polypeptide subunits so named because they were isolated from skeletal muscle and the brain respectively. The isoenzymes are CK 1 (BB); CK 2 (MB); and CK 3 (MM) which can be separated electrophoretically. Creatine kinase 1 is the fraction that migrates most rapidly towards the anode, CK 3 is the slowest migrating fraction and CK 2 is intermediate. Creatine kinase 1 is distributed primarily in the brain and nervous system but is also present in the lung, thyroid, gastrointestinal tract and genitourinary system. Creatine kinase 2 is predominant in the heart with a small amount in the genitourinary system. Creatine kinase 3 is present mainly in skeletal muscle but is found in most tissues.

Creatine kinase-MB appearance in the serum is widely used as a biochemical marker for pathological myocardial injury. However, elevated levels could be expected with significant muscle necrosis as in the muscular dystrophies and multiple forms of myositis.

Following an acute myocardial infarction CK-MB appears in the serum, usually within 4-8 hours, it peaks at 24 hours and returns to normal levels at 48-72 hours. Its appearance is usually reported as a percentage of total CK activity

Figure 3. The Reaction Catalyzed by Creatine Kinase (CK)



CREATINE

PHOSPHOCREATINE

+ ADP

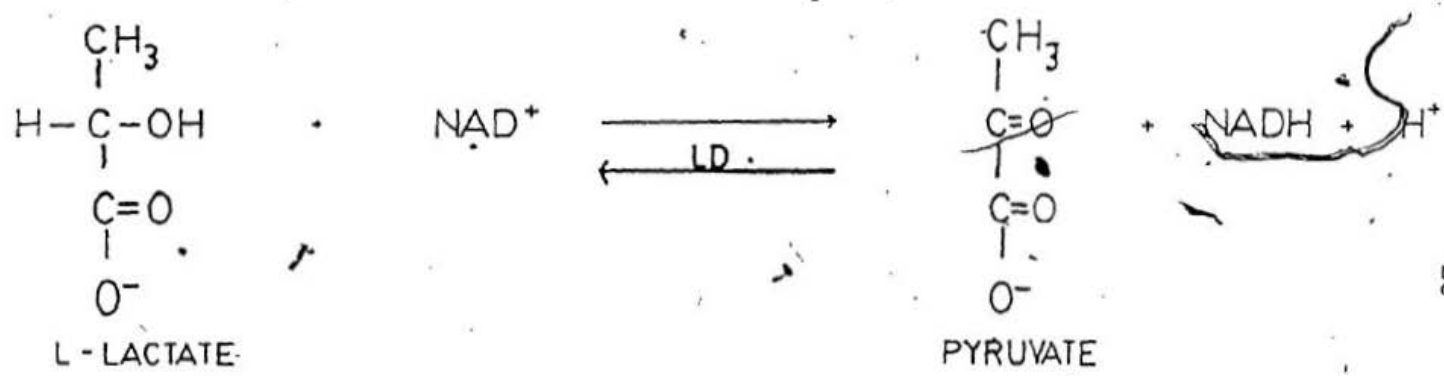
with a value greater than 5% indicative of myocardial damage (Lott and Stang, 1980). Skeletal muscle injury will cause both total CK and CK-MB elevation, however, the %CK-MB will remain less than five percent.

In 1964 Jacobs described a fourth CK isoenzyme that was associated with the mitochondria. It can be distinguished from the other isoenzymes by the fact that it moves towards the cathode during electrophoresis at pH 8.8 while the others migrate towards the anode (Hall et al., 1979). This isoenzyme is a dimer composed of 2 identical subunits. Its molecular weight is similar to that of the other isoenzymes however it differs in amino acid composition, amino-terminal amino acid, electrophoretic mobility and in certain immunological properties (Lang and Wurzburg, 1982).

1.6.2. Lactate Dehydrogenase

Lactate dehydrogenase (LD; L-lactate:NAD oxidoreductase; EC 1.1.1.27) has a molecular weight of 134 000 daltons. This zinc-containing enzyme catalyzes the interconversion of pyruvate and L-lactate with the cofactor NADH or NAD (Figure 4). Lactate dehydrogenase plays an important role in tissues that utilize glucose.

Figure 4. The Reaction Catalyzed by Lactate Dehydrogenase (LD)


CH3
H–C–OH
C=O
O⁻
L-LACTATE
NAD⁺
LD
CH3
C=O
C=O
O⁻
PYRUVATE
NADH + H⁺

The enzyme is predominant in the cytoplasm of the heart, kidney, liver, skeletal muscle and red blood cells. As a result of its wide distribution, elevated total enzyme levels are present in many disease states such as hypoxia, acute leukemias and hepatitis.

There are 5 isoenzymes of LD, each of which is a tetramer composed of 2 types of subunits designated H and M for heart and muscle respectively. The subunit compositions of the isoenzymes are: LD 1:HHHH; LD 2:HHHM; LD 3:HHMM; LD 4:HMMM; and LD 5:MMMM.

The LD 1 and LD 2 fractions are normally present primarily in tissues with aerobic metabolism such as the heart, kidney and brain. Tissues such as the liver and skeletal muscle with primarily anaerobic metabolism have a large portion of LD 4 and LD 5. Lactate dehydrogenase 3 is distributed in many tissues, for example endocrine glands, spleen and lung.

In the normal human serum LD 2 has the greatest activity followed in order by LD 1, LD 3, LD 4 and LD 5. Following an acute myocardial infarction there is a typical "flipped" LD pattern in that LD 1 is greater than LD 2 that appears within 48 hours in close to 80% of patients. This usually returns to normal values within one week although total LD

activity remains elevated.

1.6.3. Aspartate Aminotransferase

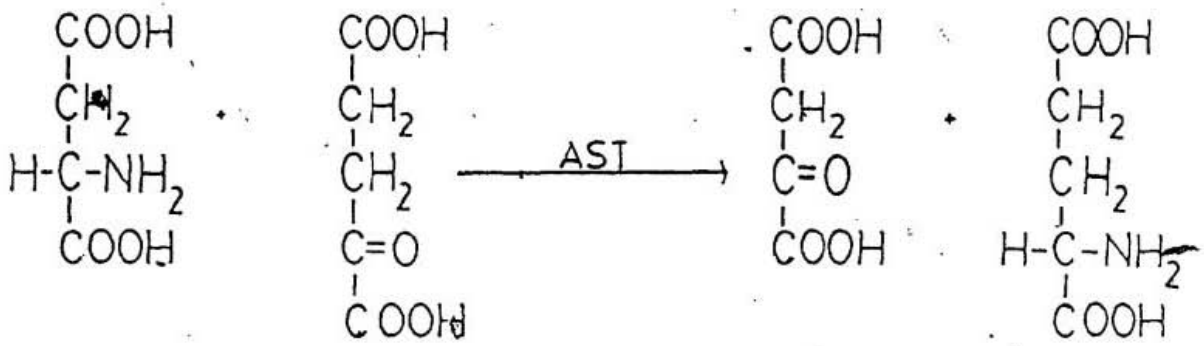
Aspartate aminotransferase (AST) is a dimer of 2 identical subunits. It is present in the cytoplasm of the liver, kidney, myocardium and skeletal muscle. This enzyme is involved in the transamination of an amino group from the donor-amino acid- L-aspartate- to an acceptor keto acid- oxaloacetate. The heart and skeletal muscle are a rich source of AST, and consequently serum AST is elevated in myocardial infarction.

Changes in serum CK, LD and AST activity are of value in the diagnosis of myocardial damage. Creatine kinase rises rapidly starting at 4-6 hours and reaching peak values at 24-36 hours. By this time AST levels are increasing and usually peaks at 2-3 days. LD tends to rise a little later and peaks at 2-3 days but usually remains elevated for up to 14 days.

1.6.4. Myoglobin

Myoglobin is a monomeric heme-containing protein that transports oxygen in mammalian muscles. It is found in smooth, skeletal and cardiac muscles. Destruction of muscle

Figure 5. The Reaction Catalyzed by Aspartate Aminotransferase (AST)



ASPARTIC ACID | KETOGLUTARIC ACID | OXALOACETIC ACID | GLUTAMIC ACID

fibers results in the release of myoglobin into the serum. This has been demonstrated following infraction of large skeletal muscles and cardiac muscle.

1.7. Objectives

The aim of investigation with the New Zealand white rabbit was to study acute enzyme changes subsequent to Doxorubicin treatment. The enzymes of interest were CK, LD and myoglobin.

Detailed monitoring of acute toxicity associated with Doxorubicin use was possible in the Sprague-Dawley rat. Serum enzyme and isoenzyme changes particularly those indicative of myocardial damage were analyzed. Detailed urine analysis was used to monitor renal injury, if any. The concentration of the parent drug in the myocardium, was determined in an attempt to correlate drug levels with toxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Doxorubicin-HCl was kindly provided by Adria Laboratories of Canada (Mississauga, Ontario). Dr. Kenneth K. Chan (Department of Pharmacy, University of California at Los Angeles, California) kindly supplied the Daunorubicin standard for the HPLC work. The male Sprague-Dawley rats (462-525 g) were obtained from Canadian Hybrid Farms, Nova Scotia. Male New Zealand white rabbits (1.85-2.50 kg) were purchased from Memorial University of Newfoundland's local vivarium. Both species were housed in the Animal Care quarters, Faculty of Medicine, where they were subject to alternating 12 hour light/darkness cycles with controlled humidity.

Methanol was purchased from J.T. Baker Chemical Company, New Jersey. Chloroform was supplied by Fisher Scientific, New Jersey. Isopropanol (propan-2-ol) and 85% phosphoric acid were obtained from Alltech Industries, Illinois. All of these chemicals were used for high performance liquid chromatography and were of HPLC grade.

2.2. Methods

2.2.1. *in vivo* Experimentation with Sprague-Dawley Rats.

The rats were permitted free access to food (Purina Mills Rat Chow # 5012, St.Louis, Mo.) and water until approximately 12 hours before treatment at which time they were withdrawn. Ethrane (Enflurane; 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether, Ohio Medical Anesthetics, Quebec) was used to induce anesthesia. Five rats in each of 3 experimental groups were administered by intraperitoneal (i.p.) injection either 3, 6, or 9 mg/kg body weight Doxorubicin. Doxorubicin was reconstituted in 0.9% saline immediately prior to administration to the animals. One set of 5 control rats were treated with an equal volume of saline via i.p. injection. Blood samples were collected by cardiac puncture. The rats were then individually placed in "metabolic cages" for 24 hour urine sample collection at which time food and water were returned. Following urine collection the animals were returned to their separate cages. At 48 hours the same procedures for producing anesthesia and blood collection were followed. At this time the rats were sacrificed by air injection into the heart. Samples of the heart were taken for measurement of Doxorubicin and its metabolites and for CK isoenzyme analysis. The tissues were kept on ice until transferred to 4°C.

2.2.1.1. Serum Analysis

The blood samples were centrifuged at 1000 rpm for 5 minutes using an Adams sero-fuge centrifuge (Clay Adams, New York). The serum was separated and assayed for:

(i) creatine kinase (CK)

(ii) lactate dehydrogenase (LD)

(iii) alkaline phosphatase

(iv) aspartate aminotransferase (AST, GOT)

(v) alanine aminotransferase (ALT, GPT)

Statistical Analysis

The Student's t-test was used to test statistical significance between the values obtained for the total enzyme activities in the individual rat at 0 and 48 hours. This statistical test was also performed to determine any significant difference between the 4 groups; the 3 experimental and 1 control group.

2.2.1.1.1. CK Isoenzyme Analysis

The CK isoenzymes were electrophoretically separated on Super Sepraphore cellulosic membranes (Gelman Sciences, Michigan). The Gelman CK Isozyme U.V. Reagent Set (Product

No.51914, Gelman Sciences, Michigan) was used to visualize the CK isoenzymes.

PRINCIPLE

Creatine kinase catalyzes the reaction between creatine phosphate and ADP to yield creatine and ATP. The latter product is coupled to glucose to produce glucose-6-phosphate and ADP. The reaction between glucose-6-phosphate and NADP is catalyzed by glucose-6-phosphate dehydrogenase yielding 6-phosphogluconate and NADPH. NADPH fluoresces when excited by UV light in the 340-375 nm range. The intensity of fluorescence is directly proportional to enzyme activity.

$$\text{Creatine phosphate} + \text{ADP} \xrightleftharpoons{\text{CK, } Mg^{2+}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{6-phosphogluconate} + \text{NADPH}$$

PROCEDURE

1. Soak a Super Sepraphore strip in 100 ml High Resolution Buffer for at least 10 minutes.

2. Remove the strip from the buffer and blot on an absorbent pad.
3. Place serum samples and CK isoenzyme control serum (Roche Diagnostics Systems, New Jersey. Item 37326) in individual wells of the applicator block using capillary tubes.
4. The strip is then loaded onto a bridge and placed into an electrophoresis chamber filled with buffer.
5. The samples are transferred onto the membrane.
6. Connect power supply and electrophorese at 225 V for 20 minutes.
7. During the last 5 minutes of the run a substrate transfer strip is floated in 1 ml of CK Isozyme U.V. Reagent (Product No. 51914) on a glass slide.
8. At end of run a second glass slide is pulled across the surface of the first at a 45° angle to expel air bubbles and remove excess substrate.
9. The Super Sepraphore membrane is laid flat onto the substrate transfer strip and a second glass slide is pulled across to remove air bubbles.
10. It is then incubated at 37°C for 30 minutes.
11. The transfer strip and membrane are separated.
12. The membrane is dried using a hair dryer at low temperature and viewed at 375 nm for qualitative examination.

2.2.1.1.2. LD Isoenzyme Analysis

The LD isoenzymes were separated by electrophoresis on Super Sepraphore membranes (Gelman Sciences, Michigan). Visualization of the isoenzymes was based on the reversible reaction between NADH and pyruvate to form lactate and NAD, which is catalyzed by LD.

$$\text{L-lactate} + \text{NAD} \xrightarrow{\text{LD}} \text{pyruvate} + \text{NADPH} + \text{H}$$

PROCEDURE -

1. A Sepraphore III electrophoresis membrane (Product No. 62092) is soaked in 100 ml High Resolution Buffer (Product No.51104) at least 10 minutes.
2. The membrane is removed from the buffer and blotted on an absorbent pad.
3. The serum samples and control are placed in individual wells of the applicator block using capillary tubes.
4. The membrane is loaded onto a bridge, and placed in an electrophoresis chamber filled with buffer.
5. The samples are transferred onto the membrane.
6. The power supply is connected and electrophoresis is conducted at 225 V for 20 minutes.
7. During the last 5 minutes of the run a substrate transfer strip is floated in 1 ml of the LD reagent on a glass slide.

8. At the end of the run a second glass slide is gently run across the surface of the first at a 45° angle.
9. The membrane is held horizontally and laid flat onto the substrate transfer strip. A second glass slide is pulled across the surface of the first at a 45° angle.
10. This is incubated at 37°C for 30 minutes.
11. The transfer strip and membrane are separated and the membrane is placed in a 5% Acetic Acid bath for 5 minutes.
12. Next it is placed in a cool water bath for 5 minutes.
13. The membrane is dried between 2 absorbent pads for approximately 1 hour.
14. The membrane is visually examined.
15. The isoenzymes were quantitated on the Beckman Model CDS-200 Computing Densitometer at 575 nm.

2.2.1.2. Tissue Analysis

2.2.1.2.1. Tissue Extraction

Heart tissue samples were analyzed for measurement of Doxorubicin and its metabolites by high performance liquid chromatography.

The method for tissue extraction was a modification of that of Cummings and coworkers (Cummings et al., 1984). The tissues were allowed to thaw at room temperature.

Approximately 1.0 g of tissue was washed in 3 ml of buffered 0.9% NaCl and minced with dissection scissors. A homogenate was produced using a Ten-Broeck tissue homogenizer (Fisher Scientific, Nova Scotia). To 1 ml of homogenate was added 5 ml of chloroform/isopropanol (2:1) mixture which was then vortexed for 1 minute using a vortex mixer (Fisher Scientific, Nova Scotia), then centrifuged at 1000 rpm for 15 minutes using the CU-5000 Centrifuge (Beckman Instruments, Ontario). This produced 3 phases: an upper aqueous phase, a middle tissue pellet and a lower organic phase. The upper phase was discarded by aspiration. The lower phase was retained by aspiration through the tissue pellet and evaporated to dryness in an analytical evaporator at 40°C and 25 mm pressure nitrogen. The dry extracts were reconstituted in 75 µl of methanol, a known amount of Daunorubicin was added to the sample and injected onto the HPLC.

2.2.1.2.2. High Performance Liquid Chromatography

The HPLC system consisted of a model 421 Controller, Model 112 Solvent Delivery Module and a Model 340 Organizer (Beckman Instruments, California) fitted with an Econosphere 300 C18 column 250 mm in length, and an internal diameter of 5 µm. A 25 µl Hamilton syringe (Hamilton, Nevada) was used to inject the sample into the

Altex 210 sample injector and onto the column.

The organic phase consisted of 99.85% isopropanol in 85% phosphoric acid (0.15 M). It was filtered through a millipore filter of pore size 0.50 µm (Millipore Corp., Massachusetts). The aqueous phase was 99.85% water in 85% phosphoric acid (pH 7.6). The ratio of organic to aqueous phase was 2:1. This was filtered through a millipore filter of pore size of 45 µm. The solvent system was pumped through the column at a flow rate of 0.50 ml/minute.

The peaks were detected with a Model 121 Gilson fluorometer (Mandel Scientific, Ontario) fitted with filters specific for detection of Doxorubicin.

2.2.1.2.3. Myocardial Total CK Analysis

Rat hearts were homogenized with 1% Triton X 100 in 0.155 M KCl in Ten-Broeck tissue homogenizers. A ratio of 0.5 g tissue to 2 ml medium was used. The resulting crude homogenate was centrifuged at 4°C at 24 000 rpm for 15 minutes in the International Portable centrifuge. The supernatant was retained and ultracentrifuged at 40 000 x g for 5 minutes at room temperature in a Beckman L5-65 Ultracentrifuge. The supernatant was analyzed for total CK activity on the Automated Hitachi Analyzer.

2.2.1.2.4. Mitochondrial Isolation

Rat livers were removed for the purpose of mitochondrial isolation. The tissues were rinsed with 30 ml of ice cold Preparation Medium which consists of 0.25 M sucrose, 3.4 mM tris-HCl, pH 7.4 at 20°C, 1 mM EGTA (ethylene-glycol-bis-(aminoethyl) tetra-acetate). The medium was decanted. The livers were finely minced with scissors in another 30 ml of medium. This medium was decanted. This washing procedure was repeated once more. A crude homogenate was prepared in a hand held Dounce homogenizer which was filled with medium for a total volume of about 55 millliliters. The resulting homogenate was poured into a 250 ml conical flask on ice to which an additional 50 ml of medium was added. The homogenate was centrifuged in a Sorvall ultracentrifuge (DuPont, New Jersey) at 6500 rpm at 4°C for 10 minutes. The supernatant was decanted into clean centrifuge tubes and centrifuged at 25 000 rpm for 7 minutes. The supernatant was decanted. The pellet was gently resuspended with a glass rod and then pooled. Eighty millliliters of Preparation medium was added. This was poured into 2 centrifuge tubes and centrifuged at 25 000 rpm for 7 minutes. The pellets were resuspended as before into 1 tube and centrifuged at 25 000 rpm for 7 minutes. The pellet was gently resuspended and about 3 ml of medium was

added. This solution was then frozen and thawed several times and then used for CK isoenzyme analysis purposes.

2.2.1.3. Urine Analysis

The sodium and potassium content of the urines were measured on the Astra-8 Automated Analyzer. The technique employed "ion selective electrode" methodology. Total LD activity was assayed on the Hitachi Automated Analyzer. The The Chemstrip 9 Dipstick test was purchased from Boehringer Mannheim, Quebec. The osmolality of the samples was determined using the Automated Osmette purchased from Boehringer Mannheim, Quebec. The procedure for assay of total protein is as follows:

1. Label tubes as BLANK, STANDARD #1, STANDARD #2, and URINE TEST #1, 2...etc.
2. Add 9.0 ml 1.5% sulfosalicylic acid to each tube.
3. Add 1.0 ml urine to the test samples.
4. Add 1.0 ml of standard to standard tubes and 1.0 ml of 0.85% saline to the blank tube. Commercially available control standards were used and appropriately diluted to give solutions of 0.64 and 0.32 g/L.

