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TISSUE RESPONSES OF CALVES TO GRADED DOSAGE LEVELS OF MECHLORETHAMINE·HCL AND URACIL MUSTARD

by

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INTRODUCTION

Historically, the ultimate goals of cancer chemotherapy have been to depress the growth of neoplasms, alleviate suffering and prolong the life of the host. The ability of currently used cancer chemotherapeutic agents to achieve these goals is in part limited by their toxic manifestations. In addition to controlling the growth of neoplasms, these compounds also act on any other rapidly proliferating tissues in the body such as hematopoietic tissues, lymphoid tissues, the epithelial lining of the gastrointestinal tract and reproductive cells. Thus the clinician must walk a thin line between death of his patient due to the tumor and death due to the toxic properties of the therapeutic agent.

Assay systems for the anticancer properties of compounds have utilized laboratory animals with inducible neoplasms. Assays for the toxic manifestations of potential anticancer agents, to date, have been of secondary importance. Possibly if extensive assay systems for the toxic properties of potential cancer chemotherapeutic agents were also devised, then information from both types of assays could bring cancer chemotherapy closer to its ultimate goals.

The philosophy guiding these investigations was: if an assay system for the other toxic properties of anticancer compounds can be devised which is sensitive enough, it could be used to detect minute differences in toxic activities of compounds due to slight molecular structural differences. Then it may be possible to engineer away from the undesirable toxic structures by the addition or subtraction of chemical radicals while retaining their desirable anticancer properties.

Studies were initiated to determine if the calf assay system could be further refined so as to be able to detect differences in toxic activities of closely related structural analogues. Attempts were made to equate graded dosage levels of alkylating agents with responses of the calf other than the time of occurrence of death.

LITERATURE REVIEW

Biological Assay Responses of Animals

The biological assay is a technique in which unknown substances and/or unknown amounts of substances are administered to animals (6). The responses of the animals are compared qualitatively and quantitatively to the responses of animals to known substances.

The responses of animals which can be measured are numerous. Life or death of animals in response to compounds is one of the easier measurements made. Other responses which can be measured are changes in blood pressure and varying degrees of growth, increase and regression, of the animal body or a single organ (6). Many attempts have been made to determine if the animal's responses can be equated to graded dosage levels of an experimental substance. Such attempts have been successful only when the responses measured were dose related.

Graded responses of animals to compounds

Laboratory animals have long been used in numerous and varied procedures for determining graded responses of physiologically active compounds or agents. These investigations generally have been preceded by other investigations with the same compounds in which the presence or absence of a response had been noted.

Young rats have been used as the biological assay animal for vitamin A determinations (30). The rats are allowed to become vitamin A depleted. They are then fed vitamin A-free diets to which graded amounts of the

unknown substance are added. The rate of growth of the rats is then compared to that of rats fed known amounts of vitamin A and the amount of vitamin A activity in the unknown substance is determined.

Biological assay techniques are also prominent in endocrine research. Graded amounts of follicle stimulating hormone (FSH) administered to immature female rats have been shown to elicit a graded growth response in the ovary (53). One of the more sensitive biological assay techniques in endocrine research is the ovarian ascorbic acid depletion technique used for the detection of graded amounts of lutenizing hormone (LH) (10). The LH is injected into specially prepared immature female rats and the ovarian ascorbic acid is determined after four hours.

There are also toxins which produce a graded response in laboratory animals. Aflatoxin is a toxin from certain strains of Aspergillus flavus which mimics actinomyocin D and inhibits DNA-dependent RNA synthesis (4). Newberne, et al. (33) have shown that graded amounts of this toxin produce a graded amount of bile duct proliferation and hepatocarcinoma formation in the livers of rats.

Although the small laboratory animals are generally used for biological assays, the graded response is not limited to these laboratory species. Shupe, et al. (51) have shown that graded amounts of fluorine produce graded amounts of damage to the teeth of cattle. The adult bovine animal would probably not be used as the assay system for fluorine, but this illustrates that both large and small animals are capable of showing graded responses.

General responses of animals and man to hematopoietic tissue poisons

The literature abounds with details of agents of industrial or therapeutic importance and of feeding stuffs which have been shown to be potent hematopoietic tissue poisons. Wintrobe (62) lists many varied agents which have this toxic property including benzene, antibiotics, ionizing radiation and anticancer agents. There are also feeding stuffs such as bracken fern (13,19) and trichloroethylene-extracted soybean oil meal (19,37) which in the past have caused a tremendous amount of economic damage to the cattle industry because of their hematopoietic tissue poisoning properties.

Benzene is used as a solvent in many commercial industries (62). This agent causes a pancytopenia or a depletion of all of the cellular elements in the circulating blood. Benzene is an example of a known toxic compound which is still used in industry. Elaborate safety precautions have been devised to allow the continued use of this industrially important compound.

Chloramphenicol is a broad spectrum antibiotic which is widely used in the chemotherapy of microbial infections (62). This compound is a very potent hematopoietic tissue poison especially in certain humans where it also causes a pancytopenia. Although chloramphenicol can be a very toxic compound, its usefulness often outweighs its disadvantages. Experimental findings in dogs (42) and clinical findings (12) have suggested that the toxic manifestations due to this compound occur with chronic use, but do not appear to be dose related. There are no reports available in which attempts have been made to determine if graded responses occur with this agent.

Ionizing radiation has proven to be very toxic not only for hematopoietic tissue but also any other rapidly proliferating tissues of man and animals (2,7,26). This is an example of a laboratory and industrial poison which also has therapeutic usefulness. The dog, pig, burro (2) and calf (43) have been shown to be extremely sensitive to ionizing radiation. All animals have been shown to give the characteristic "aplastic anemia" response of thrombocytopenia, leukopenia, hemorrhage and death to this agent. Schultze, et al. (48) have shown that the calf is the most sensitive animal to gamma radiation.

Many species of animals; mice, rats, dogs and monkeys, in addition to man, are sensitive to the hematopoietic tissue poisoning properties of cancer chemotherapeutic agents (5,16,36,41,45,46,50). The time of occurrence of death of animals in response to cancer chemotherapeutic agents is the most quantitative response established to date and is the basis of toxicity assays for these compounds (45,46). The time of occurrence of signs of hematopoietic tissue poisoning has been at most a qualitative criterion in these toxicity assays. Sternberg, et al. (54) have reported that lymphoid depletion is one of the main microscopic changes in tissues due to the alkylating types of anticancer compounds. They reported an apparent increase in lymphoid depletion with an increase in dosage levels but no attempts were made to quantitate the graded lesions.

Schmidt, et al. (45,46) did attempt to equate clinical and gross changes with graded dosage levels of mechlorethamine·HCl (HN2) given to dogs and monkeys. Their findings suggest that graded amounts of tissue damage occur with graded amounts of HN2. However, changes reported in

lymphoid tissues consisted of suggestions of gross decreases in size of the lymph nodes and the spleens of dogs and monkeys.

Several reports suggest that the dog is the most sensitive assay animal for the toxic properties of anticancer compounds (16,41,45,46,64). Bailey (1) has presented evidence that the calf is 2-5 times more sensitive than the dog to HN2 and uracil mustard (UM). These findings suggest that the calf may be a better assay animal for certain alkylating types of cancer chemotherapeutic agents.

A forage substance which will induce "aplastic anemia" in cattle is bracken fern, Pteridium acquilinum (13,14,15). This toxic plant affects both young and old cattle, but the condition occurs generally when there is little other forage present for the animals to eat. No attempts have been reported to determine if graded responses occur in cattle with this substance.

A feed ingredient which was once of much concern to the cattle industry of Scotland, Germany and the United States is trichloroethylene-extracted soybean oil meal (TCESOM) (37). This toxic meal can induce in cattle a classical response of hematopoietic tissue poisoning or "aplastic anemia" very similar to that produced by ionizing radiation in animals (48). This toxic entity has been shown to induce a graded response in cattle. Picken, et al. (38) and Pritchard, et al. (40) have shown that the young calf is a good biological assay animal for this toxic meal.

Many investigators have shown that hematopoietic tissue poisons are capable of inducing a graded response in various species of animals. The quantitative response of the calf to TCESOM and the greater sensitivity

of the calf to ionizing radiation and certain cancer chemotherapeutic agents suggest that the calf may be a more sensitive assay animal for hematopoietic tissue poisons.