5. Allow to stand at room temperature 10 minutes and read absorbances at 420 nm against the blank.

CALCULATION: $\frac{\text{Absorbance Test}}{\text{Absorbance Standard}} \times \text{Concentration standard}$

= concentration of protein in test fluid

Principle: The protein is precipitated with sulfosalicylic acid and the turbidity is measured.

2.2.2. *in vivo* Experimentation with New Zealand White Rabbits

Two groups of rabbits were studied. The five animals in the experimental group were administered 10 mg/kg Doxorubicin in saline by i.p. injection. The control group of 5 animals were treated with an equal volume of saline by the same route. At predetermined intervals blood samples were collected from the marginal ear veins using a winged needle infusion set. The samples were centrifuged at 1000 rpm for 5 minutes using an Adams sero-fuge centrifuge (Clay Adams, New York). The separated serum was used for measurement of the total activities of CK and ED on the Hitachi Automated Analyzer. The remaining serum was stored at 4-8°C for myoglobin assay and CK isoenzyme analysis.

2.2.2.1. Myoglobin Assay

The Rapitex Myoglobin kit (Behring Institut, West Germany)

was used to demonstrate myoglobin in the serum. This assay is based on an immunochemical reaction between myoglobin and antibodies to myoglobin bound to polystyrene particles producing visible agglutination.

PROCEDURE

1. The serum samples are transferred from 4°C to room temperature.
2. Fifty µl serum and 10 µl absorption solution (consisting of an antibody solution) are placed on a zone on the test plate.
3. Twenty five µl of the RapiTex Myoglobin solution (consisting of polystyrene particles with antibodies sensitized to myoglobin) are added to the serum-absorption solution mixture.
4. The plate is slowly rotated for 3 minutes and examined for agglutination.
5. Agglutination implies presence of myoglobin in sample.

2.2.2.2. CK Isoenzyme Analysis

The CK isoenzymes were analyzed by the same method as used to analyze the rat serum and quantitated as a percentage of total CK on the Beckman Model CDS-200 Computing Densitometer with the following settings:

(a) Zero control for setting chart zero value: "0".

(b) Peak Height control for setting maximum pen deflection for the most dense fraction scanned: " - "

(c) Delimit Sensitivity control for selection of delimit sensitivity value in the automatic peak detection: "9 o'clock position".

(d) Mode control: "fluorescence".

(e) Trace Length for selection of chart trace length: "normal".

(f) Slit Control: slit size of 0.30 x 2.0 mm (small).

(g) To select the filter wavelength, the filter wheel should be set at position 5, UV.

(h) When operating in the fluorescence mode, the photodetector should be covered with a magnetic cover.

CHAPTER 3

RESULTS

3.1. Experimentation with Sprague-Dawley Rats

3.1.1. Serum Analysis

3.1.1.1. Total Enzyme Activities

The activities of CK, LD, ALT, AST and alkaline phosphatase were assayed at 0 and 48 hours following treatment with one dose of saline or Doxorubicin. Each line in figures 6-21 represents the data obtained at 0 and 48 hours posttreatment from one rat. The Student's t-test was used to test statistical significance between the values obtained in the individual rat at 0 and 48 hours. If the results were demonstrated to be significantly different at the 0.05 level this is indicated in the description accompanying each figure. This statistical test was also performed to determine any significant difference between the means of the 4 groups. The results of this test are included following the data of each specific enzyme assay.

Figure 6. Total CK Activity Following Saline Treatment. At 0 hour the activities were high and widely distributed. Note the decrease in CK activity at 48 hours as compared to 0 hour.

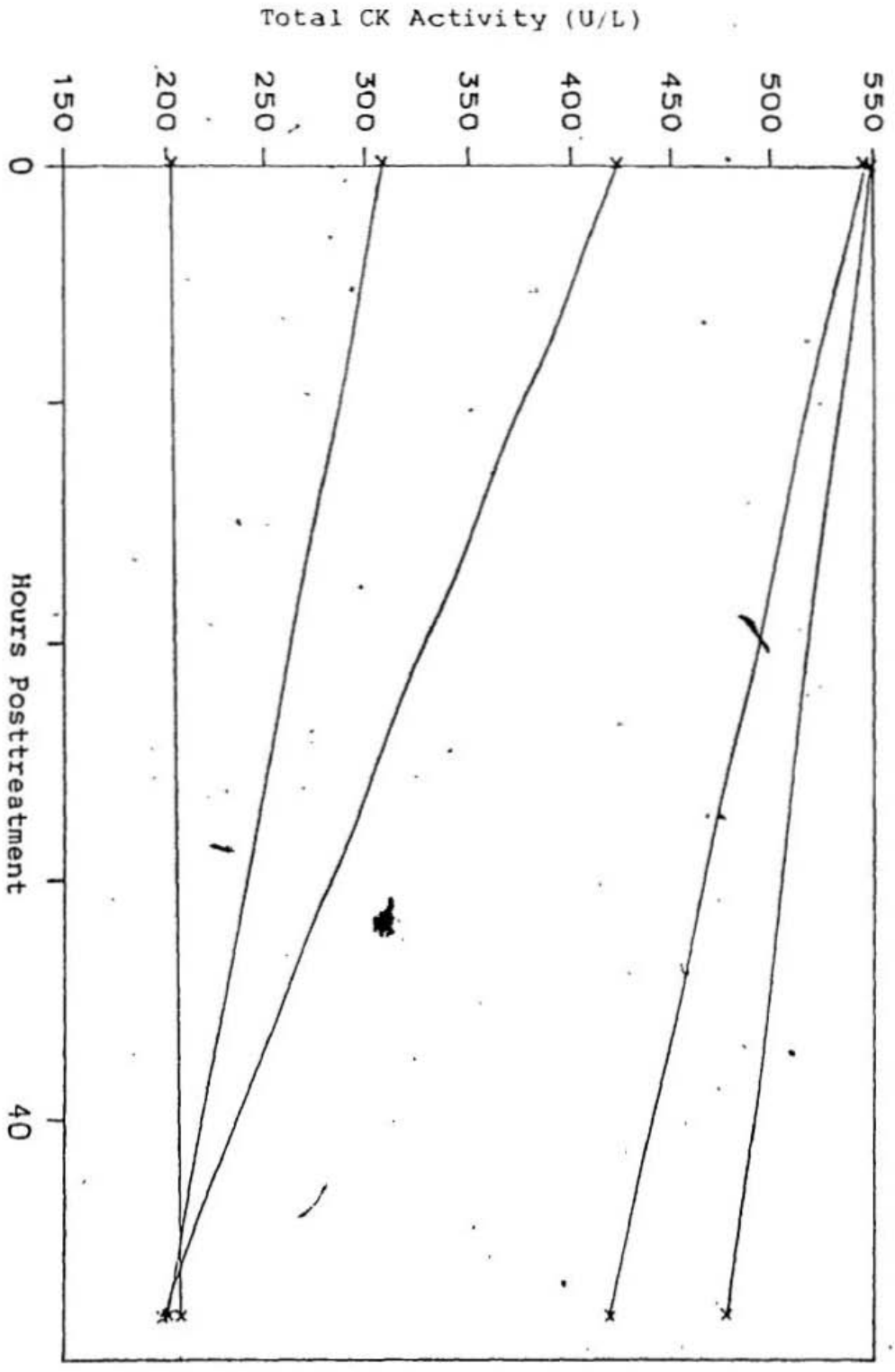

Total CK Activity (U/L)
150
200
250
300
350
400
450
500
550
0
Hours Posttreatment
40

Figure 7. Total CK Activity Following 3 mg/kg Doxorubicin. There was a statistically significant increase in activity at 48 hours as compared to 0 hour ($p < 0.05$).

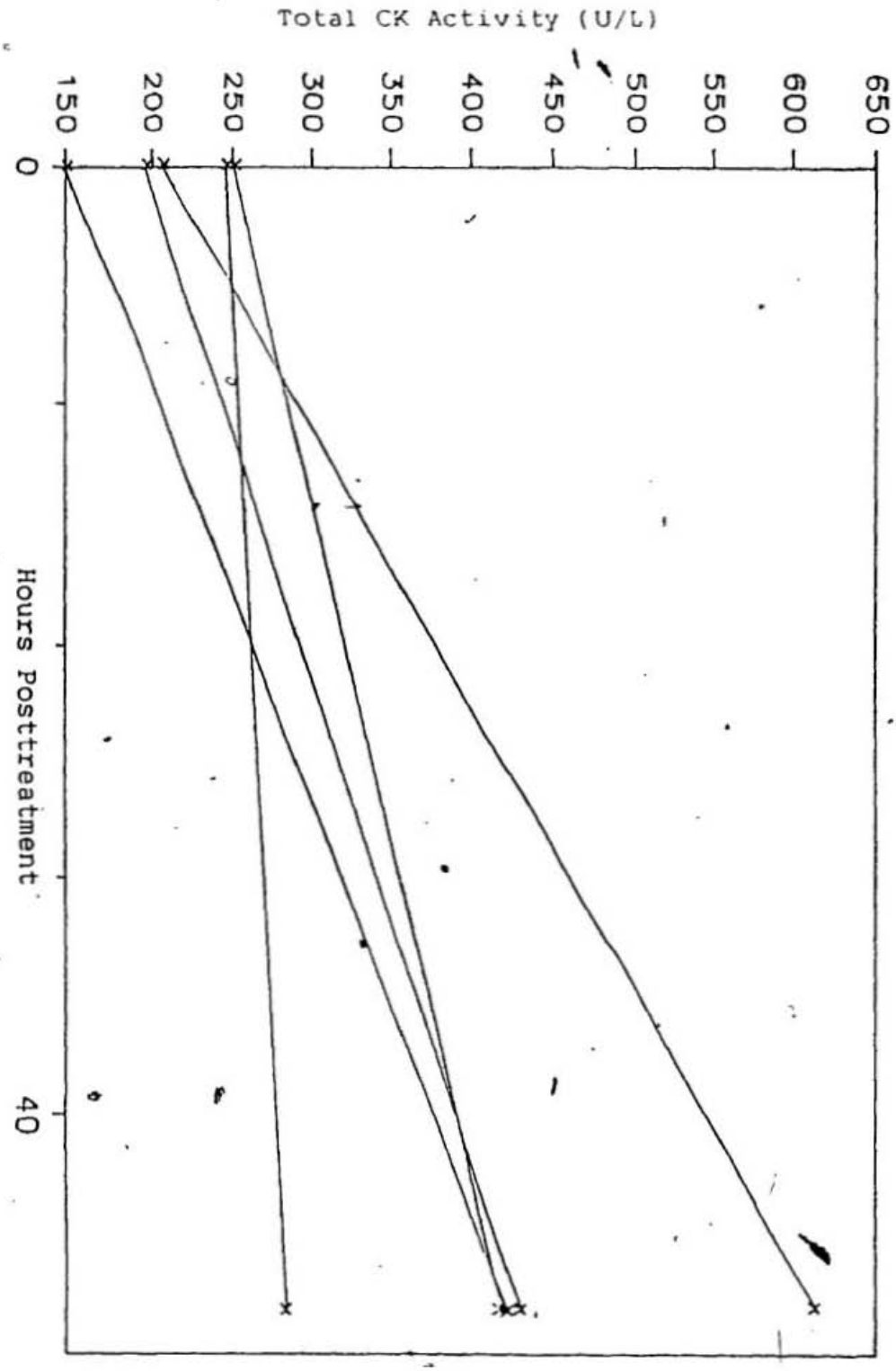

Total CK Activity (U/L)
150
200
250
300
350
400
450
500
550
600
650
0
40
Hours Posttreatment

Figure 8. Total CK Activity Following 6 mg/kg Doxorubicin. There was a statistically significant difference in the means of the values obtained at 48 hours as compared to that at O hour ($p < 0.05$)

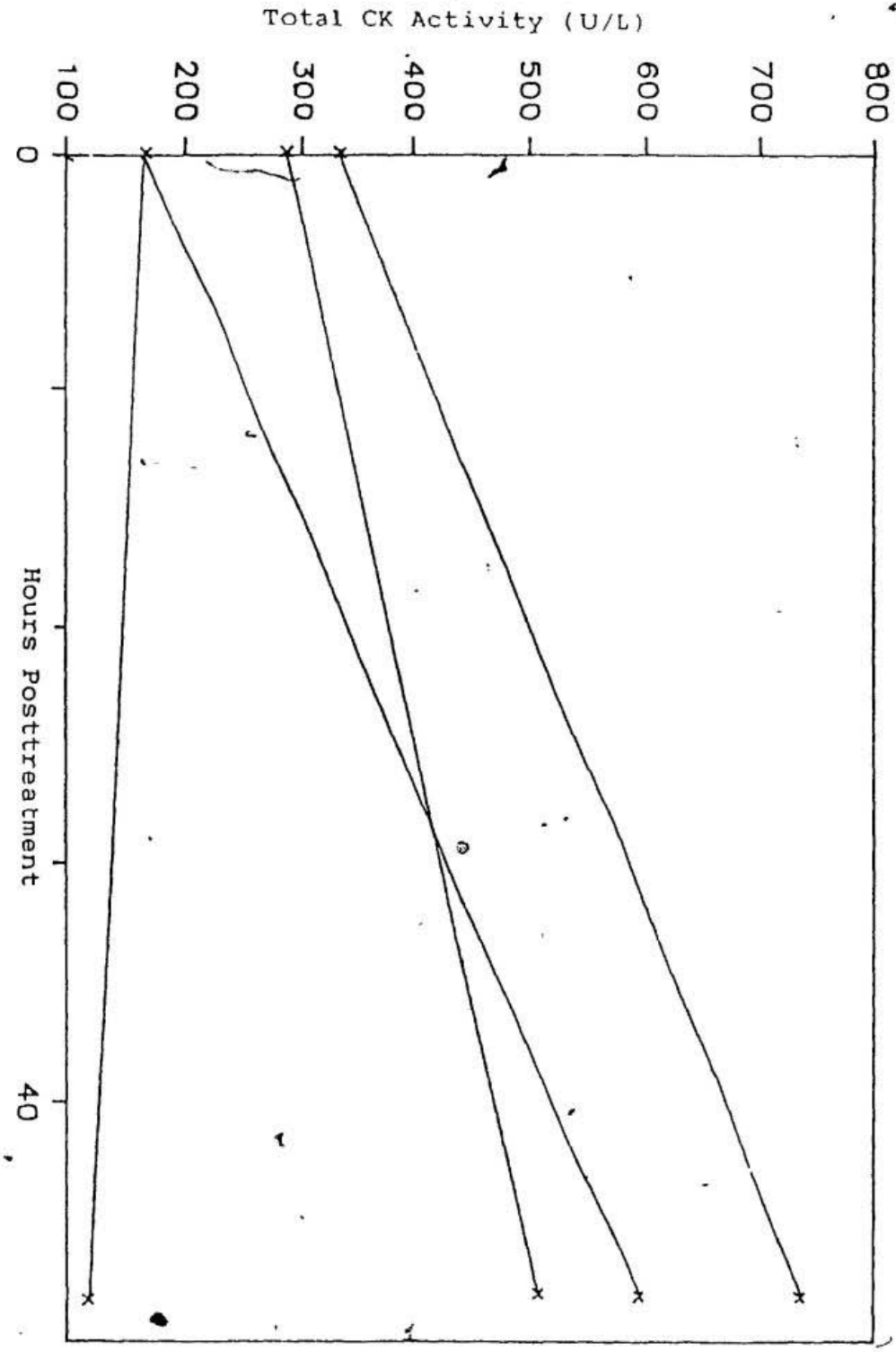
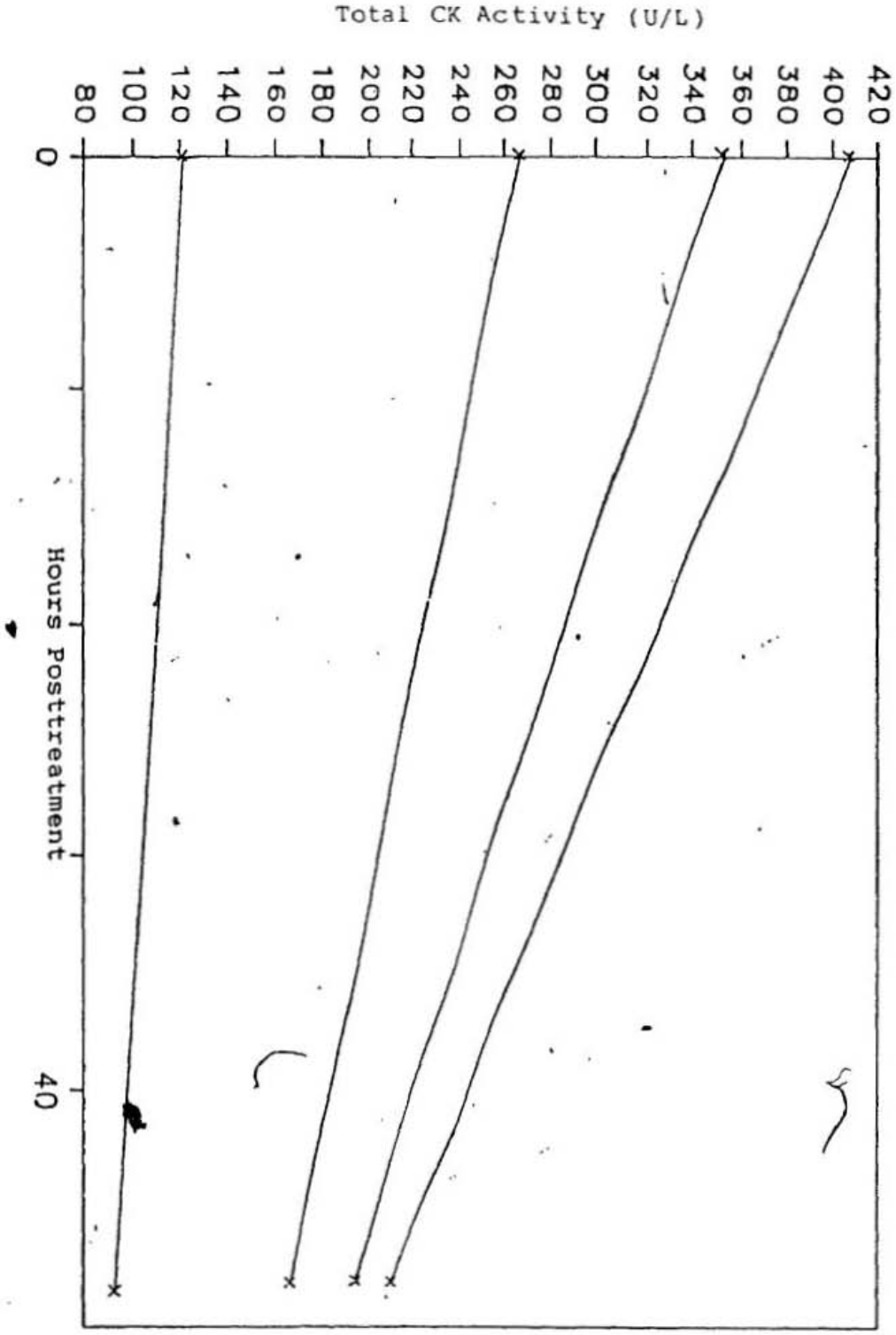

Total CK Activity (U/L)
100
200
300
400
500
600
700
800
0
Hours Posttreatment
40

Figure 9. Total CK Activity Following 9 mg/kg Doxorubicin. A significant decrease in CK activity was noted at 48 hours ($p < 0.05$).


Total CK Activity (U/L)
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
0
40
Hours Posttreatment

Statistical Analysis of CK Activity

With respect to CK total activity there was a difference in the mean 0 hour values between the saline-treated controls and those treated with the 3 mg/kg dose and also between the control group and those administered 6 mg/kg. At 48 hours post injection there was a significant difference between the 3 mg/kg and 9 mg/kg groups and between the 6 and 9 mg/kg treated groups.

Figure 10. Total LD Activity Following Saline Treatment. At 48 hours as compared to 0 hour three rats showed a marginal decrease and two a marginal increase.

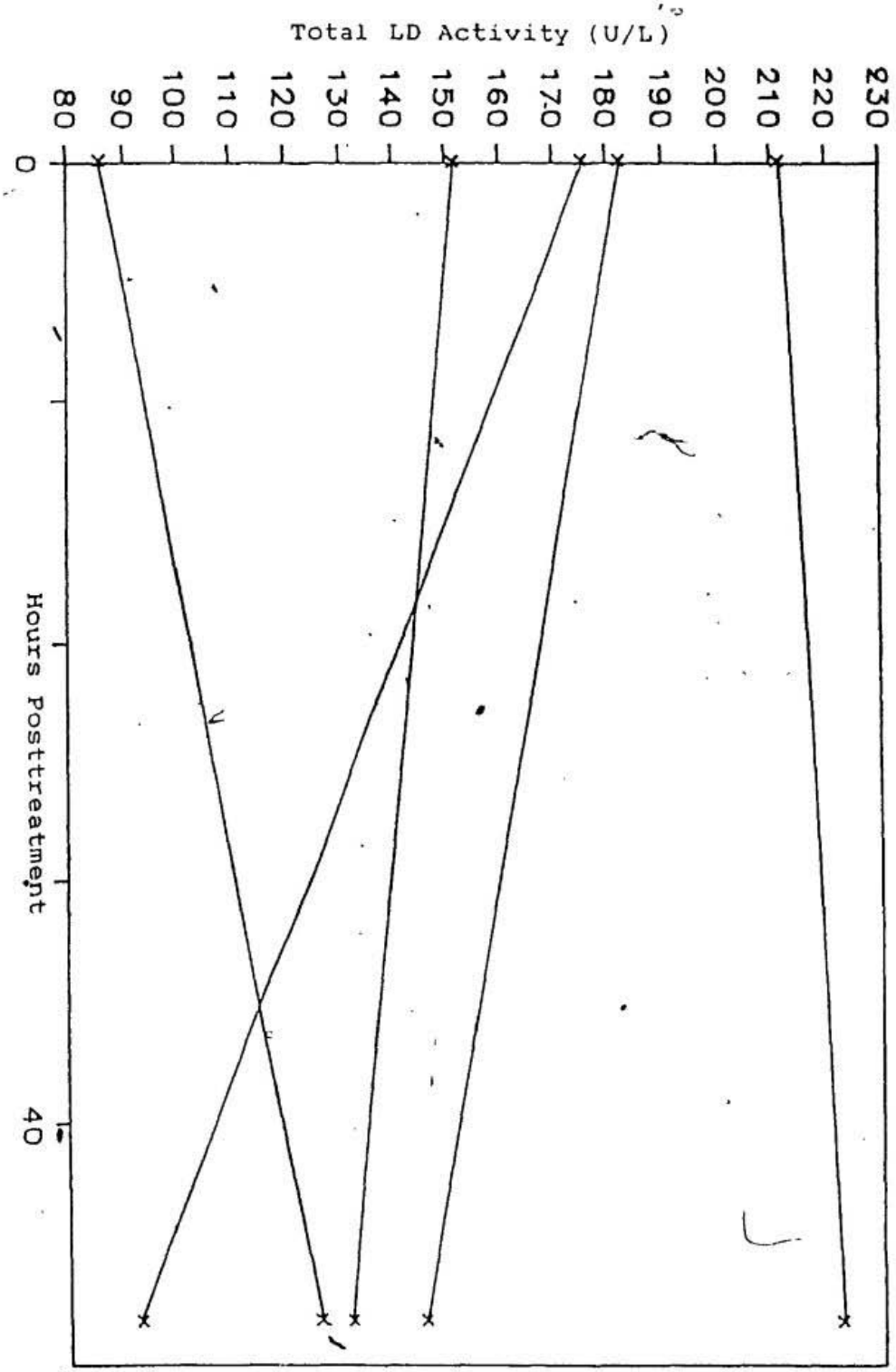

Total LD Activity (U/L)
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
0
Hours Posttreatment
40

Figure 11. Total LD Activity Following 3 mg/kg Doxorubicin. Four of five rats demonstrated an increase in activity at 48 hours as compared to 0 hour. The one rat which demonstrated a decrease in activity had a very high 0 hour value as compared to the others.

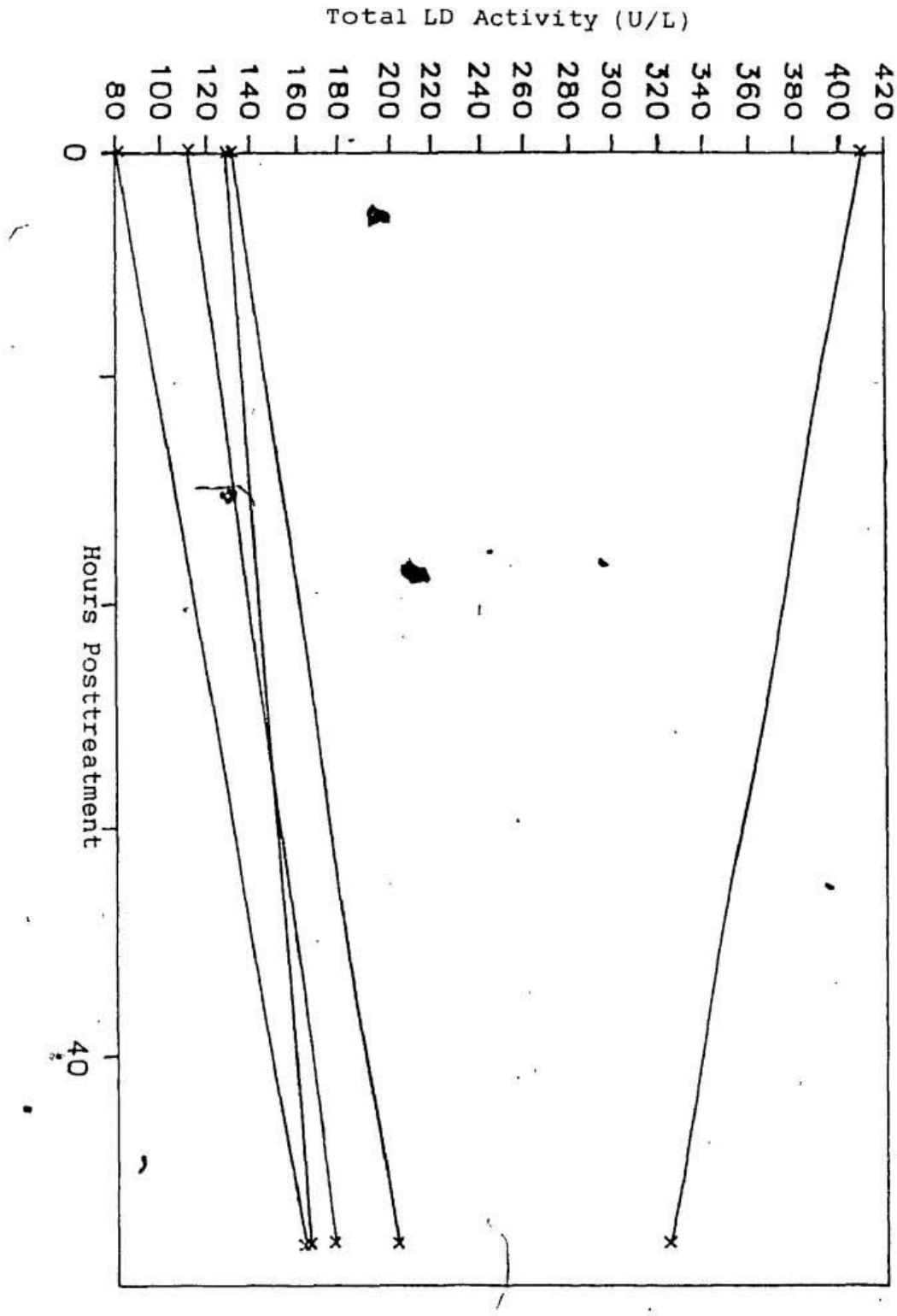

Total LD Activity (U/L)
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
0
Hours Posttreatment
40

Figure 12. Total LD Activity Following 6 mg/kg Doxorubicin. A statistically significant increase in LD activity was noted at 48 hours ($p < 0.05$).

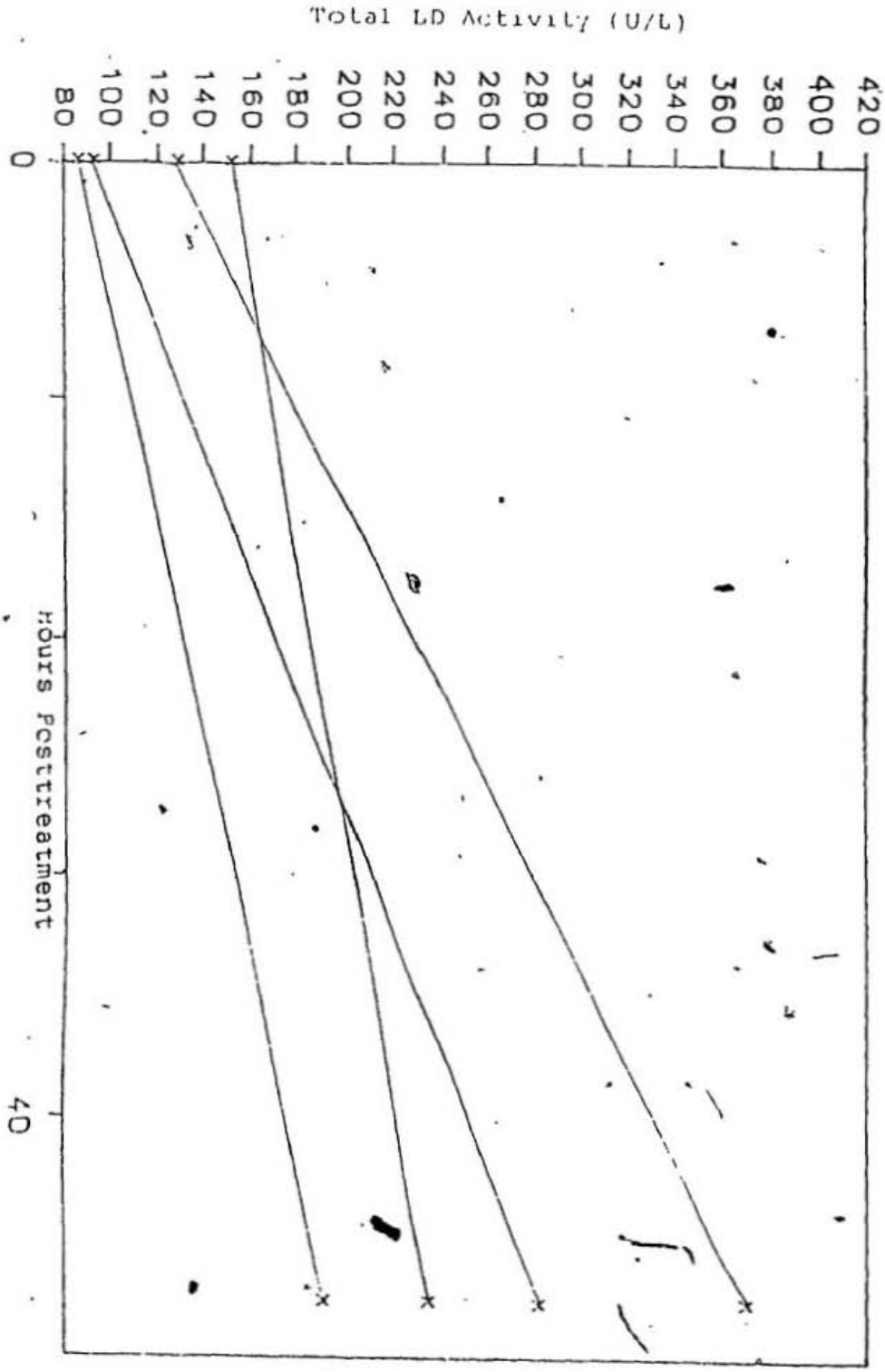
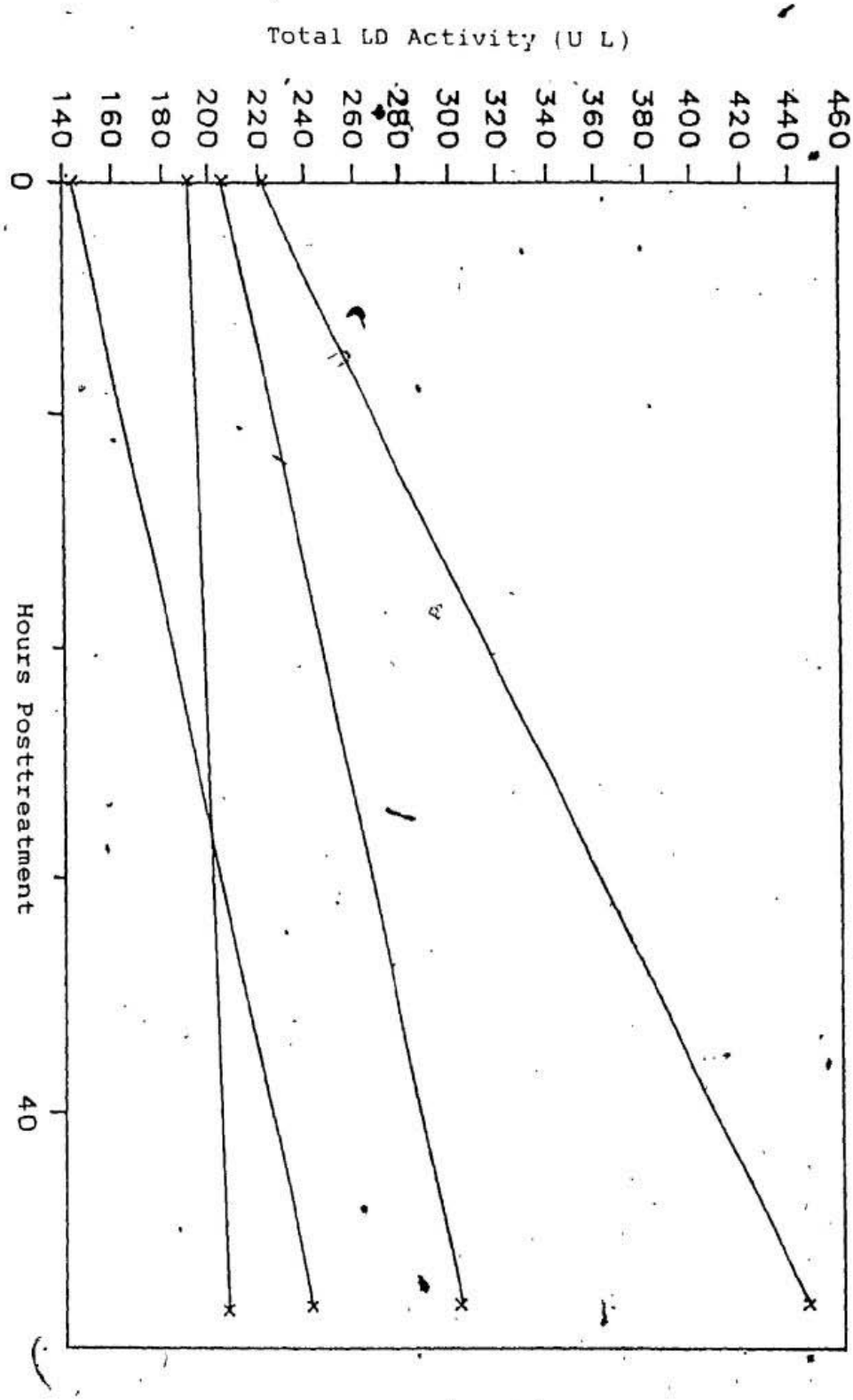

Total LD Activity (U/L)
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
0
40
Hours Posttreatment

Figure 13. Total LD Activity Following 9 mg/kg Doxorubicin. Note the increase in activity at 48 hours.


Total LD Activity (U L)
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
0
Hours Posttreatment
40

Statistical Analysis of LD Activity

At time 48 hours the means of the saline and 6 mg/kg treated groups and the means of the saline and 9 mg/kg treated groups were demonstrated to be statistically different.

Figure 14. Total AST Activity Following Saline Treatment. The rats demonstrated either minimal change or a decrease in AST activity at 48 hours as compared to 0 hour

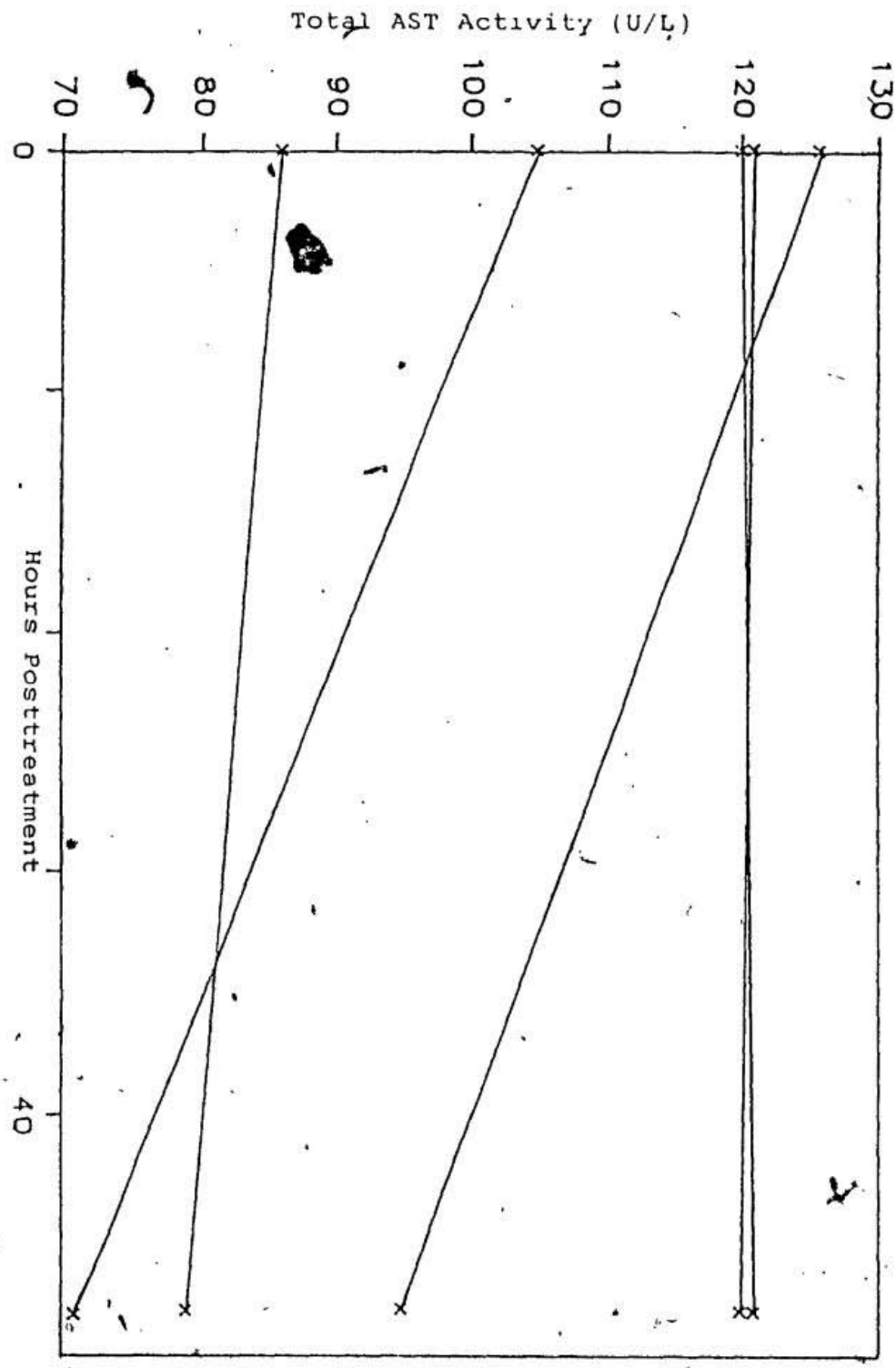

Total AST Activity (U/L)
70
80
90
100
110
120
130
0
40
Hours Posttreatment

Figure 15. Total AST Activity Following 3 mg/kg Doxorubicin. All rats demonstrated an increase in activity at 48 hours as compared to 0 hour.