Responses of calves to hematopoietic tissue poisons

The young calf was first shown to be sensitive to hematopoietic tissue poisons when it was determined that bracken fern, Pteridium acquilinum, was the cause of an "aplastic anemia" in cattle while on forage especially during late summer (13,14,15). The toxic principle of bracken fern is unknown but has been shown to be thermolabile (15). Although there have been a considerable number of investigations (13,14, 15,19) dealing with bracken poisoning, there have been no reported attempts to quantitate the responses of calves to this toxic forage.

Perman, et al. (35), Picken, et al. (38) and Pritchard, et al. (40) have shown that the young calf is a good, easily housed experimental animal for the assay of the toxic properties of TCESOM. They have shown that the calf is sensitive and quantitative in its response to graded dosage levels of TCESOM. The times of occurrence of the events of thrombocytopenia, leukopenia, relative lymphocytosis, visible hemorrhages, temperature spike and death have been quantitative in their response to graded dosage levels of this toxic meal.

McKinney, et al. (27) synthesized two compounds, S-(dichlorovinyl)-L-cysteine (DCVC) and S-(dichlorovinyl)-L-glutathione (DCVG), which were capable of inducing a syndrome in calves similar to that reported for TCESOM. Derr, et al. (8), McKinney, et al. (28,29), Perman, et al. (35) and Schultze, et al. (47), using DCVC in a variety of dosage regimens;

single dose, multiple dose, oral and intravenous, have shown that the young calf responds in a quantitative manner to graded dosage levels of DCVC. McKinney, et al. (27) also reported that DCVC was more toxic to the calf than its structural analogue, DCVG. Strafuss and Sautter (58) reported that gradations of lymphoid depletion and bone marrow hypoplasia were produced by DCVC but no quantitation was attempted.

The quantitative response of the young calf to TCESOM and DCVC and the report of Schultze, et al. (48) showing that the calf was more sensitive than other animals to the hematopoietic tissue poisoning properties of ionizing radiation, suggested to Bailey (1) that the calf might be a good assay animal for other hematopoietic tissue poisons. His studies involved investigating the responses of the calf to the toxic properties of several cancer chemotherapeutic agents. Bailey (1) gave to calves graded daily dosage levels of mechlorethamine.HCl (HN2), uracil mustard (UM), 5-fluorouracil (5-FU) and amethopterin (AMETH). His findings showed that the time of occurrence of death of the calves treated with graded dosage levels of the two alkylating agents, HN2 and UM, was quantitative. His results also suggested that the calf was 2-5 times more sensitive to these compounds than the reportedly sensitive dog and 5-10 times more sensitive than the rat. Other findings included data showing that UM on a molar basis was 2 times more toxic to the calf than its analogue, HN2. Bailey (1) reported that the calf was sensitive but not quantitative in its response to 5-FU and not at all sensitive to AMETH.

Bailey's (1) studies suggest that the calf may be a more sensitive assay animal for the toxic properties of certain closely related alkylating agents. If the calf is sensitive enough, then it might be used to exploit the possibility of using more highly developed assays to aid in the development of new and better cancer chemotherapeutic agents.

Expression of Dose-Response Relationships

Drug dosages in human and animal medicine have been used to define the quantity of a therapeutic compound required to produce a given response (55). The size of the animal as indicated by its weight has been a useful criterion by which to govern the dosage levels. The problem has been in the transposition of dosage levels between species of animals because the smaller species require more compound per unit of body weight to produce a desired effect than do the larger species (55). Recently, Freireich, et al. (17) and Pinkel (39) have shown that the use of body surface area has been a useful criterion for the adjustment of drug dosages between different species for cancer chemotherapy. The body surface area is a variable which can be expressed as a function of weight (17). Even though investigators are continually searching for other means by which to establish drug dosages, body weight appears to be the most reliable criterion for adjusting dosage levels.

The amount of a physiologically active agent or compound per unit of body weight required to induce a measurable response in an experimental animal is an expression of a dose-response relationship (6,56). Several complex mathematical manipulations have been devised to more adequately

define the dose-response relationship. One technique is to compare the intensity of a response expressed as a percentage with the dosage level which has undergone a logarithmic transformation (55). Other methods have used Lineweaver-Burk plots in attempts to further define the dose-response relationships (21).

Regardless of the means of expressing dose-response relationships, none of the presently used methods, elaborate though they may be, of expressing dose-response relationships gives an adequate definition of the therapeutic effectiveness or the damage to the tissues of interest produced by an administered compound. Blood levels of therapeutic compounds have been widely used to give an indication of the therapeutic effectiveness of compounds (22,57,61). In some instances, dosage levels per unit of body weight of presently used therapeutic agents have been established to maintain certain blood levels. This is the case with penicillin in that dosage levels which produce and maintain barely detectible blood levels appear to be sufficient for therapeutic effectiveness (22). In the field of cancer chemotherapy, Tan and Cole (59) have recently suggested that blood levels of anticancer agents may be used as indicators of therapeutic effectiveness.

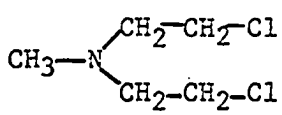
Simon (52) and Weinstein (61) theorize that blood levels of compounds should indicate to some extent the concentration of the compounds in the tissue fluids bathing each individual cell. Since many therapeutic compounds are present in the blood in complexes with proteins in addition to the free forms (3,55), Bonneycastle (3) suggests that plasma or blood levels have little meaning. Bonneycastle (3) and Stowe (57) suggest that

the blood concentration of a compound is only a rough estimate of the amount of drug remaining in the body regardless of form.

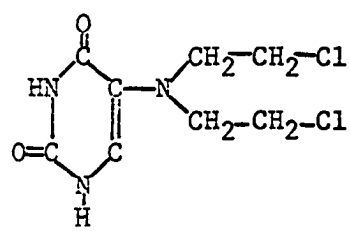
To date, none of the methods of expressing dose-response relationships involves an adequate definition of the actual damage produced in the tissues due to an administered compound. Blood or plasma levels of therapeutic compounds, even though there is disagreement about their validity or usefulness, are still the only means by which attempts can be made to adequately establish the amount of a compound or substance which elicits a certain observable response such as tissue damage.

Mechlorethamine·HCl and Uracil Mustard

Mechlorethamine·HCl (2,2'-dichloro-N-methyl-diethylamine) (HN2) and uracil mustard (5-[bis(2-chloroethyl)amino]-uracil) (UM) are alkylating agents which have widespread use in cancer chemotherapy (34). The structures of these compounds are as follows:



Mechlorethamine·HCl



Uracil Mustard

These two compounds are structural analogues and differ only in the carrier portions of the molecule, the methyl group on the HN2 and the uracil moiety on the UM (23). These compounds are polyfunctional alkylating agents in that they are capable of inducing the phenomena of alkylation

two or more times before becoming inactive (23,24). Both of these compounds have a crystalline structure. HN2 is extremely soluble in water but UM is insoluble. Schumacher and O'Connell (49) have used the adjuvant, dimethylacetamide (DMA), to increase the solubility of UM in water. Bailey (1) did not observe toxicity differences in calves treated with UM due to the presence of small quantities of DMA. The DMA did appear to enhance the solubility of the UM in water (1).

Mode of action

Alkylation can occur in any electron rich center in the body, but most alkylating agents of therapeutic value appear to have a prediliction for several sites (24). The main site of action is thought to be at the 7' position of guanylic acid. The ensuing reactions are thought to be cleavage of the bond with the sugar moiety and disruption of the DNA chain. Johnson and Bergel (23) suggest that other possible sites of action are the cleavage of the imidazole ring of purines and the causing of interactions between two separate DNA chains, between DNA and certain proteins and also between two separate proteins.

Shanbrom, et al. (50) have postulated that UM is capable of acting as an antimetabolite due to its uracil moiety but there is no evidence to substantiate this thesis. Their thesis could be a possible explanation for Bailey's (1) findings that UM was two times as toxic to the calf as HN2. However, Johnson and Bergel (23) present evidence that different carrier portions of the alkylating agent molecule vary the efficiency of the alkylation reaction. This may indicate that the uracil moiety induces more efficient alkylation than the methyl moiety.

Determination of Alkylating Agents in Tissue Fluids

The extensive use of alkylating agents in cancer chemotherapy has demanded the development of sensitive assay techniques for these agents in tissue fluids. Several methods for the detection of alkylating agents have been developed.