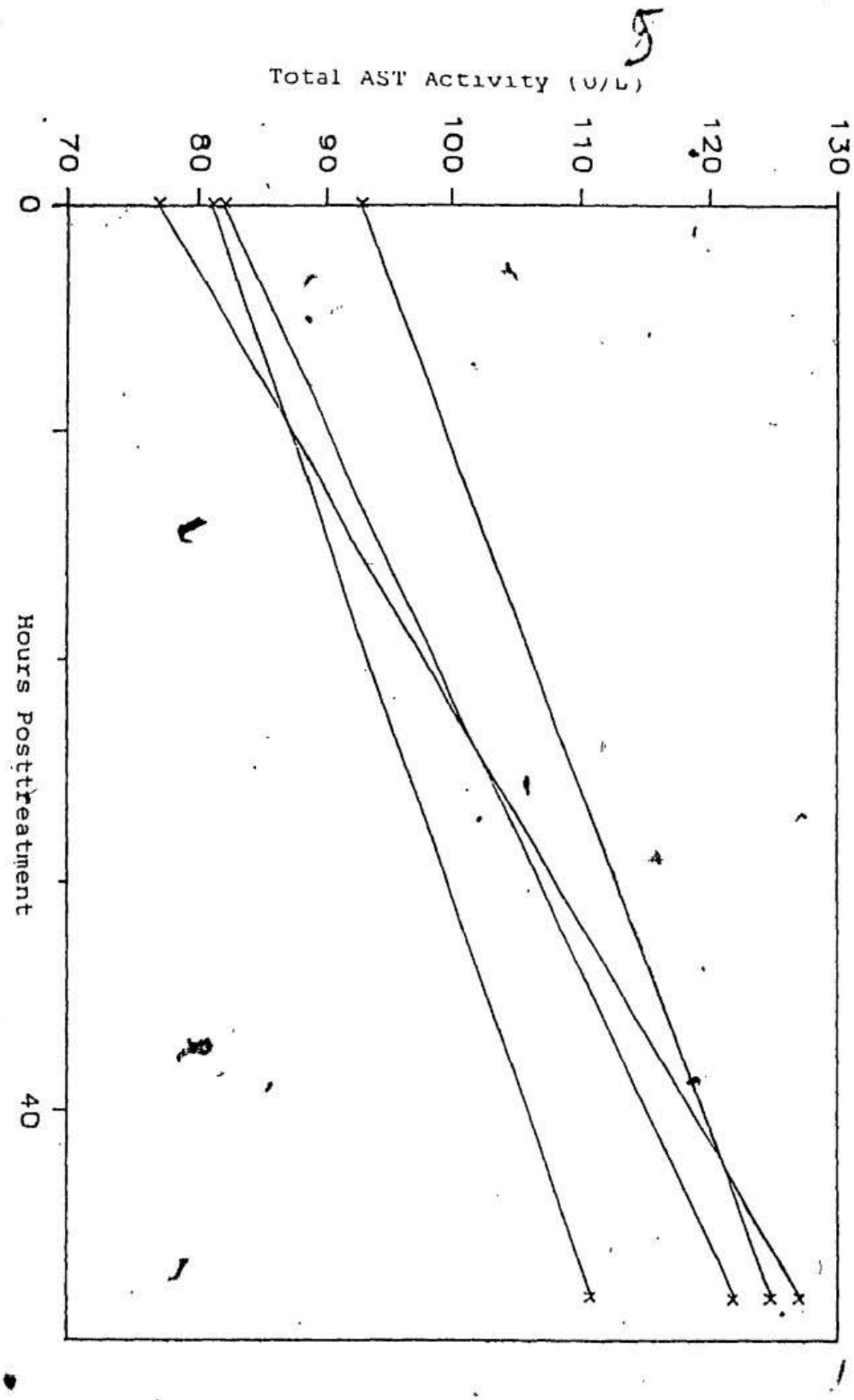

Total AST Activity (U/L)
70
80
90
100
110
120
130
0
Hours Posttreatment
40

Figure 16. Total AST Activity Following 6 mg/kg Doxorubicin. There was a statistically significant increase in activity at 48 hours as compared to 0 hour ($p < 0.05$).

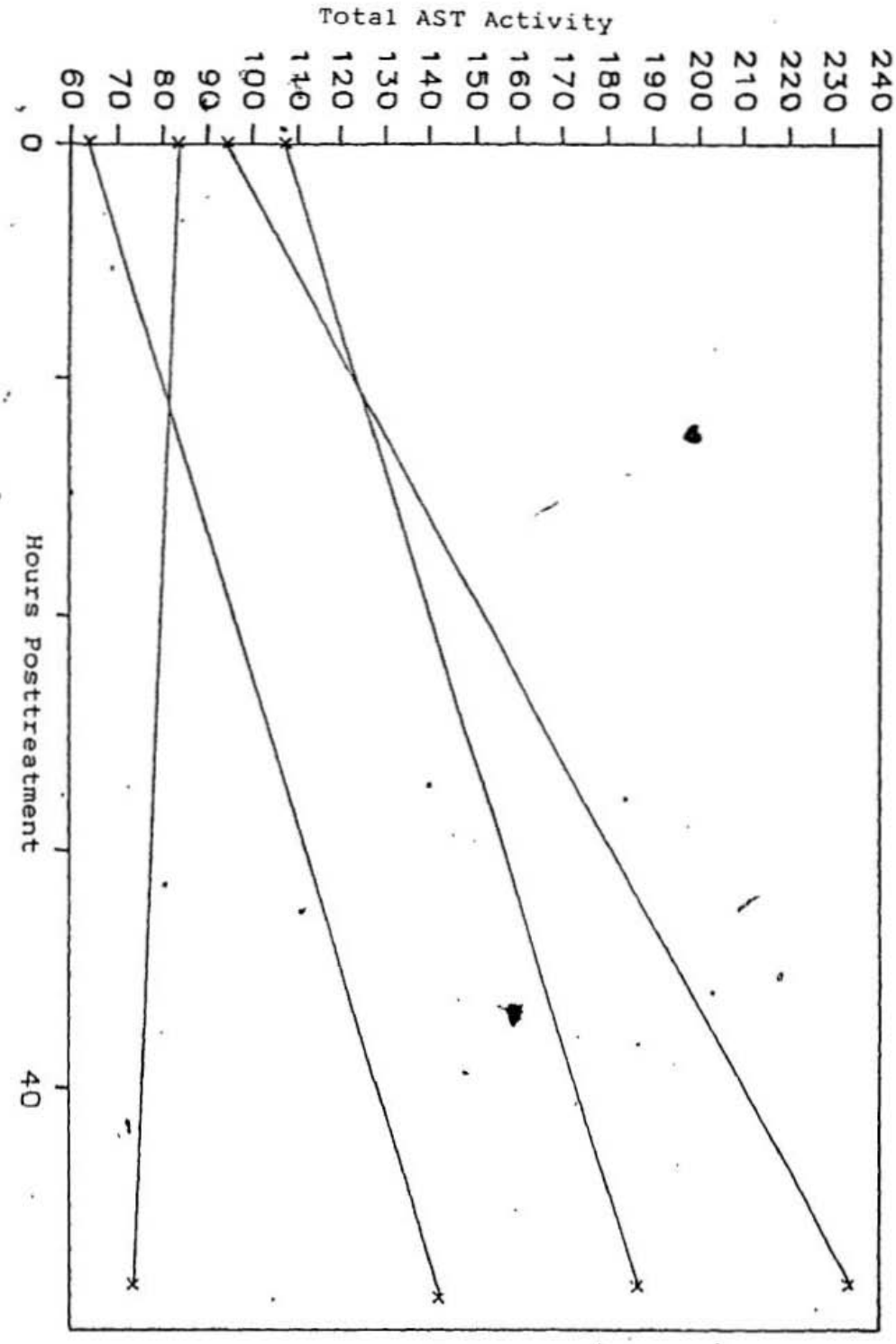
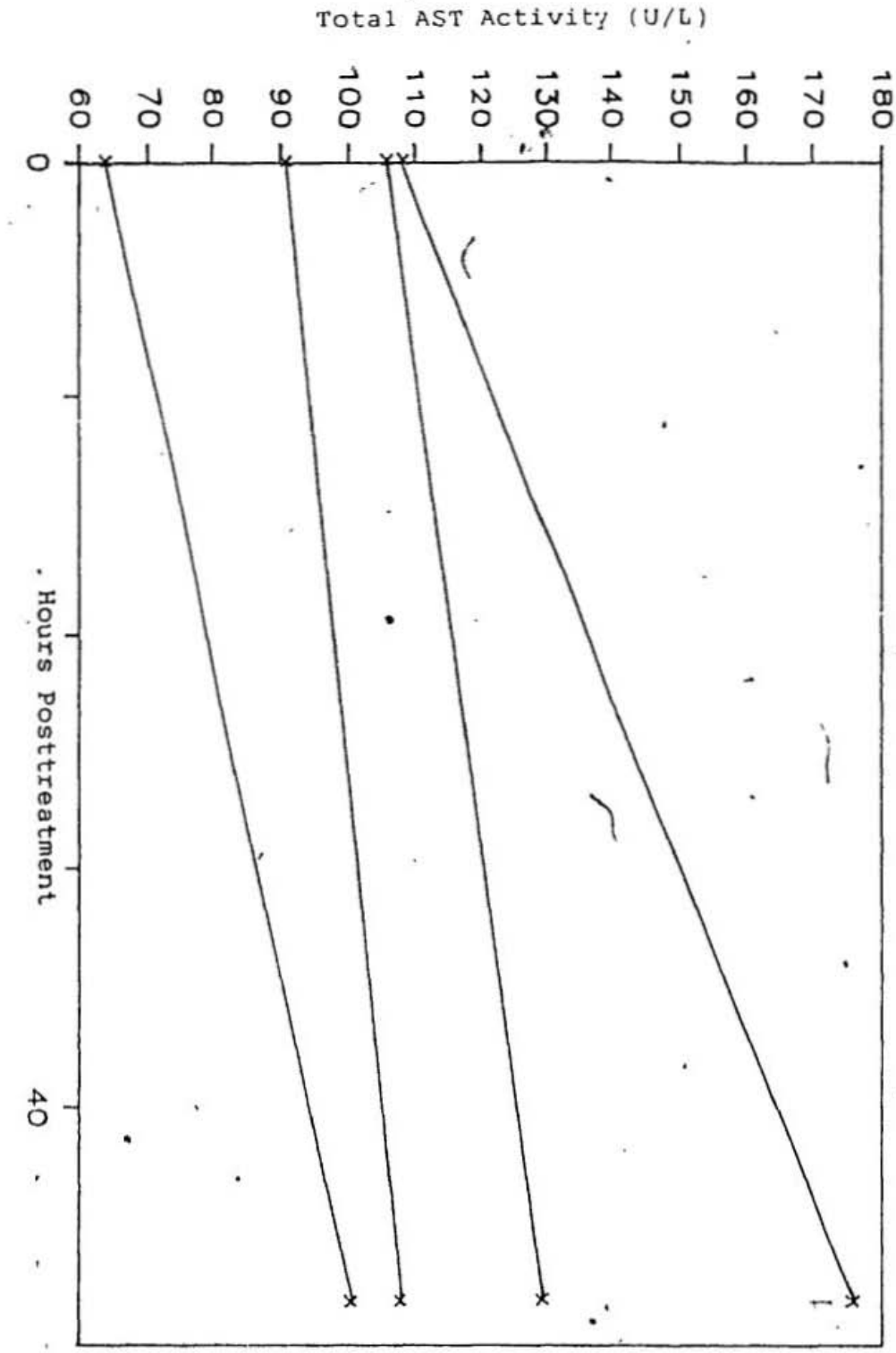

Total AST Activity
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
0
Hours Posttreatment
40

Figure 17. Total AST Activity Following 9 mg/kg Doxorubicin. All rats demonstrated an increase in activity at 48 hours.


Total AST Activity (U/L)
60
70
80
90
100
110
120
130
140
150
160
170
180
0
Hours Posttreatment
40

Statistical Analysis of AST Activity

A trend for an increased activity with Doxorubicin treated animals was noted though this was not shown to be statistically significant.

Figure 18. Total ALT activity Following Saline Treatement. With one exception in which there was an increase in activity all other rats demonstrated minimal or no change in activity.

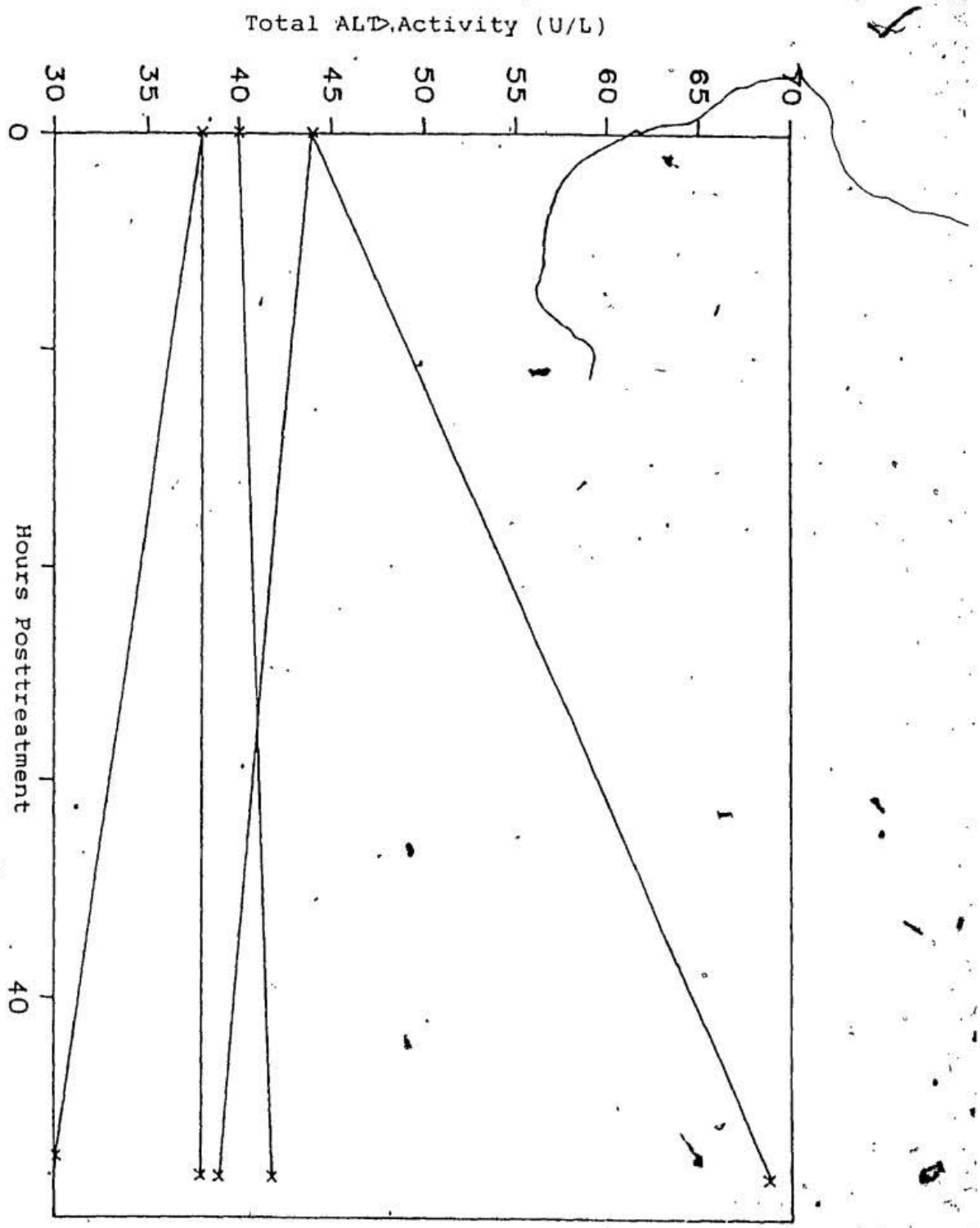

Total ALT Activity (U/L)
30
35
40
45
50
55
60
65
70
0
Hours Posttreatment
40

Figure 19. Total ALT Activity Following 3 mg/kg Doxorubicin. There was a minimal decrease in activity at 48 hours subsequent to treatment in all animals.

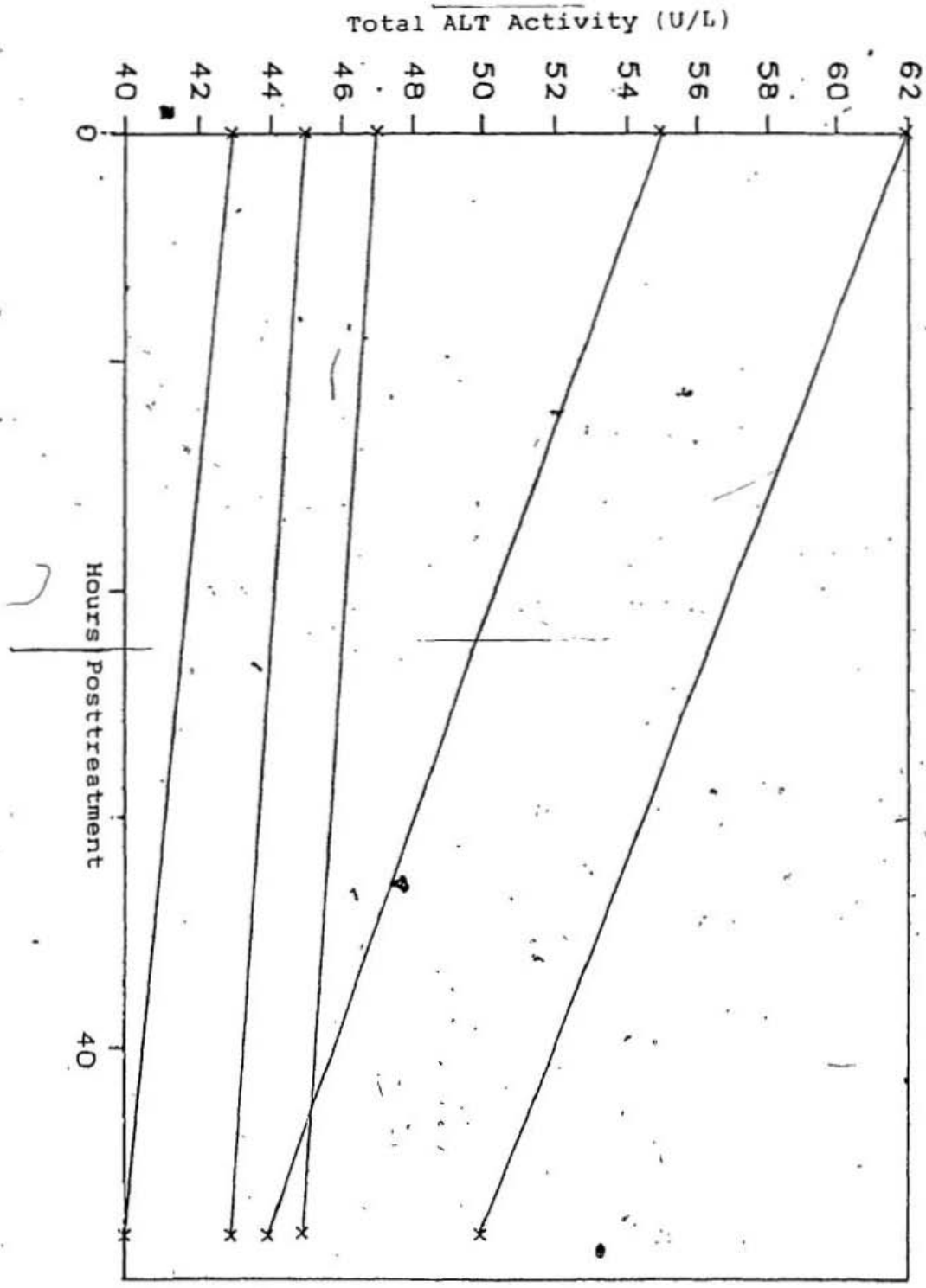

Total ALT Activity (U/L)
40
42
44
46
48
50
52
54
56
58
60
62
0
Hours Posttreatment
40

Figure 20. Total ALT Activity Following 6 mg/kg Doxorubicin. With one exception there was minimal increase in activity.