^{14}C labeled mechlorethamine·HCl (HN2) has been used as a method for determining the tissue levels and distribution of this compound (32). Nadkarni, et al. (32) have shown that over 90 percent of the activity leaves the blood within 30 seconds in mice. Mellett and Woods (31) suggest that a limitation of this method is that the labeled carbon stays with the break-down products and does not give an adequate indication of the unchanged parent compound remaining in the tissues.

Mellett and Woods (31) have reported a fluorometric technique for determining alkylating agents in tissue fluids. This technique is reported to be specific for the unchanged parent compound and appears to be the most sensitive technique reported in the literature. Blood and plasma concentrations detected with this procedure have been as low as 0.02 µg. of HN2 per ml. of blood or plasma. The authors (31) have shown that 90 percent of the HN2 activity disappears from the blood of dogs within 4 minutes in contrast to the 30 seconds reported in mice using labeled compounds (32). The fluorometric technique of Mellett and Woods (31) is a multistage procedure and requires a large volume of plasma, 5 ml., for each determination. Tan and Cole (59) suggest that because of its complexity and large volume of plasma required, the fluorometric technique is not designed for routine clinical procedures. Mellett and Woods (31)

report that time is an important factor in their procedure, but they do not define the limitations.

Friedman and Boger (18), Tan and Cole (59) and Klatt, et al. (25) have reported the use of gamma-(4-nitrobenzyl)pyridine (NBP) for determining the presence of alkylating agents in tissue fluids. This reagent is a nucleophilic agent which when combined with an alkylating agent under alkaline conditions produces a blue color (18). The amount of color has been shown to have a linear relationship with increasing concentrations of HN2 between 1 and 10 μg. per ml. of plasma (18).

Epstein, et al. (11) first described the use of NBP in a method for the quantitative estimation of alkylating agents in an aqueous solution. Friedman and Boger (18) described a technique for detecting alkylating agents in tissue fluids using NBP. Their method involved deproteinization with 1N hydrochloric acid followed by development of the color in an aqueous solution. The colored pigment was extracted with an equal volume of ethyl acetate and determined in a colorimeter. They found that the color formed was stable only for 3-5 minutes. The sensitivity of this procedure was reported to be 1 μg. of HN2 per ml. of tissue fluids.

Tan and Cole (59) described a procedure with NBP for detecting alkylating agents in a small volume of plasma. This method involved deproteinization with zinc sulfate and barium hydroxide. The color pigment was formed in the protein-free filtrate and the colored compound was concentrated and extracted in a small volume of ethyl acetate. The solution was centrifuged, the ethyl acetate upper layer placed in a micro-

cuvette and the optical density of the solution was determined. The color was reported to be stable for 3-5 minutes. The sensitivity of the technique was reported to be 0.05 μg. of HN2 per ml. of plasma.

Klatt, et al. (25) also have reported a procedure utilizing NBP in determining alkylating agents in plasma. This technique involves deproteinization and interaction with the NBP in the same step. The color is read in an aqueous solution. The color is reported also to be stable for only 3-5 minutes. In contrast to the method of Tan and Cole (59), there are no centrifugation and extraction manipulations between color formation and determining the optical density of the colored solution. The sensitivity of this technique is reported to be 0.05 μmoles of HN2 per ml. of plasma or about 0.1 μg. per ml. of plasma.

The techniques reported in the literature for determining alkylating agents are multistep, complicated procedures. The fluorometric procedure appears to be the most sensitive technique, but it is also the most complicated. The procedures utilizing NBP as the color reagent are less sensitive than the fluorometric technique by factors of 1/5 to 1/2 and are less complicated. Time appears to be an important factor in both the fluorometric and NBP techniques for determining alkylating agents.

MATERIALS AND METHODS

Experimental Animals

Calves of mixed breeds and sexes were purchased from several local sources. These calves weighed from 37 to 74 kg. The animals were housed, two per stall, in clean, well ventilated concrete stalls. In good weather, the animals had access to a large fenced outside area.

The rations consisted of a commercial powdered milk preparation[1] with grain, grass hay and fresh water available at all times.

After receipt, the calves were conditioned for a period of one to two weeks. This conditioning period was essential to establish the state of health of the calves. This period was also used to measure the physiological parameters to be determined during the experimental period in order to establish the base line values for each individual animal.

During the experimental period, clinical examinations were made daily on each calf to detect signs which could be attributable to the test compounds. The calves were weighed weekly to establish the growth responses and to allow adjustment of the dosage levels.

The hair on the necks of the calves was kept closely clipped over the jugular veins to allow adequate disinfection of the areas. Blood samples were collected and the test compounds were administered via the jugular veins. The test compounds were given on the basis of a predetermined amount per unit of body weight.

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[1]Dari Dri; Milk Specialties, Inc., Dundee, Illinois.

One ml. blood samples were collected with siliconized[1] needles and syringes for thrombocyte counts. Ten ml. of blood were collected in evacuated containers[2] with the dipotassium salt of ethylenediaminetetraacetic acid (K_2EDTA) as the anticoagulant for the remaining hematologic procedures and for determining plasma levels of alkylating agents. Five additional ml. of blood were collected in evacuated containers[2] with no anticoagulant. Serum was harvested from this blood sample and frozen for future serum protein evaluations.

Test Compounds

The cancer chemotherapeutic agents used in this investigation were obtained from a local retail source.

Mechlorethamine·HCl

The mechlorethamine·HCl[3] was furnished in sterile, 10 mg. vials. This compound was reconstituted daily with 10 ml. of sterile water to make a final concentration of 1 mg. per ml.

Uracil mustard

The uracil mustard[4] was supplied in one mg. capsules. The contents of the capsules were emptied into sterile volumetric flasks of sufficient

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[1]Siliclad; Clay-Adams, Inc., New York, N. Y.

[2]Vacutainer; Becton, Dickinson and Co., Rutherford, N. Y.

[3]Mustargen hydrochloride; Merck, Sharp and Dohme, Division of Merck and Co., West Point, Pa.

[4]Uracil mustard; The Upjohn Co., Kalamazoo, Mich.

size. Sterile saline (0.85% NaCl) was added to make a final concentration of 1 mg. per ml. Solutions of uracil mustard with a concentration of 1 mg. per ml. were also prepared with dimethylacetamide as the solvent. All solutions were prepared daily and the excesses were discarded.

Peripheral Blood Evaluations

The physiological parameters measured in the peripheral blood were total thrombocyte count, total erythrocyte count, total leukocyte count, differential leukocyte count, packed cell volume and hemoglobin determination.

Thrombocyte counts

Thrombocyte counts were made with a disposable dilution system[1] described by Gerarde (20). The dilutions were made at the time of obtaining the blood sample. A drop of blood from a siliconized syringe was placed on a paraffin block to facilitate the use of the disposable dilution system. The diluted blood was used to charge a hemocytometer and the counts were made with the use of a microscope.

Erythrocyte and leukocyte counts

Erythrocytes and leukocytes were enumerated using a disposable dilution system[2] and an electronic particle counter[3] as described by

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[1]Unopette No. 5805; Becton, Dickinson and Co., Rutherford, N. J.

[2]Unopette No. 5865; Becton, Dickinson and Co., Rutherford, N. J.

[3]Coulter Counter Model F; Coulter Electronics, Inc., Hialeah, Fla.

Gerarde (20). The particle counter was calibrated for calf blood cells using methods reported by Weide, et al. (60) and Wisecup and Crouch (63).

Differential leukocyte counts

Blood smears were made and stained by the Wright's method as described by Schalm (44). The stain was applied for a three minute period. A similar quantity of the buffer solution was mixed with the stain and allowed to remain on the slide for an additional five minute period. The slide was washed with tap water, air dried and examined by the oil immersion technique. One hundred cells were differentiated for each differential count.

Packed cell volume

The packed cell volume was determined by the microhematocrit method (44). A microhematocrit[1] centrifuge and reader were used in this procedure.

Hemoglobin determinations

The cyanmethemoglobin method for hemoglobin determination was used (9,44). The disposable dilution system[2] described by Gerarde (20) was utilized. The developed color was read in a spectrophotometer[3] at 540 mμ.

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[1]Clay-Adams, Inc., New York, N. Y.

[2]Unopette No. 5857; Becton, Dickinson and Co., Rutherford, N. J.

[3]Spectronic 20; Bausch and Lomb, Inc., Rochester, N. Y.