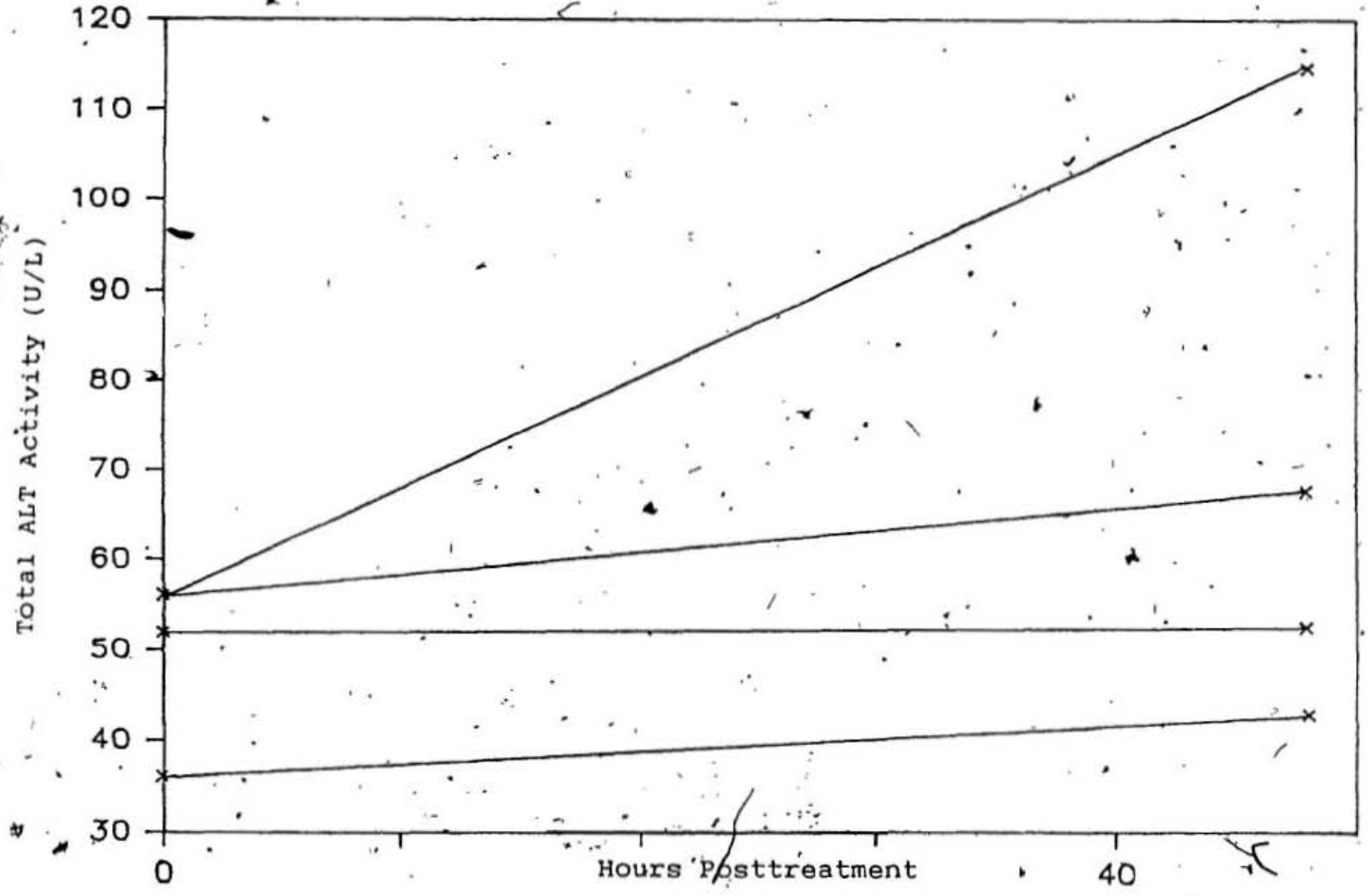
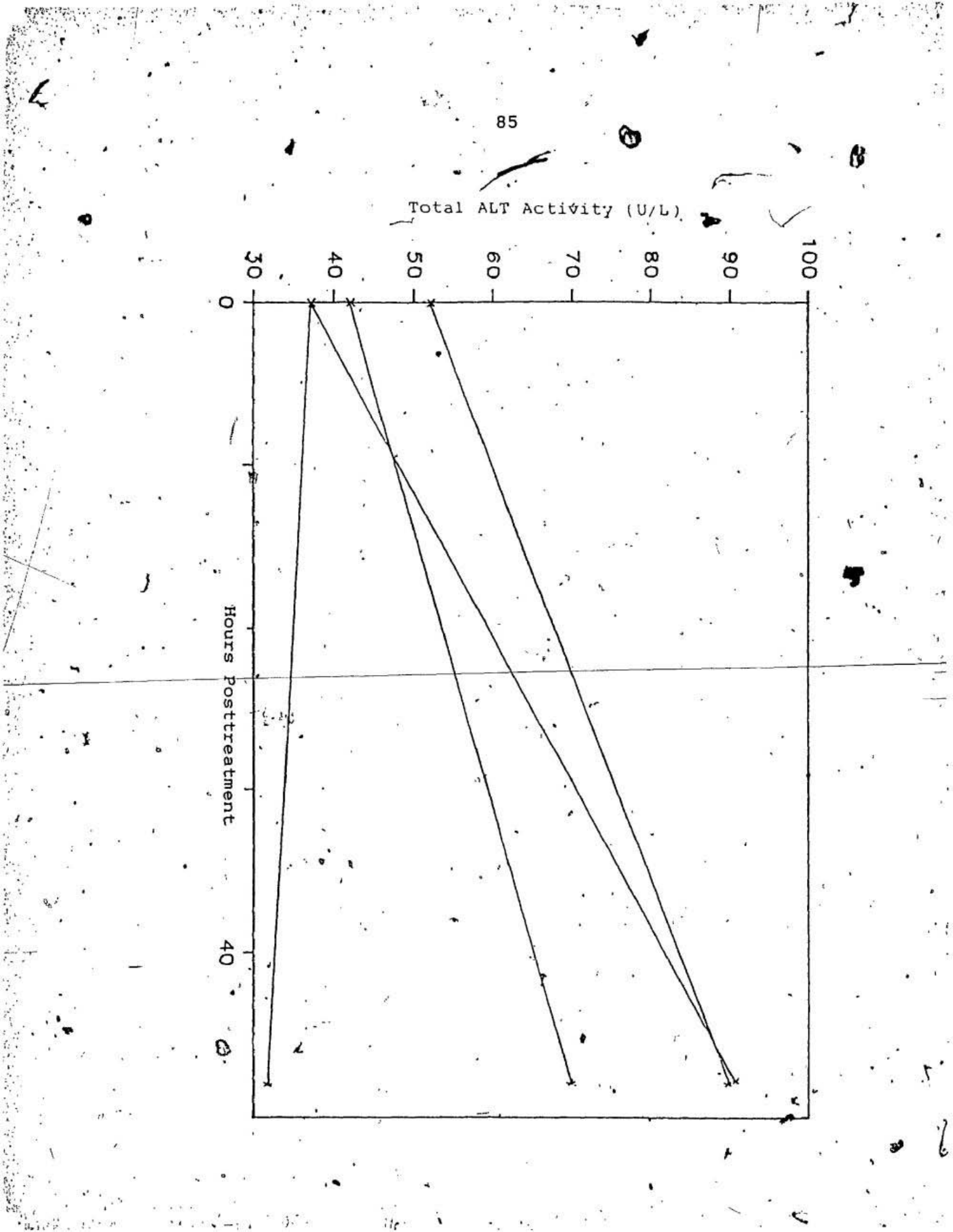

120
110
100
90
80
70
60
50
40
30
Total ALT Activity (U/L)
0
Hours Posttreatment
40

Figure 21. Total ALT Activity Following 9 mg/kg Doxorubicin. A statistically significant increase in activity was demonstrated at 48 hours as compared to 0 hour ($p < 0.05$).

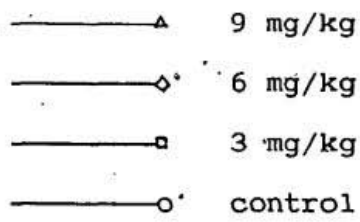

Total ALT Activity (U/L)
30
40
50
60
70
80
90
100
0
Hours Posttreatment
40

Statistical Analysis of ALT Activity

No statistically significant difference was demonstrated between the control and experimental groups.

3.1.1.2. LD Isoenzyme Analysis

Figure 22. LD-1 Activity Following Treatment with Saline, 3, 6 or 9 mg/kg Doxorubicin. Note the increased activity at 48 hours with increasing Doxorubicin dose. The 0 hour value represents the mean of values obtained from 14 rats. The 48 hour value of each dose group represents the mean of values for 5 rats. This is so with figures 22-26.



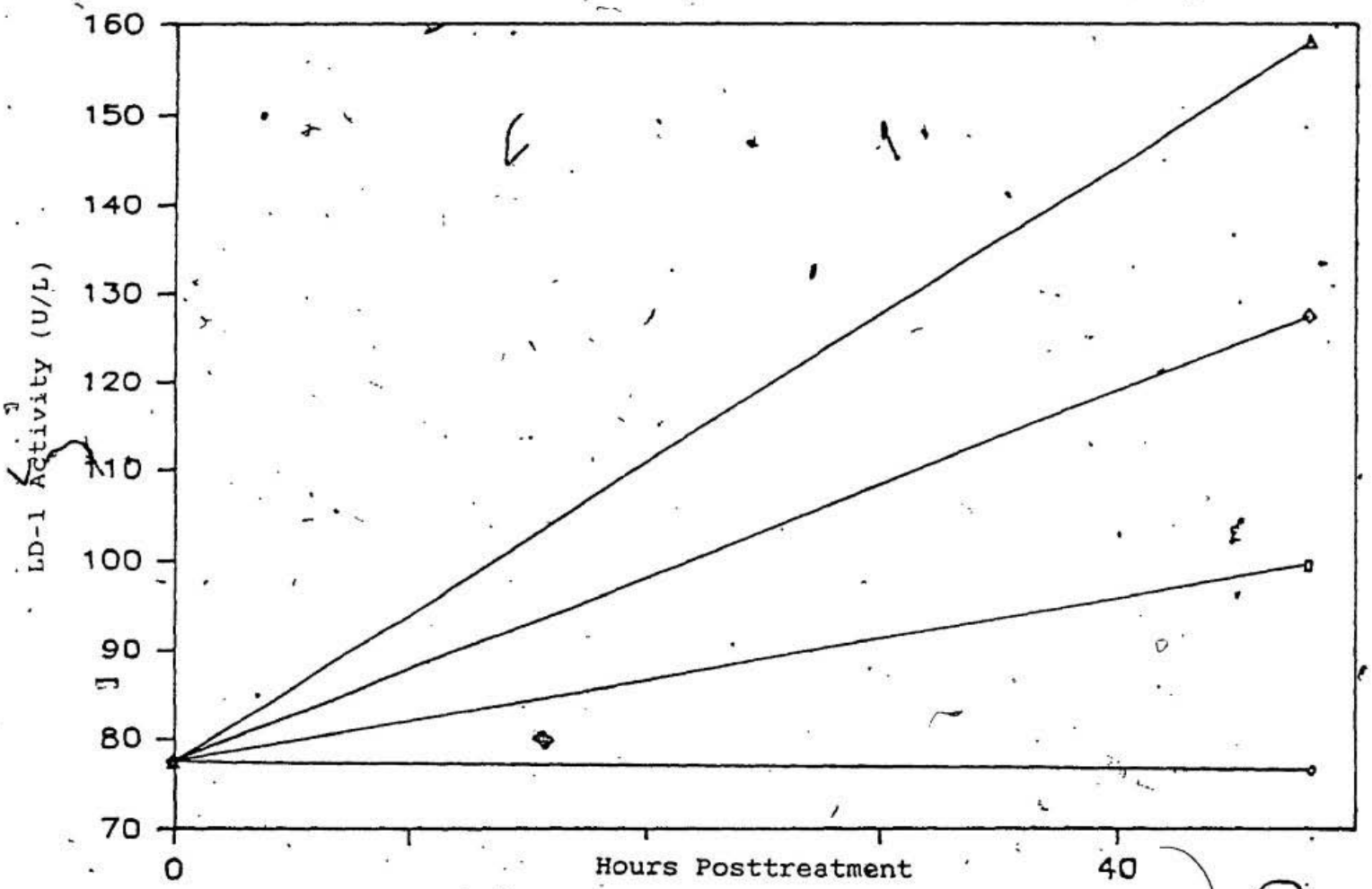
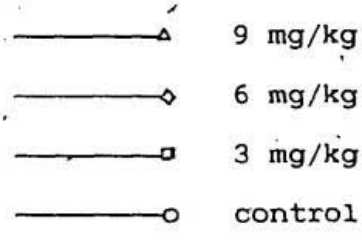

160
150
140
130
120
110
100
90
80
70
LD-1 Activity (U/L)
0
Hours Posttreatment
40

Figure 23. LD-2 Activity Following Treatment with Saline, 3, 6 or 9 mg/kg Doxorubicin. Note the minimal increased activity at 48 hours with increasing dose.



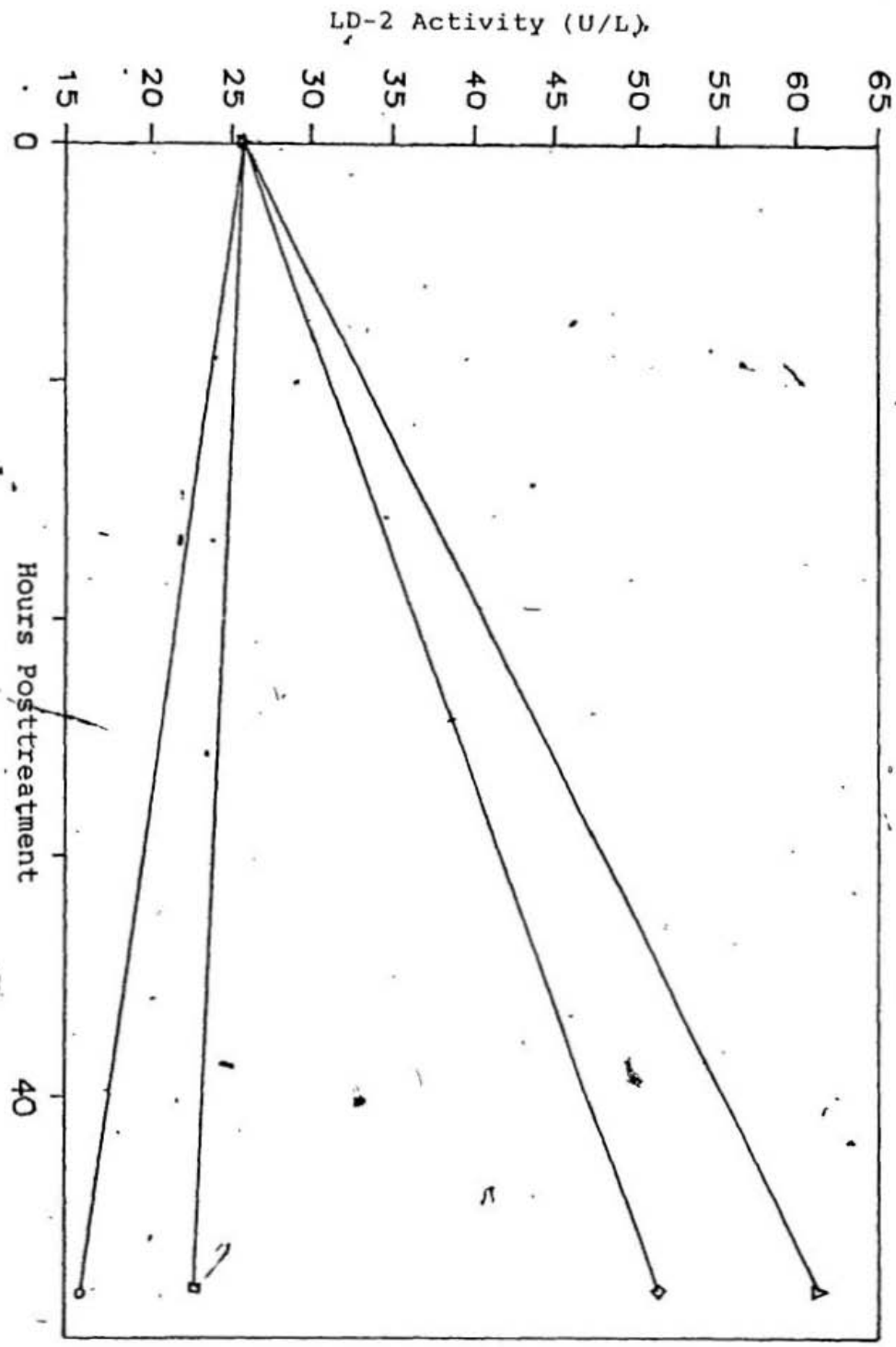
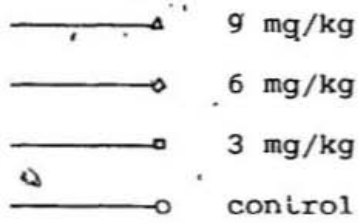

LD-2 Activity (U/L)
15
20
25
30
35
40
45
50
55
60
65
0
Hours Posttreatment
40

Figure 24. LD-3 Activity Following Treatment with Saline, 3, 6 or 9 mg/kg Doxorubicin. A significant increase was noted only with the 9 mg/kg drug dose.