Serum Protein Evaluations

Total serum proteins

The total serum proteins were determined by the Biuret method using a commercially prepared reagent[1]. Duplicates were run on each serum sample. Six ml. of the Biuret reagent were added to 0.1 ml. of serum, mixed and placed in a water bath at 37° C for 30 minutes. The optical density was determined at 540 mμ with a spectrophotometer[2]. Standard curves were prepared using a commercial protein standard[3].

Electrophoresis of serum proteins

The serum protein fractions were evaluated with a cellulose acetate electrophoresis system[4]. A buffer system of pH 8.6 and ionic strength of 0.05 was utilized in this procedure. The cellulose acetate strips with serum applied were placed in the electrophoresis chamber for 90 minutes with an amperage of 1.5 ma. per strip. The strips were stained with Ponceau S and cleared with a solution of 13 percent acetic acid in absolute methanol. The strips were scanned with a densitometer[4]. The densitometer readings were plotted on graph paper and the area under the curve for each peak was determined by planimetric techniques. The albumin-globulin ratios were determined from these values.

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[1]Hycel, Inc., Houston, Texas.

[2]Spectronic 20; Bausch and Lomb, Inc., Rochester, N. Y.

[3]Dade Reagents, Miami, Fla.

[4]Gelman Instrument Co., Ann Arbor, Mich.

Determination of Plasma Levels of Alkylating Agents

Reagents

1. $ZnSO_4$ (reagent grade), 6 percent (w/v) in water.
2. $Ba(OH)_2$ (reagent grade), 65 gm. per liter of boiled water, filtered and stored under soda lime.

The $ZnSO_4$ and $Ba(OH)_2$ solutions were titrated against each other to a phenolphthalein end point of pH 8.2. The titration was confirmed with a pH meter[1].

3. Acetate buffer, glacial acetic acid was diluted and neutralized with 1N NaOH to pH 4.6. The concentration of the acetate ion was adjusted to 1N.
4. Gamma-(4-nitrobenzyl)pyridine, 5 percent (w/v) in fractionally distilled methyl-ethyl ketone (boiling point range of 78-80° C).
5. Triethylamine (reagent grade), 50 percent (v/v) in acetone.
6. Methyl acohol (reagent grade).
7. Plasma for the subsequent preparation of standard curves was harvested from adult bovine blood and frozen in 40 ml. aliquots.

Preparation of alkylating agent standard curves

Ten mg. of mechlorethamine·HCl (HN2) was diluted to 100 ml. with deionized water making a final concentration of 100 ug. per ml. Standard solutions were prepared by the addition of a sufficient amount of the

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[1]Model GS; Beckman Instruments, Inc., Fullerton, Calif.

diluted HN2 to the stock bovine plasma. Concentrations used were 0.5, 1.0 and 2.0 μg. of HN2 per ml. of plasma. A standard curve was prepared for each assay.

Method for determination of HN2 in calf plasma

A predetermined amount of alkylating agent was administered to a calf via the jugular vein. Blood samples were taken at zero time and at intervals of 1, 2, 5, 10 and 20 minutes following the administration of the compound with the previously described evacuated 10 ml. tubes with K_2EDTA, mixed and placed in an ice bath. The blood samples were centrifuged and two 2 ml. aliquots were taken from each plasma sample. To each 2 ml. sample of plasma, 2.3 ml. of the $ZnSO_4$ solution, an equivalent amount of the $Ba(OH)_2$ solution and 0.4 ml. of methyl alcohol were added. The solutions were thoroughly mixed[1] following the addition of each reagent. The mixtures were centrifuged and 4.0 ml. of the clear supernatants were placed into clean test tubes and placed in an ice bath. 0.2 ml. of the acetate buffer and 0.4 ml. of the NBP reagent were added to each of the cooled tubes. The solutions were thoroughly mixed and glass marbles were placed on top of the tubes. The samples were digested at 80-90° C for twenty minutes and then cooled in an ice bath. The cooled mixtures were centrifuged and 3.0 ml. of the supernatants were placed into previously calibrated spectrophotometer[2] tubes. Two ml. of the triethylamine (TEA) reagent were added to each sample and the solutions

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[1]Vortex Jr. Mixer; Scientific Industries, Inc., Springfield, Mass.

[2]Spectronic 20; Bausch and Lomb, Inc., Rochester, N. Y.

were mixed by inversion. Optical density readings were made starting five minutes after the addition of the TEA reagent to the first tube with a spectrophotometer[1] set at 565 mμ. All readings were completed within 45 minutes following the digestion period. All samples and standards were assayed in duplicate.

Necropsy Techniques

Necropsies were performed on all experimental animals. Animals not dying due to the experimental procedures were electrocuted. All observed gross lesions were noted and recorded for subsequent evaluation. Tissues with gross lesions and representative lymphoid tissues, spleen, thymus and the right popliteal lymph node, were placed in buffered 10 percent formalin for preservation. All preserved tissues were processed for staining with hematoxylin-eosin and examined microscopically.

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[1]Spectronic 20; Bausch and Lomb, Inc., Rochester, N. Y.

EXPERIMENTAL

The purpose of the investigation was to determine the nature of the relationships between graded dosage levels of two administered alkylating agents, mechlorethamine·HCl (HN2) and uracil mustard (UM), and the responses produced in the calf. Major emphasis was to be placed on the tissue responses. A secondary phase of the investigation was to determine if plasma levels of the alkylating agents, HN2 and UM, administered to the calf could be detected and characterized. Attempts would be made to relate the plasma levels to the dosage levels and the tissue responses observed.

Determination and Characterization of Plasma Levels of Alkylating Agents

Selection and refinement of the method

Characterization of plasma levels of compounds can be accomplished by defining the magnitude of the peak levels, duration of the levels or possibly defining the areas under the plasma level curves. Many blood samples are required to adequately characterize a plasma level of a compound. This requirement placed an immediate limitation on the technique for alkylating agent determination to be employed.

The fluorometric technique of Mellett and Woods (31), the most sensitive technique for alkylating agent determination reported in the literature, was obviously the method of choice based on its reported sensitivity. It was also the most complex technique reported for the determination of alkylating agents in plasma. This complexity was not desirable because of the number of samples required for the proposed studies.

The less complex group of techniques for determining alkylating agents in plasma all employed gamma-(4-nitrobenzyl)pyridine (NBP) as the color reagent. These techniques were reported to be less sensitive than the fluorometric method. A decision was made to examine all of the NBP procedures and try to determine which procedure readily lended itself to the particular needs of the proposed studies. A review indicated that all of the techniques utilizing NBP as the color reagent followed a similar pattern: a deproteinization step, a step involving reaction of the alkylating agent with the NBP reagent at elevated temperatures, development of the color under alkaline conditions and determination of the color produced by comparison with a standard curve. All authors (17,24,59) suggest that the color should be developed and read within 45 minutes following digestion.

The most sensitive method utilizing NBP reported, that of Tan and Cole (59), was selected for the initial studies. This method involved the use of a plasma filtrate. The deproteinization step was by-passed for the initial studies and an aqueous solution of HN2 was prepared to simulate the plasma filtrate. An appropriate number of samples and blanks were used. The procedure of Tan and Cole (59) was followed except that all amounts were doubled. The NBP, 5 percent in acetone, plus the pH 4.5 acetate buffer were added and the solutions were heated in boiling water for 20 minutes. The solutions were cooled and acetone, ethyl acetate and sodium hydroxide (NaOH) were added. The samples were shaken and centrifuged.

A blue color could be seen in the ethyl acetate upper layer immediately after the addition of the NaOH but the bulk of the color appeared to dissipate before the steps of centrifugation and transfer to the microcuvettes could be accomplished. The ethyl acetate upper layers were removed with Pasteur pipettes and placed in microcuvettes. All optical density (O. D.) readings obtained were comparable to the blank O. D. readings indicating complete loss of the color in the initial experiment.

Three additional trials were undertaken. Efforts were made to lower the manipulation times by using a smaller number of samples but there were no real improvements. The results indicated that the pigment was not stable for the 3-5 minute period as the authors (59) had suggested. The preliminary findings suggested that this procedure would not be desirable for the projected experiments.

The method of Klatt, et al. (25) was the next NBP procedure examined. This procedure was reported to be one half as sensitive as the method of Tan and Cole (59). In this procedure, NBP reagent, water and acetic acid were added to the plasma and the mixture was heated at 80-90° C for 15 minutes. The solutions were cooled and centrifuged. An aliquot of the supernatant was drawn off and added to an equal volume of triethylamine, 50 percent in acetone, for color development. There were no centrifugation and extraction steps after the color development.

Following the literature examination of the method of Klatt, et al. (25), it was decided to attempt to modify the method of Tan and Cole (59) by developing and reading the color in an aqueous solution as described by Klatt, et al. (25) in order to avoid the time consuming extraction

procedures. Acetone was retained as the NBP solvent as indicated by Tan and Cole (59), but triethylamine (TEA), 50 percent in acetone was used as the alkalifying reagent as suggested by Klatt, et al. (25).

Simulated plasma filtrates were prepared by using aqueous solutions of HN2 with concentrations of 0.5, 1.0 and 2.0 µg. per ml. Two ml. of each concentration were used in triplicate and appropriate blank solutions were prepared. Appropriate volumes of acetate buffer and NBP reagent were added and the samples were placed in a water bath at 80-90° C for 20 minutes. The tubes were cooled in an ice bath, the TEA reagent added and the color was determined. Despite several problems, a suprisingly straight line relationship was observed using this modified technique.

The problems were bumping of the solutions during digestion with physical loss of contents, precipitate formation after digestion which hampered accurate pipetting, and high blank readings. An additional observation made was that the blue color had an initial surge of deep color and then it appeared to stabilize for a period of time.

It was suggested that the high temperatures of digestion caused the evaporation of the NBP solvent and this was the reason for the bumping and precipitate formation. A change in the procedure was made to use methyl-ethyl ketone (MEK) (boiling point of 79.6° C) as the NBP solvent as suggested by Klatt, et al. (25). Bumping and precipitate formation were decreased to some extent but there were no appreciable improvements in the high blank readings. The surge of color persisted. The use of MEK with a boiling point of 79.6° C which was near the water bath temperature of 80-90° C appeared to decrease the problems.

It was thought that some low boiling fractions in the MEK may have been the cause of the bumping and high blank readings. A second experiment was run in which fractionally distilled MEK (boiling point range of 78-80° C) was used as the NBP solvent. The blank readings were lowered by 0.01 O. D. units (0.06 to 0.05) but some bumping and precipitate formation still occurred.

Klatt, et al. (25) had reported the use of short-stemmed funnels in the mouths of the test tubes during digestion. Another experiment was designed in which the fractionally distilled MEK was used as the NBP solvent and a marble was placed on top of each tube during digestion. It was found that the bumping stopped and the precipitate formation was essentially alleviated.

Further attempts were made to alleviate the occasional precipitate formation. An experiment was designed using fractionally distilled diethyl ketone (DEK) with a boiling point range of 101-103° C as the NBP solvent. The DEK was observed to be relatively insoluble in the aqueous solution. The addition of the TEA reagent caused the formation of a turbid solution instead of a clear, colored solution. No standard curves were obtained following this experiment.

The results of the preliminary experiments with aqueous solutions of HN2 indicated that the modified Tan and Cole (59) method for alkylating agents gave a satisfactory straight line relationship between the concentration of the HN2 and the color produced. The use of fractionally distilled MEK as the NBP solvent and the TEA reagent as the alkalifying reagent appeared to give the best results. The blank readings obtained

with this procedure were high. These O. D. readings were 0.05, but were very consistent from run to run.

Preparation of standard curves for HN2 in bovine plasma

The use of calves in following plasma levels of alkylating agents had not been previously reported. Attempts were made to prepare working standard curves for HN2 by adding known amounts of HN2 to bovine plasma.

The Tan and Cole (59) method for alkylating agent determination in plasma called for deproteinization of the plasma with zinc hydroxide. Equal volumes of bovine plasma and 5 percent zinc sulfate ($ZnSO_4$), an equivalent amount of a saturated barium hydroxide [$Ba(OH)_2$] solution and a small volume of methyl alcohol (MeOH) were mixed and processed as described by Tan and Cole (59). A clear plasma filtrate could not be obtained using these reagents. The equivalency of the $ZnSO_4$ and $Ba(OH)_2$ solutions was rechecked, following titration to a phenolphthalein end point of pH 8.2, with a pH meter. Several attempts at deproteinization of the bovine plasma with the rechecked solutions were unsuccessful and clear filtrates were not obtained.

A 6 percent solution of $ZnSO_4$ was prepared and titrated against the saturated $Ba(OH)_2$ solution. Clear plasma filtrates were still not obtained consistently. A further adjustment of the suggested volumes was undertaken. The use of 2.3 ml. of the 6 percent $ZnSO_4$ solution, an equivalent amount of the $Ba(OH)_2$ solution and 0.4 ml. of the MeOH were found to give satisfactory plasma filtrates with 2.0 ml. of bovine plasma. In addition, it was found that the solutions must be added to the bovine

plasma in the order mentioned and that the solutions must be thoroughly mixed following addition of each reagent before clear plasma filtrates could be routinely obtained.

Several standard curves were prepared using concentrations of HN2 as low as 0.1 µg. per ml. of plasma to as high as 10.0 µg. per ml. of plasma. The curves obtained were satisfactory straight line curves, but there was some day-to-day variation.

The amount of change of O. D. units due to 0.1 µg. of HN2 per ml. of plasma was only 0.003 O. D. units. This change in O. D. units was easily within the normal fluctuation of the spectrophotometer[1] being used. This suggests that errors ranging from 20 to 100 percent could be obtained with HN2 concentrations below 0.5 µg. per ml. of plasma.

If the majority of the detected plasma levels in the proposed animal studies were found to be above 0.5 µg. of HN2 per ml. of plasma, the error, even though large, could be acceptable. However, if the majority of the detected plasma levels were less than 0.5 µg. of HN2 per ml. of plasma, the magnitude of the errors could render any interpretation of the results invalid.

Decay of the color pigment

The labile nature of the color pigment was still of concern even though the preliminary results indicated that it had not caused trouble. An experiment was designed to determine the decay curves for the color pigments from plasma filtrates. A single concentration of HN2, 1 µg. per

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[1]Spectronic 20; Bausch and Lomb, Inc., Rochester, N. Y.

ml. of bovine plasma, was used in these experiments. The experiments involved determining the O. D. at 0.5, 1, 2, 5, 10 and 20 minutes following the addition of the TEA reagent while keeping within the 45 minute time limit. The results of three trials are presented in Table 1.

Table 1. Change in O. D. readings following the addition of TEA reagent

Experiment	0.5 min.	1 min.	2 min.	5 min.	10 min.	20 min.
1	0.020	0.019	0.019	0.015	0.014	0.014
2	0.021	0.020	0.017	0.017	0.012	0.015
3	0.023	0.019	0.016	0.018	0.011	0.011
Average	0.021	0.019	0.017	0.016	0.012	0.013

The results indicated that there was a steady loss of color over the first 10 minutes. Since there was no apparent early plateau after adding the TEA reagent, there was no appropriate time to make the O. D. readings. A decision was made to make all O. D. readings starting 5 minutes after the addition of the TEA to the first sample. Following initiation of this procedure, it was found that all tubes could be read within 5-6 minutes after the addition of the TEA reagent.

Decay of HN2 activity

The use of animals and multiple sampling techniques in the proposed experiments indicated that there would be a time lapse between drawing the blood samples and determining the plasma levels of HN2. It was not known if this time interval was critical. An experiment was designed to

determine the decay of HN2 activity in both plasma and water. A concentration of 1 µg. of HN2 per ml. of plasma and water was used. The solutions were prepared to allow the HN2 to be in solution in either the plasma or water for 0, 60 or 120 minutes. The 0 time solutions were the last to be prepared and as soon as the dilutions were completed, all samples were taken through the previously described procedure of deproteinization, digestion with the NBP reagent, color formation and determination of the O. D. Triplicate samples were run for each time interval. Duplicate determinations were made for each HN2 solvent, plasma and water. The results of this experiment are presented in Table 2.

Table 2. Average O. D. readings for decay of 1 µg. of HN2 per ml. of plasma or water

Solvent	0 min.	60 min.	120 min.
Plasma	0.018 ± 0.003	0.018 ± 0.003	0.020 ± 0.002
Water	0.020 ± 0.002	0.020 ± 0.002	0.018 ± 0.002

These experiments indicate that there was no apparent loss of HN2 activity in either plasma or water over a two hour period. To assure minimum loss of HN2 activity, it was decided to keep all blood samples in an ice bath until the samples were centrifuged, the plasma harvested and the plasma levels of HN2 were determined.