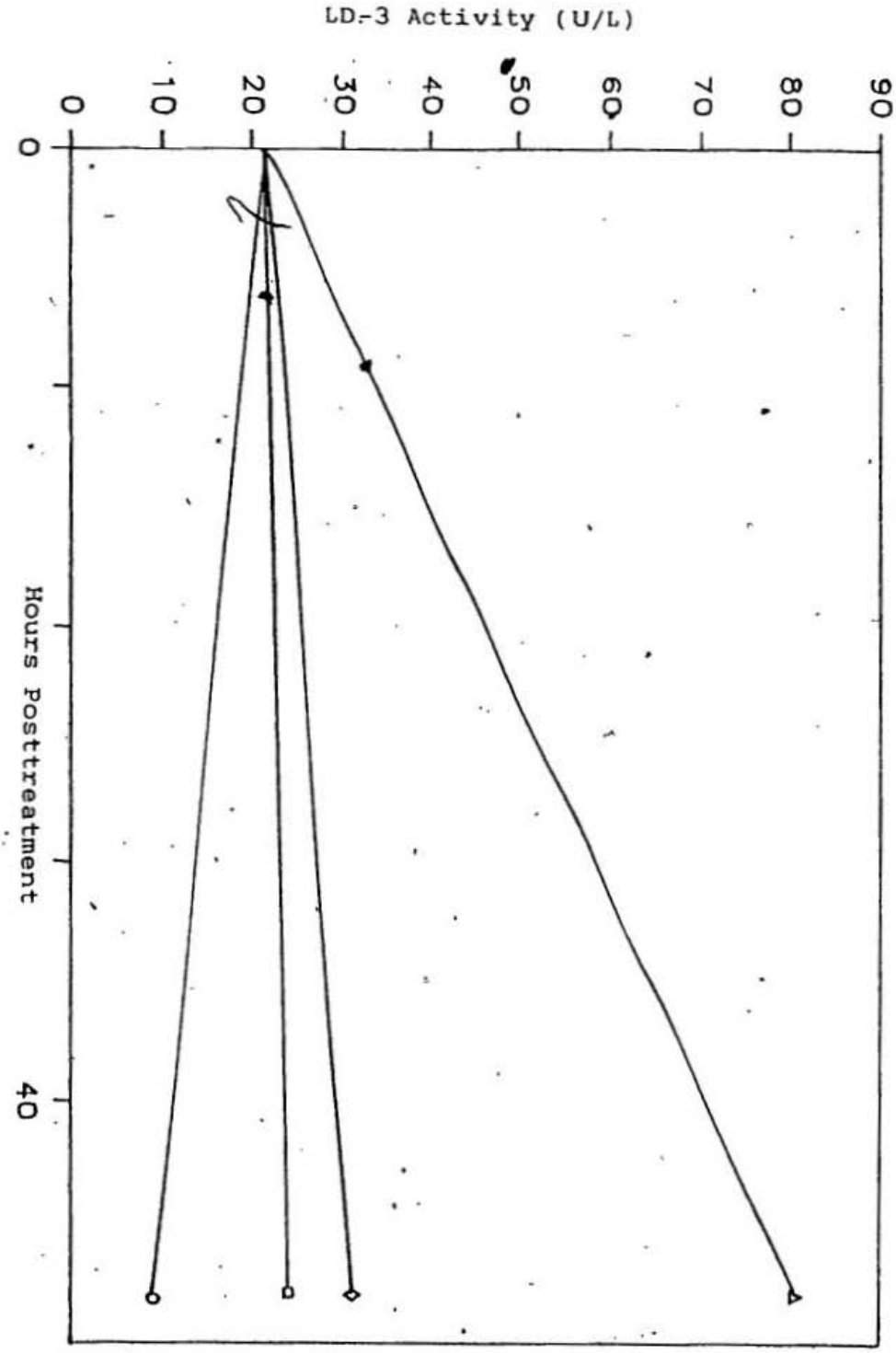
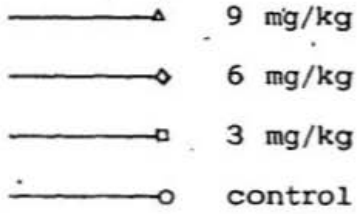
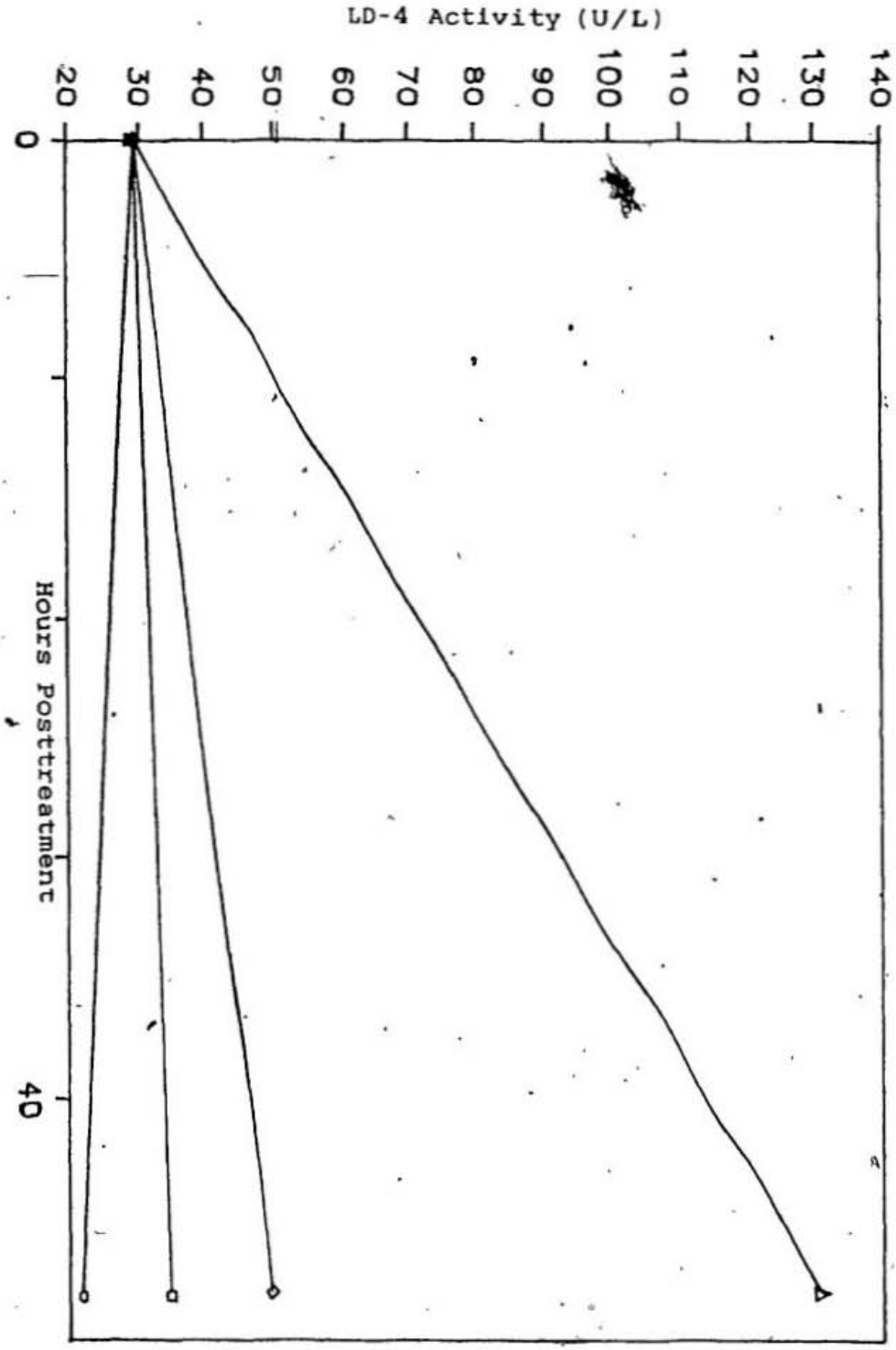

LD-3 Activity (U/L)
0
10
20
30
40
50
60
70
80
90
0
Hours Posttreatment
40

Figure 25. LD-4 Activity Following Treatment with Saline, 3, 6 or 9 mg/kg Doxorubicin. A significant increase was noted with 9 mg/kg only.




LD-4 Activity (U/L)
20
30
40
50
60
70
80
90
100
110
120
130
140
0
40
Hours Posttreatment

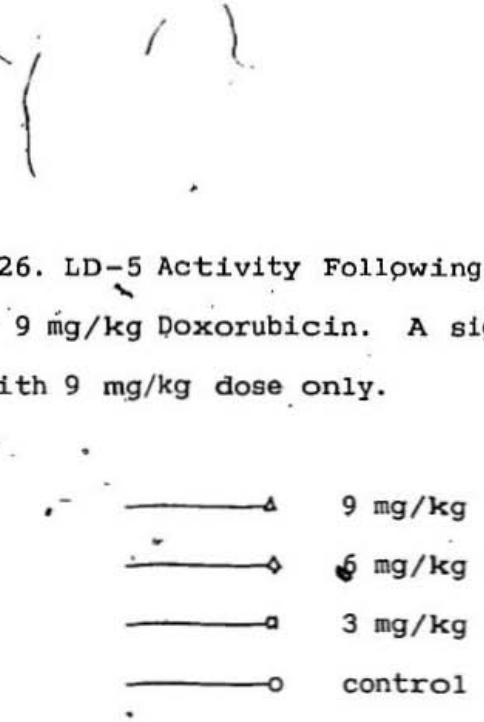


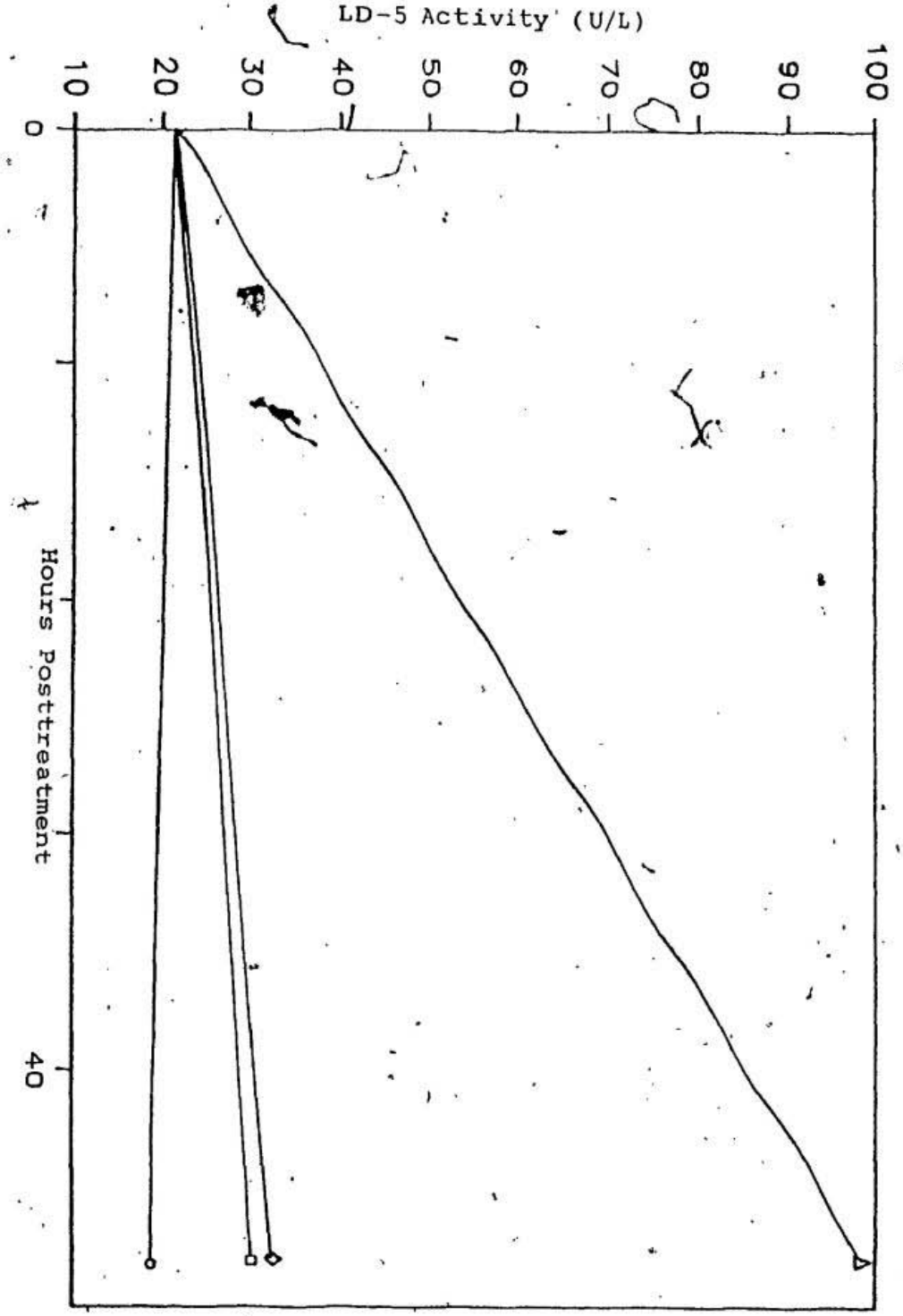
Figure 26. LD-5 Activity Following Treatment with Saline, 3, 6 or 9 mg/kg Doxorubicin. A significant increase was noted with 9 mg/kg dose only.

—△— 9 mg/kg

—◇— 6 mg/kg

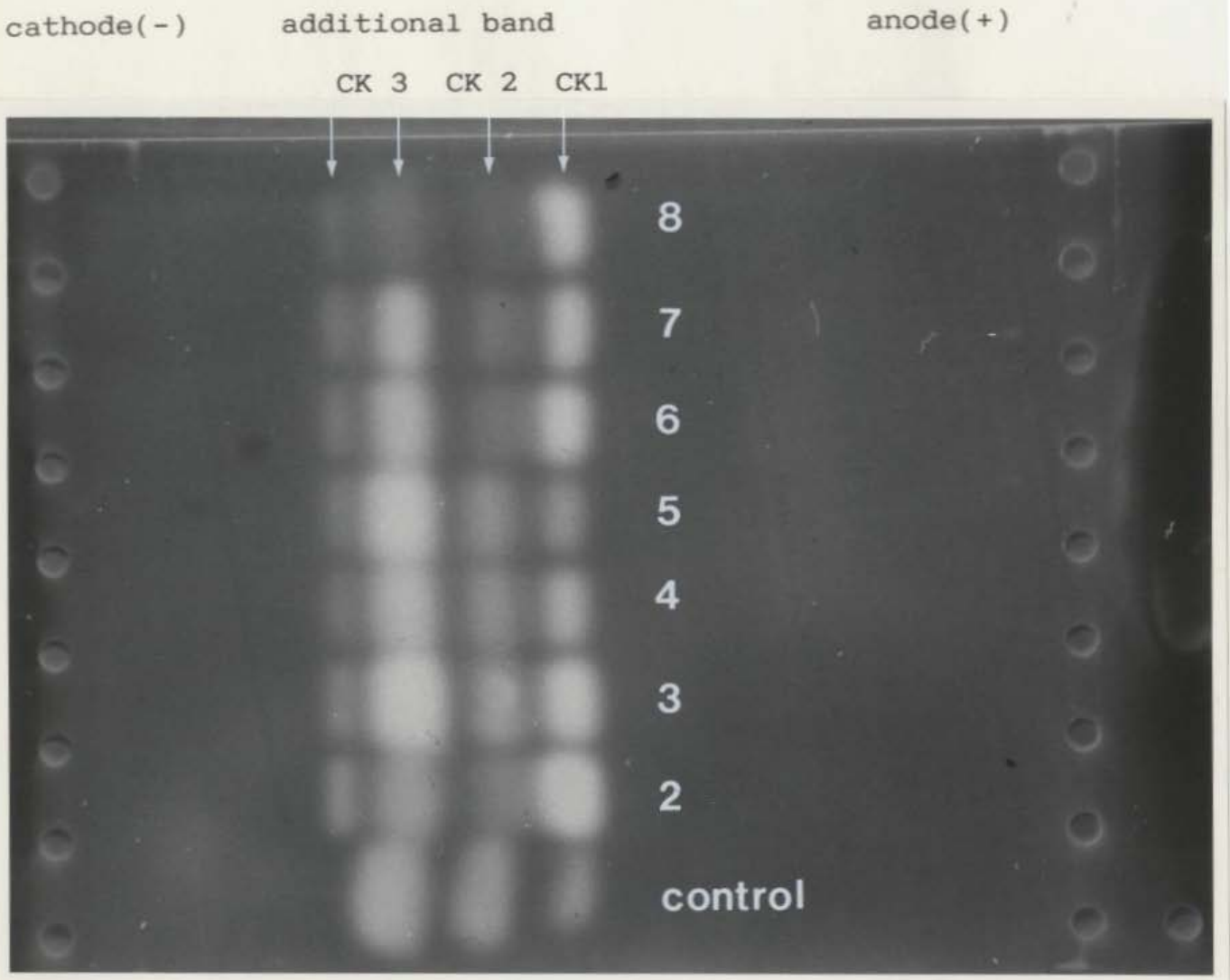
—□— 3 mg/kg

—○— control


LD-5 Activity (U/L)
10
20
30
40
50
60
70
80
90
100
0
Hours Posttreatment
40

3.1.1.3. CK Isoenzyme Analysis

Figure 27. Serum CK Isoenzyme Pattern Following Treatment with 6 or 9 mg/kg Doxorubicin. Note the additional band cathodic to CK 3 as compared to the saline injected controls which demonstrated the 3 cytosolic isoenzymes CK 1, CK 2 and CK 3 corresponding to the standards seen at the bottom of the photograph. This fourth band was believed to represent mitochondrial CK. To demonstrate that this was indeed so, mitochondria were isolated and analyzed as to its CK isoenzyme pattern (Section 3.1.2.2.).



Samples 2 to 5 were obtained from rats administered the 9 mg/kg dose of the drug. Samples 6 to 8 were from rats treated with 6 mg/kg.

3.1.2. Tissue Analysis

3.1.2.1. Tissue Measurement of Doxorubicin

3.1.2.1.1. Standard Curves

Two standard curves were necessarily constructed for determination of Doxorubicin concentration in various tissues of rats administered the drug.