Acceptable standard curves were obtained from plasma containing various concentrations of HN2. These curves were established with the use of plasma filtrates and following the final technique which is listed

in the materials and methods. There was some slight day-to-day variation in the curves but the slopes were constant. It was decided to prepare standard curves for each determination. The concentrations of HN2 to be used in the preparation of the standard curves were 0.5, 1.0 and 2.0 µg. per ml. of stock plasma.

Uracil mustard standard curves

Klatt, et al. (25) have previously shown that their NBP procedure for alkylating agents could be used to detect uracil mustard in addition to HN2 in plasma. Attempts to prepare standard curves for uracil mustard (UM) using the procedure developed for HN2 were undertaken.

A single trial was undertaken using both water and plasma as solvents for the UM. UM concentrations of 0.5, 1.0 and 2.0 µg. per ml. of plasma or water were prepared. Uracil mustard could not be detected at any of the concentrations used. Two additional trials were made after rechecking all reagents. Again, no alkylating agent activity could be detected in the UM standard solutions. No answer was readily apparent for this lack of success since Klatt, et al. (25) had reported the detection of UM with their technique.

A decision was made not to pursue further any attempt to prepare a UM standard curve since plasma levels of alkylating agents were an area of secondary importance. UM would still be utilized in the main studies and attempts to monitor plasma levels of alkylating agents would be done only with HN2.

Validity of the assay method

The results of all experiments to date indicated that acceptable standard curves for the alkylating agent, HN2, in plasma could be prepared using the modified technique. This modified technique appeared to give consistent results for plasma concentrations of HN2 above 0.5 µg. per ml.

The validity of this modified technique for determining plasma levels of HN2 below 0.5 µg. per ml. appeared to be questionable. A decision was made to go ahead and monitor the plasma levels of HN2 in the graded dosage-tissue response animal studies with the modified technique. No data appeared to exist relating graded dosages of HN2 to the plasma levels obtained. Data obtained from the proposed monitoring could give worthwhile insight into the nature of the relationships.

Preliminary animal experiments for determining HN2 plasma levels

The animal experiments described in this section were designed to answer three questions.

1. Can plasma levels of HN2 be detected in the calf following intravenous administration?
2. Can graded plasma levels of HN2 be detected in calves given graded dosage levels?
3. If graded plasma levels of HN2 are detected, can they be related to the responses of the calves to HN2?

Preliminary experiments were undertaken to determine what dosage levels of HN2 were required to produce detectible plasma levels of HN2 in calves. These experiments were designed to determine when the peak levels of HN2 occurred and what were the durations of the plasma levels.

Using the same calf to eliminate as much individual variation as possible, two different dosage levels of HN2, 0.114 and 0.343 μmoles per kg. were administered. Each dosage level was given on a separate day and the animal was allowed 2 days rest between administrations. Blood samples were obtained at 0 time and 1, 2, 5, 10 and 20 minutes following the administration of the HN2. The blood samples were kept in an ice bath until centrifuged. Plasma was harvested from these blood samples and plasma levels of HN2 were determined. The results of these experiments are presented in Table 3.

Table 3. Duration of plasma levels of HN2 (μg./ml.) following administration to the same calf

Dosage Level μmoles/kg.	0 min.	1 min.	2 min.	5 min.	10 min.	20 min.
0.114	0.00	0.00	0.00	0.00	0.00	0.00
0.343	0.00	0.68	0.38	0.31	0.15	0.20

These results show that all of the plasma levels were within the questionable range of the assay method except for the 1 minute sample with the higher dosage level. The findings suggested that a dosage level of 0.114 μmoles of HN2 per kg. was insufficient to produce a detectible plasma level with this method and 0.343 μmoles of HN2 per kg. was sufficient.

A second experiment was undertaken to establish the plasma levels produced by a much higher dosage level of HN2. The dosage level selected was chosen because it approximated dosage levels which had been reported

to produce detectible tissue damage in the dog (45). The dosage level chosen was 2.288 μmoles of HN2 per kg.

The same calf was used in this experiment that had been used in the previous experiments after a 7 day period of rest. The compound was administered on 2 separate days, two days apart. The plasma levels were monitored over a two hour period following each injection of HN2. The results of this experiment appear in Table 4.

Table 4. Duration of plasma levels of HN2 (μg./ml.) following administration of 2.288 μmoles per kg. to the same calf

	Time (minutes)								
Injection	0	1	2	5	10	20	30	60	120
1	0.00	0.10	0.35	0.00	0.32	0.10	0.40	0.40	0.55
2	0.00	0.50	0.50	0.20	0.28	0.28	0.28	0.12	0.00

These findings show that all of the plasma levels detected appear to be in the questionable range of the standard curve. The maximum peak levels with both injections were comparable in magnitude but differed in the time of appearance. It is not known whether the variability was real or a reflection of the inadequacy of the analytical method.

The plasma levels of HN2 detected in the preliminary experiments all approached or were well within the questionable area of the standard curve. However, a decision was made to continue to monitor the HN2 plasma levels in all of the proposed HN2 dosage-response studies to see if any relationships or trends might be suggested.

Single dose studies with HN2

The experiment involved two calves which were part of the HN2 dosage-response studies. This experiment entailed administering single injections of HN2 to calves. The dosage levels selected were 2.288 and 4.576 µmoles per kg. One level was administered to each calf. The plasma levels were monitored for 120 minutes. The results of this experiment are presented in Table 5.

Table 5. Duration of plasma levels of HN2 (µg./ml.) following administration of two dosage levels

Dosage Level µmoles/ kg.	Time (minutes) 0	1	2	5	10	20	30	60	120
2.288	0.00	0.40	0.30	0.30	0.35	0.14	0.22	0.00	0.00
4.576	0.00	0.00	0.15	0.55	0.55	0.55	0.00	0.00	0.00

Inspection of these data show that while the higher dosage level produced the higher plasma levels, all plasma levels were still in the questionable range. Additional evaluation of the plasma level data showed that the areas under the curves for both plasma levels were approximately the same. It also can be noted that the 2.288 µmole dosage level in this experiment produced a similar plasma level to that observed from injection 2 in Table 4.

Multiple graded dosage levels of HN2

An experimental plan of giving each animal 4 injections of HN2 at the same dosage level, one week apart, was designed. The plasma levels were monitored for twenty minutes with blood samples being obtained at 0 time and 1, 2, 5, 10 and 20 minutes following the administration of the HN2. Four dosage levels of HN2 were selected: 0.286, 0.572, 1.144 and 2.288 μmoles per kg. per week. The results of the initial injection of each dosage level are presented in Table 6.

Table 6. Duration of plasma levels of HN2 (μg./ml.) following the initial injections of 4 different dosage levels

Dosage Level μmoles/kg.	0 min.	1 min.	2 min.	5 min.	10 min.	20 min.
0.286	0.00	0.30	0.00	0.00	0.30	0.00
0.572	0.00	0.45	0.45	0.23	0.28	0.28
1.144	0.00	0.10	0.00	0.00	0.00	0.00
2.288	0.00	0.00	0.03	0.08	0.00	0.00

All plasma levels detected following the initial injections of the HN2 were less than 0.5 μg. per ml. of plasma. The plasma level persisted for 20 minutes only with the dosage level of 0.572 μmoles per kg. Plasma levels were detected for all dosage levels following the initial administration. There were no apparent relationships between the dosage levels and the detected plasma levels since the lower dosage levels produced the higher plasma levels.

The findings from the initial administrations of HN2 did not hold with the remaining injections of HN2. Table 7 illustrates a summary of the plasma levels detected following each administration of each dosage level of HN2 in the multiple dose experiment.

Table 7. Occurrence of detectible plasma levels of HN2 following 4 administrations of different dosage levels, one week apart

Dosage Level μmoles/kg.	Injection Number 1	2	3	4
0.286	+	-	-	-
0.572	+	-	-	-
1.144	+	+	-	+
2.288	+	+	+	+

No detectible plasma levels were obtained following the remaining three injections of the two lower dosage levels of HN2 or the third injection of the 1.144 μmole dosage level. The reason for the failure to detect these subsequent plasma levels is not known. It can be theorized at least with the two lower dosage levels that the HN2 sensitized the calf's system in some manner which enabled it to clear the plasma much more rapidly than following the initial injections.

Figures 1 and 2 indicate the actual plasma levels detected in the calves receiving the two higher dosage levels of HN2 over the 4 week period. The areas under the curves are also presented on these graphic presentations. The detected plasma levels produced by these two dosage levels give the appearance of being graded and dose related.

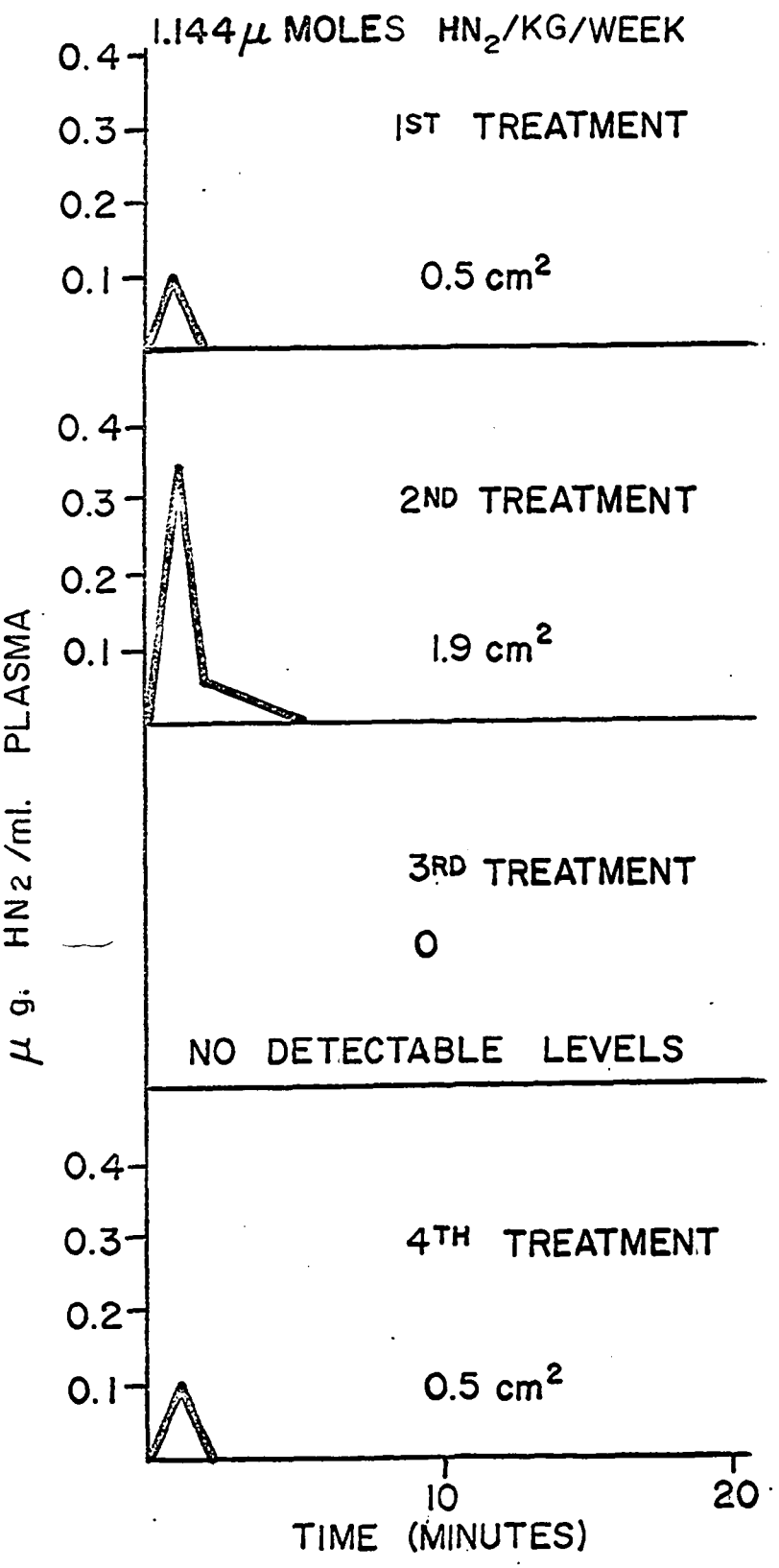


Figure 1. Duration of HN2 plasma levels in the calf

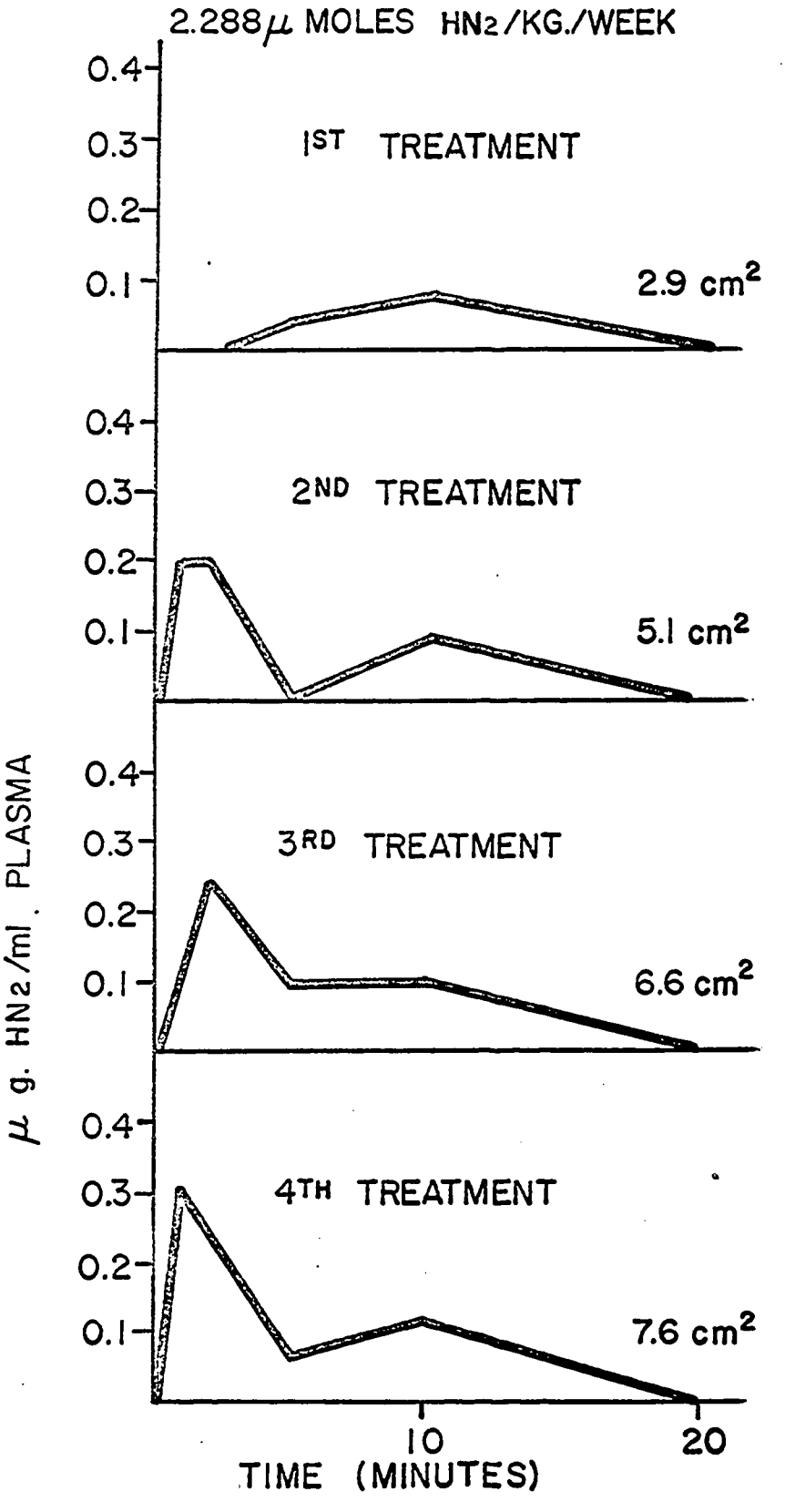


Figure 2. Duration of HN2 plasma levels in the calf

It is readily apparent from all of the experiments in which attempts were made to monitor the plasma levels of HN2 that there was a remarkable lack of consistency in the results. The plasma levels with the 1.144 and 2.288 μmole dosage levels gave indications of being graded, but overall, including the preliminary experiments with HN2, the lower dosage levels appeared to produce higher plasma levels.