Figure 28. Standard Curve of Doxorubicin Concentration

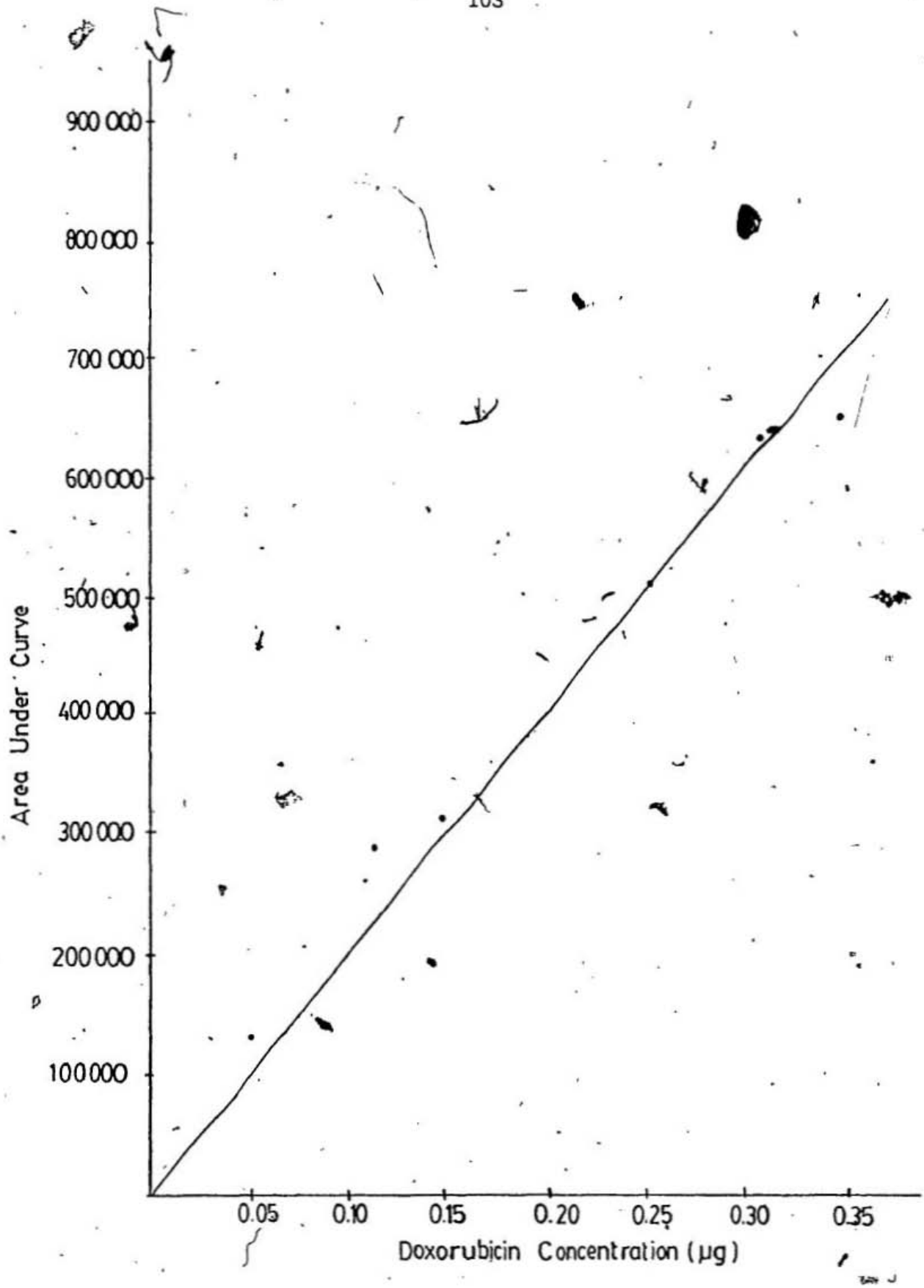

900 000
800 000
700 000
600 000
500 000
400 000
300 000
200 000
100 000
Area Under Curve
0.05
0.10
0.15
0.20
0.25
0.30
0.35
Doxorubicin Concentration (µg)

Figure 29. Standard Curve of Doxorubicin Concentration

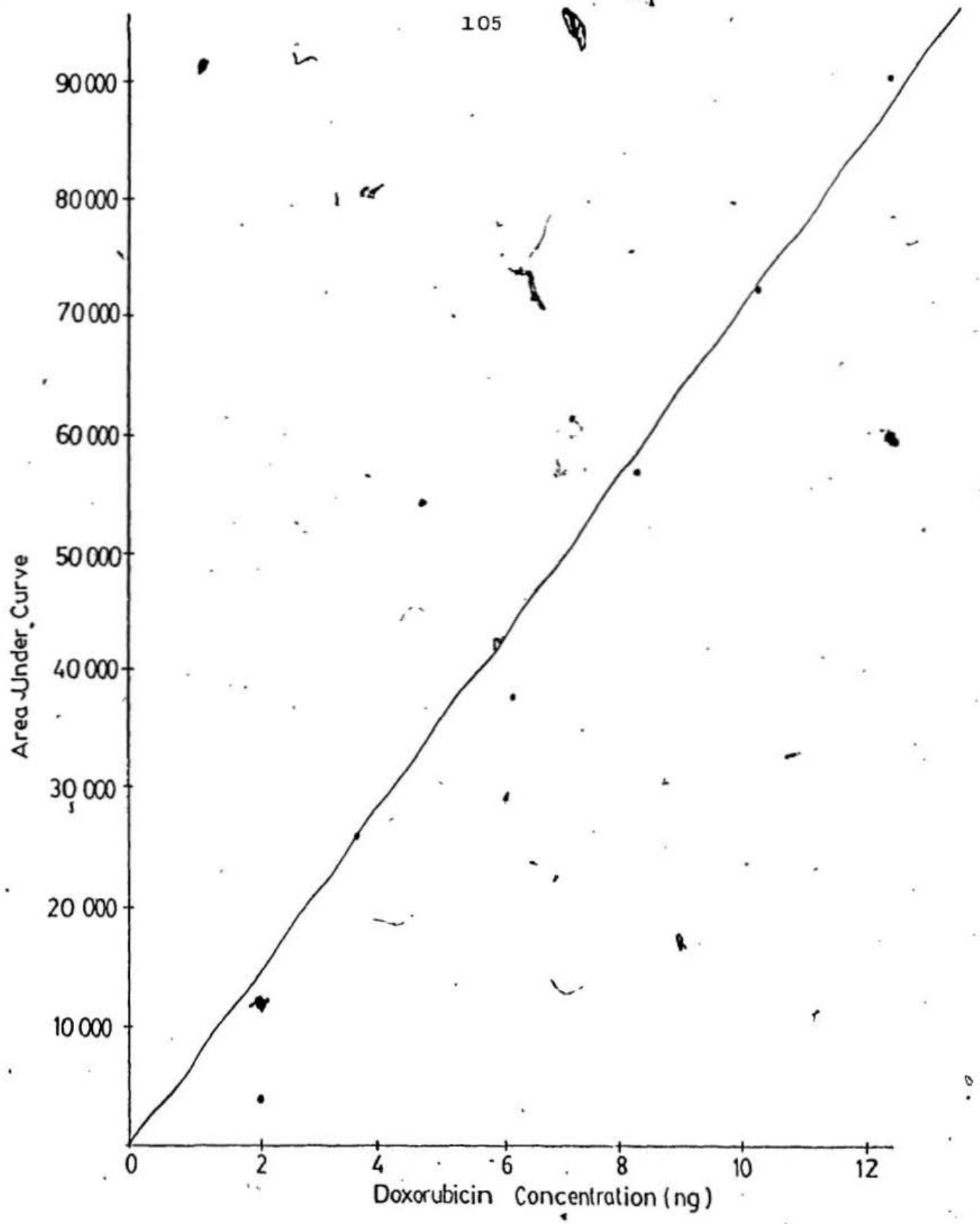

90 000
80 000
70 000
60 000
50 000
40 000
30 000
20 000
10 000
Area Under Curve
0
2
4
6
8
10
12
Doxorubicin Concentration (ng)

Figure 30. Sample Chromatogram

AUFS (absorbance units full scale) : 0.001 F.U.

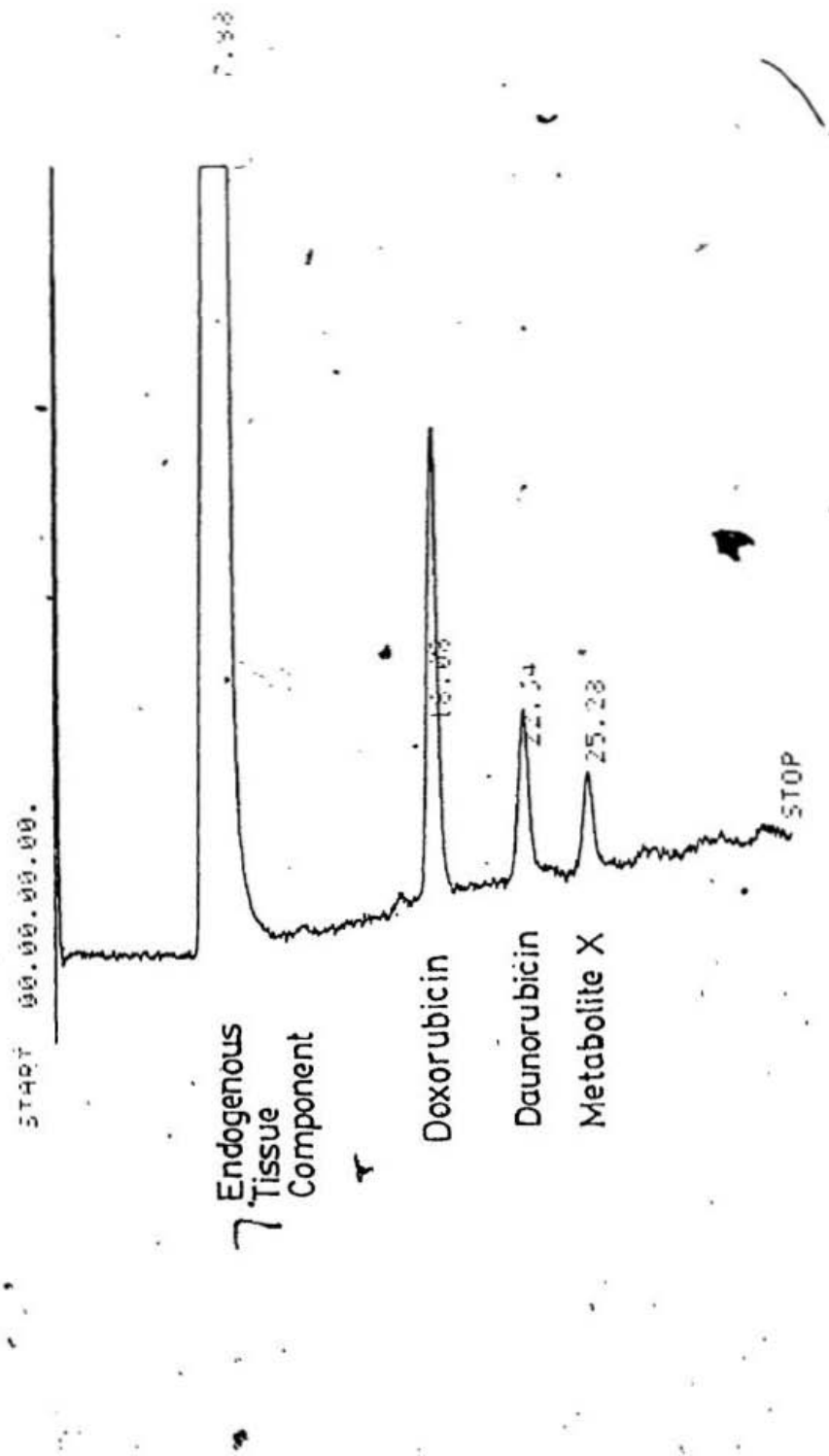
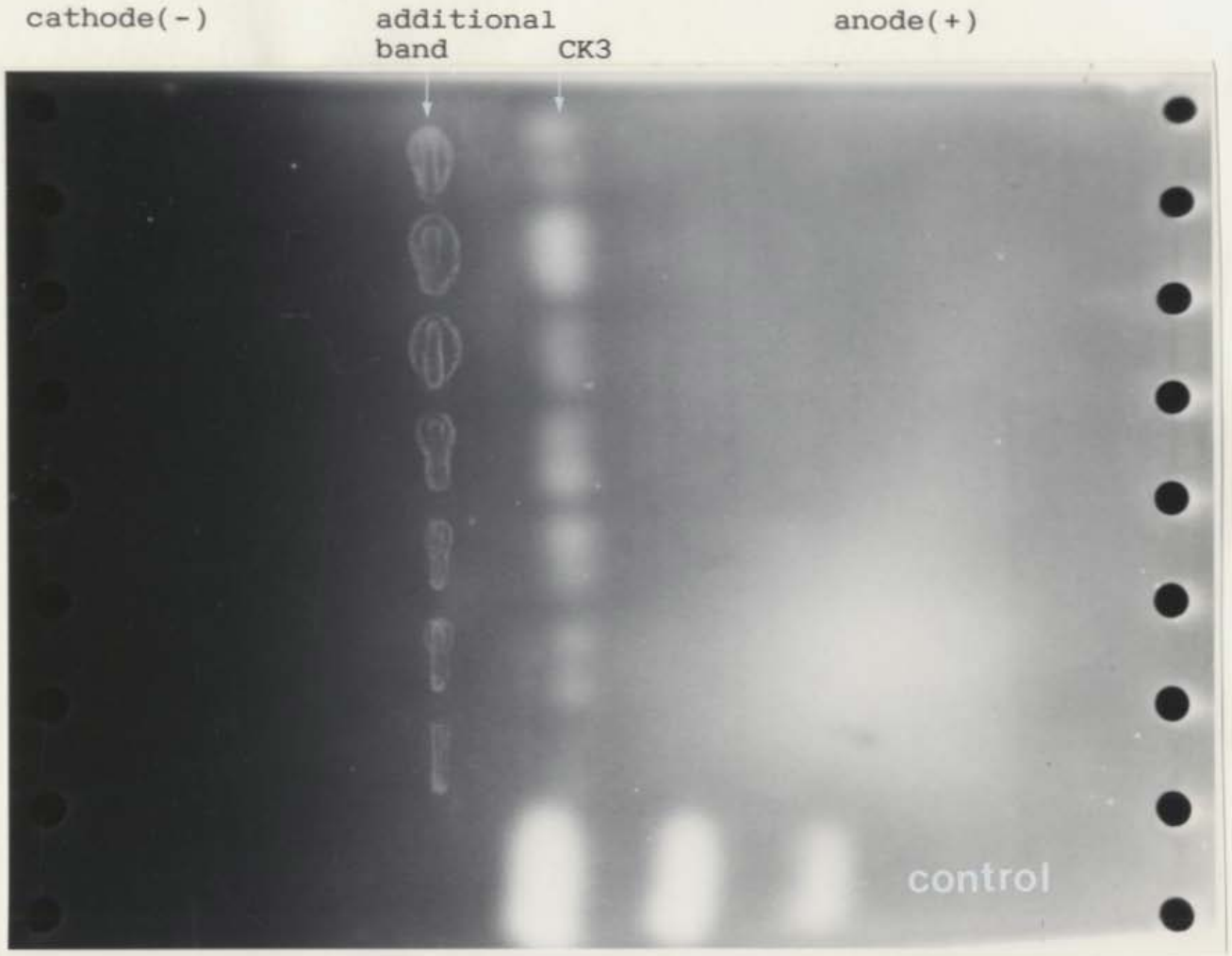


Table 1. Doxorubicin Concentration in Myocardium. Increasing drug dosage resulted in increasing myocardial tissue concentration of Doxorubicin. The values represent the mean + S.D. from 5 rats.

Dose Administered (mg/kg)	Tissue	Mean Doxorubicin Concentration (ng/g)
3	Heart	6.15 ± 1.30
6	"	50.0 ± 9.30
9	"	80.0 ± 11.1

3.1.2.2. Mitochondrial Isolation

Figure 31. Mitochondrial CK Isoenzyme Pattern. Note the presence of 2 bands, one corresponds to CK-MM of the standard (at bottom of picture). The second was cathodic to this CK-MM and quite different in appearance.


cathode(-)
additional band
CK3
anode(+)
control

3.1.2.3. Myocardial CK Total Activity

Table 2. Comparison of CK Total Activity in Mydcardium of Saline and 9 mg/kg Doxorubicin Treated Rats. There was no difference in total CK activity in the 2 groups. Each value represents the mean ± S.D. of data obtained from 5 rats.

	Dose	
	Saline	9 mg/kg
CK Activity (U/L)/ mg protein	40 771 ± 643	45 300 ± 915

3.1.3. Urinalysis

Table 3. Urine Chemistries Following Saline Treatment

Table 4. Urine Chemistries Following Treatment with 3 mg/kg Doxorubicin. There was a decreased urine volume in the 3 mg/kg treated group versus the saline administered control group.

	Dose (mg/kg)	Total Volume (ml)	Osmolality (Unit)	Na (mmol/L)	K	LD (U/L)	Total Protein (g/L)
1	Saline	45	1513	107	268	15	1.12
2	"	60	1300	87	226	17	2.77
3	"	89	712	41	107	16	0.83
4	"	40	1529	98	289	18	2.01
5	"	45	1355	81	222	17	0.36

	Dose (mg/kg)	Total Volume (ml)	Osmolality (Unit)	Na (mmol/L)	K	LD (U/L)	Total Protein (g/L)
6	3	20	-	174	448	24	2.83
7	"	30	1450	44	253	21	1.42
8	"	40	1188	41	201	16	1.30
9	"	30	1753	92	304	29	3.94
10	"	100	164	11	21	5	none detected

Table 5. Urine Chemistries Following Treatment with 6 mg/kg Doxorubicin Versus Saline Group in Twenty-Four Hour Urine Output.

Table 6. Urine Chemistries Following Treatment with 9 mg/kg Doxorubicin. Note the decreased urine output in this group versus the saline treated group.

	Dose (mg/kg)	Total Volume (ml)	Osmolality (Unit)	Na (mmol/L)	K	LD (U/L)	Total Protein (g/L)
11	6	180	-	10	16	0	0.27
12	"	19	714	54	125	1	0.63
13	"	17	1644	128	320	12	0.60
14	"	8	2268	117	329	14	1.35
15	"	3	3009	122	534	39	0.66

	Dose (mg/kg)	Total Volume (ml)	Osmolality (Unit)	Na (mmol/L)	K	LD (U/L)	Total Protein (g/L)
16	9	8	2272	154	380	32	4.39
17	"	16	1712	82	220	13	4.39
18	"	18	2283	124	366	28	3.20
19	"	5	2896	174	436	20	0.51

Table 7. Chemstrip.9 Dipstick Screen Test on 24 Hour Urine Specimens Following Saline Treatment. Several rats demonstrated glycosuria.

Table 8. Chemstrip 9 Dipstick Screen Test on 24 Hour Urine Specimens Following Treatment with 3 mg/kg Doxorubicin. There was no difference between the saline and 3 mg/kg treated groups. Several rats demonstrated glycosuria.

	Dose (mg/kg)				
	Saline	Saline	Saline	Saline	Saline
Leukocytes	-	-	-	+	+
Nitrite	+	-	-	+	+
pH	7	7	6	8	8
Protein	100	100	30-100	100	100
Glucose	1/2	normal	normal	1/2	1/4
Ketones	-	-	-	-	-
Urobilinogen	normal	normal	normal	normal	normal
Bilirubin	-	-	-	-	-
Blood	50	10-50	10	10	50

Leukocytes: leuko./ul
Nitrite: -/+
Protein: mg/dl
Glucose: g/dl
Ketones: -/+
Bilirubin: -/+
Blood: ery./ul

	Dose (mg/kg)				
	3	3	3	3	3
Leukocytes	-	10-25	-	-	-
Nitrite	-	+	+	+	+
pH	5	7	7	6	6-7
Protein	trace	100	100	500	100
Glucose	normal	1/10	1/2	1	1/2
Ketones	-	-	-	-	-
Urobilinogen	normal	normal	normal	normal	normal
Bilirubin	-	-	-	-	-
Blood	10	10	10	250	10

Leukocytes: leuko./ul
Nitrite: -/+
Protein: mg/dl
Glucose: g/dl
Ketones: -/+
Bilirubin: -/+
Blood: ery./ul

Table 9. Chemstrip 9 Dipstick Screen Test on 24 Hour Urine Specimens Following Treatment with 6 mg/kg Doxorubicin. Several rats demonstrated glycosuria.

Table 10. Chemstrip 9 Dipstick Screen Test on 24 Hour Urine Specimens Following Treatment with 9 mg/kg Doxorubicin. There was no difference between the 6 mg/kg and 9 mg/kg and saline treated groups. As in the other 3 groups several animals demonstrated glycosuria.

	Dose (mg/kg)				
	6	6	6	6	6
Leukocytes	-	500	10-25	10-25	10-25
Nitrite	-	-	-	-	-
pH	7	9	9	9	6
Protein	trace	100	30	100	100
Glucose	normal	1/20	1/20	1/20	1/20
Ketones	-	-	-	-	-
Urobilinogen	normal	normal	normal	normal	normal
Bilirubin	-	-	-	-	-
Blood	-	10	10	10	10-50

Leukocytes: leuko./ul
Nitrite: -/+
Protein: mg/dl
Glucose: g/dl
Ketones: -/+
Bilirubin: -/+
Blood: ery./ul

	Dose (mg/kg)			
	9	9	9	9
Leukocytes	10-25	-	75	10-25
Nitrite	+	-	+	-
pH	7	8	9	9
Protein	100	100	100	100
Glucose	1/20	1/20	1/20	1/20
Ketones	-	-	-	-
Urobilinogen	normal	normal	normal	normal
Bilirubin	-	+	-	-
Blood	10	10	10	10

Leukocytes: leuko./ul
Nitrite: -/+
Protein: mg/dl
Glucose: g/dl
Ketones: -/+
Bilirubin: -/+
Blood: ery./ul

3.2. Experimentation with New Zealand White Rabbits

3.2.1. Total CK Activity Analysis

Table 11. Total CK Activity Following Saline Treatment. There was a fluctuation in CK total activity. Dashed lines indicate times when blood was not collected.

Table 12. Total CK Activity Following 10 mg/kg Doxorubicin. Again note the fluctuation in CK total activity. Dashed lines indicate times when blood was not collected.

Total CK Activity (U/L)

Hours Posttreatment				
0	1	6	24	48
611	1019	410	-	-
285	49	-	248	459
1529	-	2100	1240	742
531	-	581	-	530
335	343	413	477	299
2065	2000	1631	966	578

Total CK Activity (U/L)

Hours Posttreatment							
0	1	2	3	6	12	24	48
558	-	-	-	16170	9156	11250	1380
171	330	1860	12160	-	-	4080	2120
463	661	-	-	14220	-	9570	-
344	455	-	-	5550	8620	-	2550

3.2.2. Myoglobin Assay

Table 13. Serum Myoglobin Assay Following Saline Treatment. Myoglobin was not detected in the serum at either 0 or 48 hours.

Table 14. Serum Myoglobin Assay Following Treatment with 10 mg/kg Doxorubicin. Myoglobin was detected at 48 hours posttreatment. A positive test result indicates a minimum myoglobin concentration of 100 + 20 µg/L.

Hours Posttreatment	
0	48
-	-
-	-
-	-
-	-
-	-
-	-

Hours Posttreatment	
0	48
-	+
-	+
-	+
-	+

3.2.3. CK Isoenzyme Analysis

Figure 32. Serum CK-MB Activity (% of total CK) Following Saline Treatment. There was no increase in activity from 0 to 48 hours posttreatment.

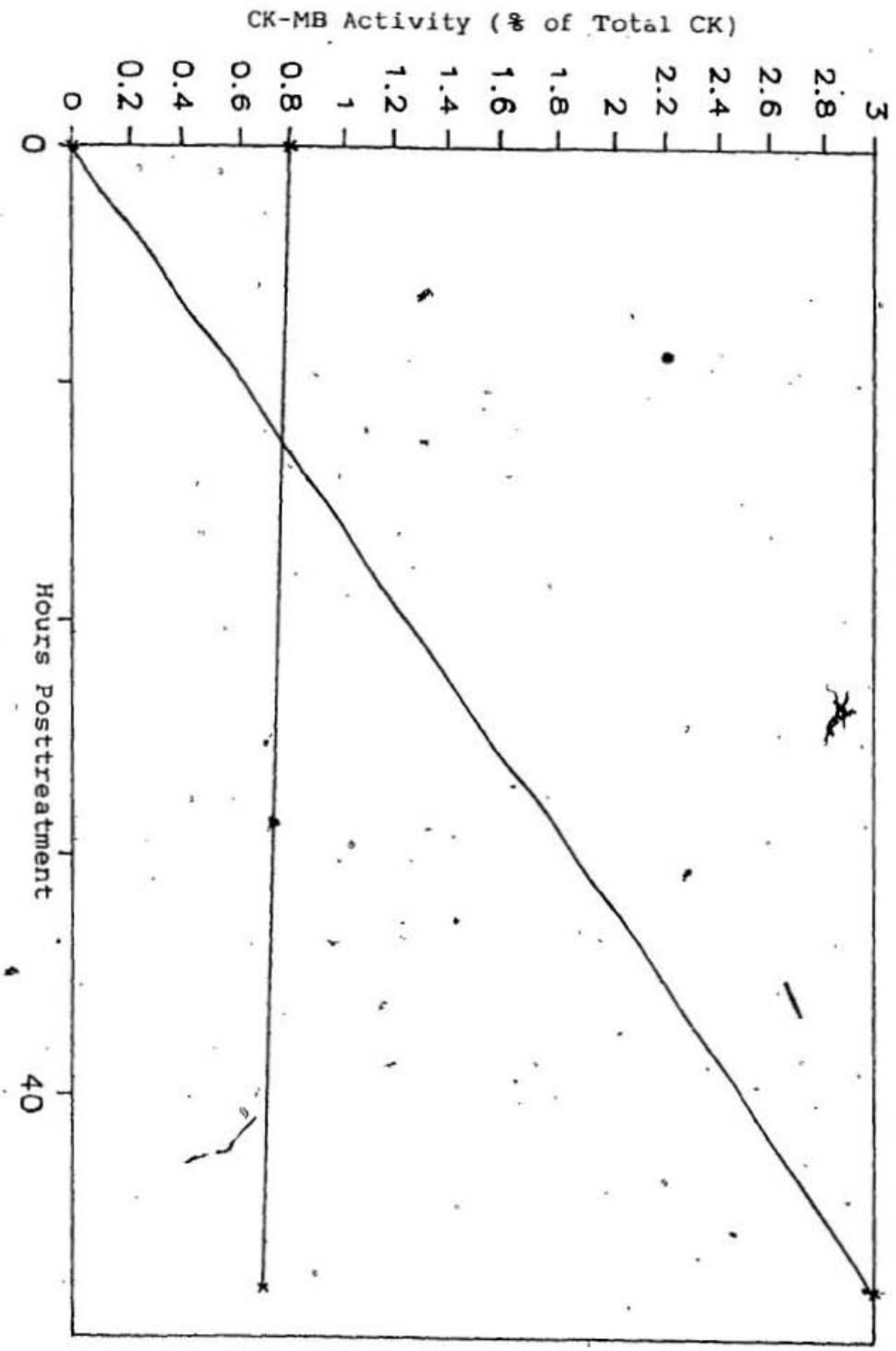
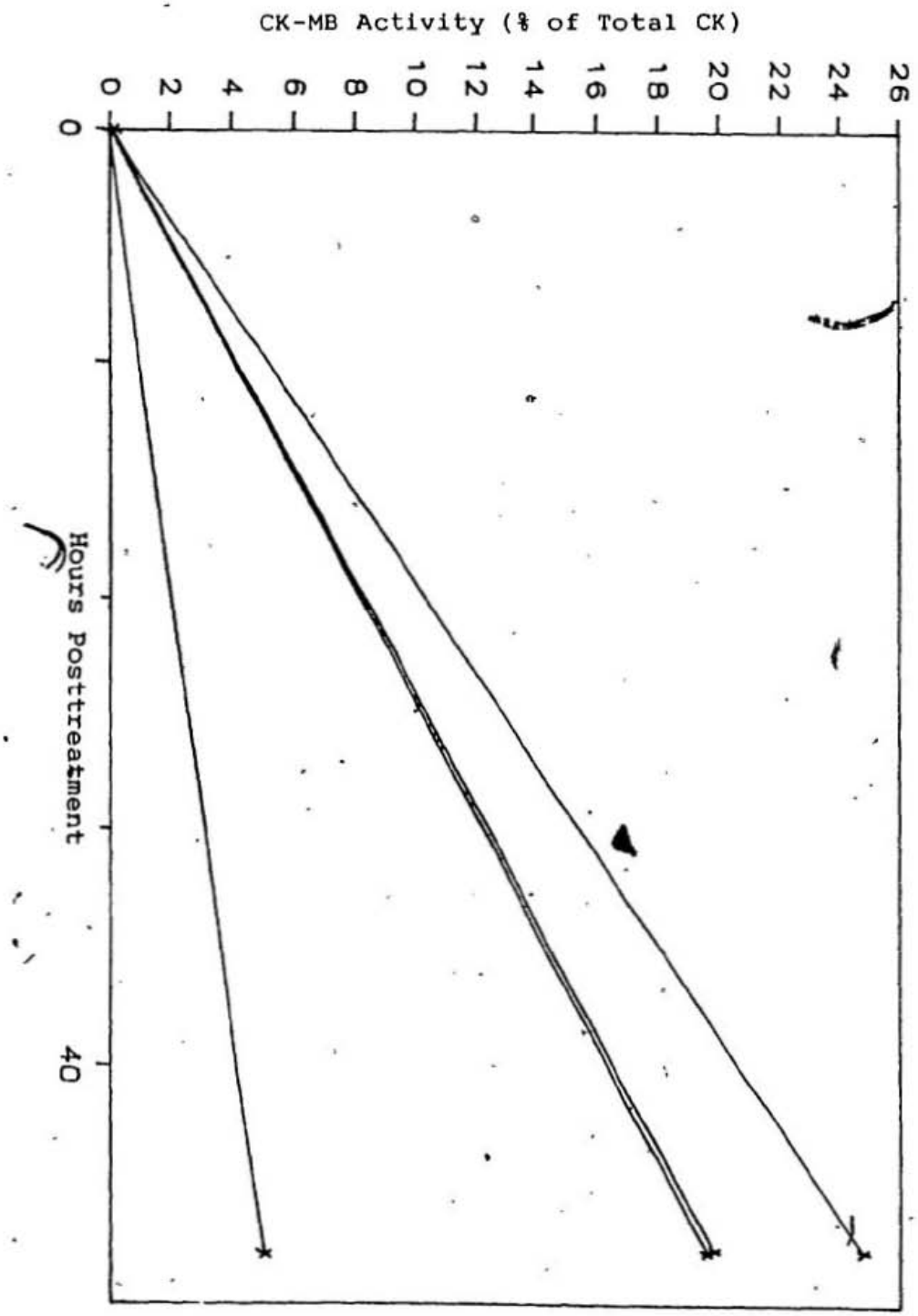

CK-MB Activity (% of Total CK)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
3
0
Hours Posttreatment
40

Figure 33. Serum CK-MB Activity (% of total CK) Following Treatment with 10 mg/kg Doxorubicin. At 48 hours posttreatment CK-MB activity was > 5% of total CK activity.


CK-MB Activity (% of Total CK)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Hours Posttreatment
0
40

CHAPTER 4

DISCUSSION

Various invasive and noninvasive techniques have been used to assess cardiac function in patients receiving Doxorubicin chemotherapy. Unfortunately no one test is reliable, in the clinical setting, in predicting which patients will develop cardiotoxicity.

Electrocardiographic monitoring of the patient is essential following myocardial infarction, however it should not and is not used as a predictive tool because alterations indicative of myocardial damage frequently do not appear prior to their clinical presentation. Sustained elevation of systolic time intervals have been suggested as an indicator of cardiac damage, however there is a high incidence of false-positive and to a lesser extent false-negative results with this test. Radionuclide angiography provides assessment of ventricular function and has been reported to be a useful marker of preclinical toxicity (Ritchie et al., 1980). Among the goals of echocardiography are to record abnormal movements of cardiac structures and the determination of cardiac dimensions. This technique has shown some promise in predicting cardiac disorders in patients treated with Doxorubicin. The histologic grade of the biopsy taken from the right ventricle via transvenous endomyocardial biopsy is usually a good correlate with cumulative drug dose, however its predictive value is in question (Ritchie et al., 1980).

4.1. New Zealand White Rabbit Study

4.1.1. Total CK and CK-MB Measurement

There was a wide distribution of total CK activity in the control and experimental animals, further to intraperitoneal injection of saline and Doxorubicin respectively. This likely represents stress related release of CK (MM component) from skeletal muscle associated with the blood collection process. A follow up of total CK activity up to 48 hours demonstrated that in the saline treated control group the enzyme activity returned to more moderate levels, whereas in the drug treated animals the enzyme activity rose sharply and in most animals tended to stay elevated. In view of the wide and variable 0 hour values firm conclusions are difficult to draw. Yet, the overall profile suggests tissue release of CK specific to drug toxicity.

In the experimental group, at 48 hours CK-MB was consistently above 5% of total CK activity i.e. CK-MB positive as compared to the control group where it was consistently negative. This finding therefore suggests more specific damage to myocardial tissue due to drug toxicity. Neri (Neri et al., 1979) investigated the value of serum CK-MB measurements as a diagnostic tool in monitoring

cardiac status following anthracycline therapy. the subjects in this study were 42 patients treated with either Doxorubicin or Daunorubicin. Serum CK-MB activity was assayed prior to and 15 hours posttreatment. At 0 hour CK-MB activity was 4.10 ± 2.70 U/L (mean ± S.D.) versus 12.57 ± 7.61 U/L at 15 hours. Creatine kinase-MB activity in 7 of 42 patients indicated cardiac abnormality in the absence of alterations in cardiac function. Further controlled clinical studies with humans will have to be conducted to determine the reliability of serial measurements of CK-MB activity as a predictive tool for those patients who will subsequently develop Doxorubicin-induced cardiotoxicity.

Though elevated CK total activity could arise from either skeletal muscle or cardiac muscle (the two tissue systems with the highest concentration of the enzyme), CK-MB if released from skeletal muscle alone would at no time constitute more than 3 to 5% of total CK activity.

4.1.2. LD Total and Isoenzyme Analysis

A fair number of blood samples from the rabbits exhibited minimum to moderate hemolysis. This was so in spite of indwelling butterfly cannulae in the ear veins and slow aspiration. As hemolysis interferes with both total and isoenzyme analysis of LD, continuation with this model

would prove futile in demonstration of myocardial damage due to Doxorubicin toxicity.

4.1.3. Myoglobin Assay

Myoglobin was detected in the serum of all rabbits 48 hours subsequent to Doxorubicin administration, but not in the saline treated controls. Since myoglobin has the greatest activity in the myocardium and skeletal muscle it could have been released from either of these sources.

The purpose of the study with the rabbit model was an attempt to identify markers of Doxorubicin cardiotoxicity. The significant serum CK-MB activity following Doxorubicin treatment indicated myocardial damage. If this technique does prove fruitful it would be an easy, painless method of monitoring patients during Doxorubicin treatment. Although myoglobin was present in the serum subsequent to drug treatment, for now it cannot be advocated as a marker of myocardial damage because its tissue origin was not further explored.

There were difficulties experienced with the New Zealand white rabbit model in blood collection. Also, there was one death in the experimental group. An autopsy revealed severe peritoneal and pleural effusions which were also observed

to a lesser degree at the time of sacrifice in all rabbits treated with the drug. Therefore we proceeded to study Doxorubicin-associated toxicity in the Sprague-Dawley rat model.

4.2. Sprague-Dawley Rat Study

The investigation with this model involved the study of biochemical parameters in serum and urine to detect acute toxicity of the drug on organ systems, specifically the heart, liver and kidney. The tissue concentrations of the drug were measured in the myocardium and correlated with the degree of toxicity reflected by the tissue.

4.2.1. Total Enzyme Activity Analysis

With administration of the 3 and 6 mg/kg doses of Doxorubicin there was a significant increase in serum CK activity at 48 hours as compared to 0 hour. On the other hand, the rats treated with 9 mg/kg of the drug showed a significant decrease at 48 hours. The control group showed a moderate decrease in CK activity at 48 hours compared to 0 hour, though the values were not significantly different from each other. Cardiotoxicity associated with Doxorubicin use has been demonstrated in the rat/rabbit/human model. Therefore we can suggest that the total CK activity increases observed with 3 mg/kg and 6 mg/kg doses

represent release of enzyme due to cardiotoxicity. The decrease in CK activity with the 9 mg/kg dose may be related to decreased protein synthesis. However, we were unable to demonstrate any significant difference between myocardial tissue activity of CK between 9 mg/kg Doxorubicin treated and control animals.

There was a significant increase in serum LD activity at 48 hours in the rats treated with 6 and 9 mg/kg Doxorubicin when compared to the controls. However in the animals treated with the 3 mg/kg dose of the drug the increase was not statistically significant. It is reasonable to postulate that the increased serum LD activity was predominantly of myocardial origin because of its high activity in cardiac tissue and the known negative effect of the drug on this tissue. However it is conceivable that it could have been released from other organ systems because of its ubiquitous tissue distribution.

Similar to LD activity, serum AST activity increased subsequent to treatment with the 3 doses of Doxorubicin. Statistical analysis indicated significant differences between the means of the control and 3 mg/kg group and between the control and 6 mg/kg group at 48 hours. The heart and liver are the 2 tissues with high AST activity. It is not possible to explain the increased serum AST

activity following drug treatment as being exclusively derived from cardiac tissue alone or hepatic tissue alone.

A significant increase in serum ALT activity was noted at 48 hours in the 9 mg/kg treated animals only. No significant difference in activity between treatment with saline and the drug was demonstrtated at 48 hours in all other groups. The liver and kidney are the two tissues with significant ALT activity suggesting its release into the serum from either or both of these sources. Serum alkaline phosphatase activity was very low (0-5 U/L) and not affected by drug treatment however this is a biliary tree enzyme whereas ALT is a hepatocellular enzyme and therefore ALT would be more sensitive to early hepatocellular damage. There was no demonstrable evidence of renal damage (see Urinalysis) and in conjunction with the results of LD isoenzyme analysis (see 4.2.4.) we postulate liver tissue as the source of increased ALT activity following the 9 mg/kg dose.

4.2.2. Creatine Kinase Isoenzyme Analysis

The CK isoenzyme pattern of saline treated controls and Doxorubicin treated animals demonstrated the 3 more commonly known "cytosolic" isoenzymes CK-MM, CK-MB and CK-BB. Creatine kinase-MM was the most predominant

isoenzyme while the other two were present in variable percentages in the individual animals. However there was no noticeable difference in the serum CK isoenzyme pattern by electrophoresis between the experimental and control groups.

Serum CK isoenzyme analysis revealed the presence of a fourth band following treatment with 6 and 9 mg/kg Doxorubicin. Based on information available in humans, this fourth cathodically migrating band was considered to be possibly of mitochondrial origin. Further experimentation was carried out to confirm this.

4.2.3. Mitochondrial Isolation

To determine the nature of this fourth band, mitochondria were isolated in tissue from untreated rats and analyzed as to its CK isoenzyme pattern. Two bands were present. One corresponded to CK-MM of the standards and was believed to originate from lysozymes (refer to page 112). The procedure followed for mitochondrial isolation did not yield exclusively mitochondria, but rather there was some lysozymal contamination. The second band at a point cathodal to CK-MM corresponded in position to the fourth band seen with the Doxorubicin treated animals, suggesting that the latter might be of mitochondrial origin (refer to

page 112). We believe that with doses of 6 and 9 mg/kg there was damage to the mitochondria resulting in the release of mitochondrial CK.

4.2.4. LD Isoenzyme Analysis

Endothelial cell damage may possibly account for the elevation of LD-3 serum activity in the present study with the 9 mg/kg dose. The increased LD-4 and LD-5 activity following treatment with the 9 mg/kg dose suggested damage to liver and/or skeletal muscle. If the LD-4 and LD-5 originated from skeletal muscle, this would have been associated with the release of CK-MM and increased serum activity of total CK while in effect there was a lowered activity with 9 mg/kg. Therefore the liver was the possible source of LD-4 and LD-5 which was supported by elevation of serum ALT activity with the 9 mg/kg dose. Olson (Olson and Shannon, 1979) quantitatively analyzed the LD isoenzymes subsequent to one dose of 20 mg/kg Doxorubicin, i.p., in female CDF rats. They observed an increase in serum LD-1 and LD-2 activity as early as 12 hours, with peak activity as 48 hours. In our study we noted an increase in LD-1 and LD-2 activity with increasing drug dose. This is another indication of myocardial damage because the heart has the highest tissue activities of these anodic LD isoenzymes.

4.2.5. Urinalysis

Urine analysis was the method used to detect signs of nephrotoxicity because the composition of the urine is largely dependent on the function and integrity of renal tissue. The urine was examined as to volume, pH, electrolyte composition, total protein and various biochemical parameters referred to in the results.

The Chemstrip 9 Dipstick Screen demonstrated no abnormalities in any of the groups except for the presence of glycosuria which was believed to be due to stress. There was no change in the 24 hour urine total protein output between the control and the 3 experimental groups. This suggested that significant glomerular damage was unlikely in the drug treated groups, as protein output is a marker of glomerular (and to a lesser extent tubular) integrity. There was a reduced 24 hour urine volume observed with the drug treated animals as compared to the controls, though this finding was not universal.

4.2.6. Drug Measurement

With increasing dose administered by i.p. injection there was increasing parent drug level in the myocardium. The measurement of Adriamycinol, the primary metabolite of

Doxorubicin thought to be cytotoxic was important in these tissues yet measurement of the metabolites was not possible because standards were not available for identification of the various peaks.

4.2.7. Conclusion

By analyzing the serum activity of enzymes specific for cardiac tissue (CK, CK-MB, LD, and LD isoenzymes, specifically LD-1 and LD-2) we have demonstrated Doxorubicin-induced cardiotoxicity. Treatment with 6 and 9 mg/kg Doxorubicin produced mitochondrial damage as evidenced by the appearance of mitochondrial CK in the serum of these rats. Following treatment with 9 mg/kg there was in addition hepatic damage as evidenced by increased serum ALT and LD-4 and LD-5 activities. Urine analysis did not reveal any renal damage as there was no major difference between the control and experimental groups, save a diminished urine volume with increasing drug dose.

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