It is of interest to note that there was one real consistency, the apparent ceiling of the peak plasma levels detected regardless of the dosage levels administered. This peak level, approximately 0.5-0.7 μg. per ml. of plasma, may be real because the analytical procedure had been shown to be able to detect levels up to twenty times this level in stock plasma. Preliminary experiments indicated that HN2 did not associate or adhere to the zinc hydroxide precipitant. The data presented in Table 2 indicate that the HN2 did not appear to associate with other plasma constituents or the plasma protein-zinc hydroxide precipitate. These findings suggest that if more HN2 had been present in the original blood than the level of 0.5-0.7 μg. per ml. detected in the plasma filtrate, it could have stayed with the erythrocytes following the harvesting of the plasma.

Responses of Calves to Graded Dosage Levels of Alkylating Agents

The major phase of the investigation was to observe the biological responses of calves to the alkylating agents, mechlorethamine·HCl (HN2) and uracil mustard (UM). Bailey (1) has previously reported that the time of occurrence of death of calves in response to graded daily dosage

levels of these compounds was quantitative. He also presented data that suggest that the calf is more sensitive to these compounds than other laboratory animals.

The present studies were undertaken to determine if changes in other measurable parameters: hematologic, serum proteins and tissues, could be found to be related to graded dosage levels of these compounds. In order to determine the presence or absence of detectible graded responses, a definite protocol was established. Graded dosage levels of the compounds were to be administered to calves for a predetermined experimental period following which the animals would be killed and necropsied.

It was decided to administer the graded dosage levels at 1, 7, 14 and 21 days. The animals would be killed on day 28 and the tissues would be examined. Four dosage levels of each compound would be chosen and there would be one calf for each dosage level. A preconditioning period would be utilized to establish the base-line parameters and to allow each animal to serve as its own control.

The physiological parameters measured two times a week in the calves during the preconditioning period and the experimental period were as follows: weight, temperature, total thrombocyte count, total erythrocyte count, total leukocyte count, differential leukocyte count, packed cell volume, hemoglobin, total serum proteins and albumin-globulin ratio. Temperature, erythrocyte, packed cell volume and hemoglobin data are not presented or discussed because no significant variations were observed in any of the experimental animals.

Preliminary experiments with mechlorethamine·HCl

The protocol calling for 4 weekly injections of HN2 indicated that preliminary probes must be undertaken to determine a range of possible dosage levels for this compound in calves. The preliminary studies were undertaken to establish dosage levels of HN2 which would produce detectible responses in calves following a single injection.

The dosage levels of HN2 chosen for the preliminary studies were 2.288 and 4.576 μmoles per kg. These dosage levels were approximately the dosage levels which had been reported to produce tissue damage in dogs (45). Each dosage level was given to a separate calf and the responses of the calves were observed. The responses and results from this experiment are presented in Table 8.

The data show that calf # 3, which received 2.288 μmoles per kg., gained weight. Calf # 2, which received 4.576 μmoles per kg., did not live long enough to show much weight change.

Thrombocytopenia was not observed in the calves. It was of interest to note that the thrombocyte counts of calf # 3 dropped from 9.9×10^5 per $mm.^3$ to 6.5×10^5 per $mm.^3$ two days following the administration of the HN2 and did not regain the magnitude of the original count until day 20.

Leukopenia was observed in both calves. Calf # 3 developed a relative lymphocytosis up to 88 percent on day 7 and this was during a lymphopenic stage. The calf recovered from the leukopenia and the leukocyte counts remained within the normal range for the remainder of the experiment.

The serum proteins were monitored only in the calf receiving 2.288 μmoles of HN2 per kg. There was a fluctuation in the total protein levels

Table 8. Responses of calves to single doses of mechlorethamine·HCl

Calf No.	3	2[a]
Dosage Data		
Amt./kg./wk. (µg.)	440	880
(µmoles)	2.288	4.576
Total Compound Given (mg.)	33.0	50.8
(µmoles)	171.6	264.2
Number of Treatments	1	1
Weight Data	Gain	Stable
Initial (kg.)	74.9	57.7
Maximum	84.4 (27)	57.7
Final	84.4	57.7
Thrombocyte counts		
Maximum (X 10^5/mm.^{3})	9.9 (-2,20)	4.5 (4)
Minimum	6.5 (2)	.....
Leukocyte Counts		
Maximum (/mm.^{3})	8,500 (13)	1,150 (4)
Minimum	2,150 (2)	.....
Serum Proteins	Stable	.....
Maximum (gm. %)	4.9 (2)	.....
Minimum	4.1 (7)	.....
Diarrhea	Yes (5-6)	Yes (2)
Death	Killed (35)	Died (5)
Gross Lesions	No	Yes[b]
Lymphoid Depletion	Yes	Yes

[a]Only one blood sample was obtained

()Indicates day of occurrence of event.

[b]Lesions not thought to be due to the compound.

and in the albumin-globulin ratios during the experimental period. No significance was attached to these fluctuations.

Diarrhea occurred in both calves. It was only transitory in calf # 3 but was very severe in calf # 2. The nature of the diarrhea of calf # 2 was very fetid, greenish-white in color and had very little solid matter present.

Death occurred in 5 days in the calf receiving 4.576 μmoles of HN2 per kg. Death could be attributed to an ionic imbalance due to the severe diarrhea and possibly due to an overwhelming secondary bacterial infection.

The calf which received 2.288 μmoles of HN2 per kg. was clinically healthy 35 days post treatment. The calf was killed by electrocution.

Gross lesions were observed only in calf # 2. These lesions were petechial hemorrhages on the epicardium and a few wide-spread patches of pneumonia in the lungs. The etiology of these lesions was thought to be due to a secondary bacterial infection resulting from the leukopenia and not primarily due to the HN2.

Lymphoid depletion was the major microscopic finding in both animals. The magnitude of the lymphoid depletion was much greater in the calf which received 4.576 μmoles of HN2 per kg. The calf which received 2.288 μmoles of HN2 per kg. showed small amounts of lymphoid depletion and may have been recovering from the effects of the HN2

It was apparent from these findings that the dosage level of HN2 of 4.576 μmoles per kg. was too high to be of any practical value for determining graded responses with the exception of the time of occurrence of

death. The apparent recovery of the calf receiving 2.288 μmoles of HN2 per kg. suggested that this dosage level could be satisfactory for the maximum dosage level in the graded dose-response studies.

Multiple dose studies with mechlorethamine·HCl

The dosage levels selected for these experiments were 0.286, 0.572, 1.144 and 2.288 μmoles of HN2 per kg. per week. The results of the graded multiple dose studies are presented in Table 9.

The weight data in Table 9 show that only calf # 8 which received the highest dosage level had a marked weight loss. Calf # 4 which received 0.572 μmoles of HN2 gained a small amount of weight and the weights of calves # 5 and # 7 remained somewhat stable but did decrease in the latter days of the experimental period. The results indicated that the HN2 was having an effect upon the growth of the calves.

The thrombocyte counts did decrease in all calves but the counts did not approach thrombocytopenic levels at any time. These findings are in contrast to the reported responses of calves to graded daily dosage levels of HN2 with which thrombocytopenic levels did occur (1).

The leukocyte counts gave the first suggestions of a marked response of the calves to HN2. The degree of leukopenia which was observed appeared to be graded with increases in dosage levels. The calves receiving the two highest dosage levels had relative lymphocyte counts above 85 percent.

The serum proteins fluctuated during the experiment and slight decreases were noted. The albumin-globulin ratios also fluctuated during the experimental period. There were no indications of graded responses of the serum proteins.

Table 9. Responses of calves to graded multiple doses of mechlorethamine·HCl

Calf No.	5	4	7	8
Dosage Data				
Amt./kg./wk. (µg.)	55	110	220	440
(µmoles)	0.286	0.572	1.144	2.288
Total Cpd. Given (mg.)	9.925	28.55	44.6	66.4
(µmoles)	51.61	148.46	231.92	345.28
No. of Treatments	4	4	4	4
Weight Data	Stable	Gain	Stable	Loss
Initial (kg.)	43.6	61.7	48.12	39.5
Maximum	45.4 (6)	69.9 (27)	52.66 (7)	39.5 (1)
Final	44.9	69.9	49.9	35.4
Thrombocyte Counts				
Maximum (X 10^5/mm.^{3})	9.3 (6)	8.3 (8)	10.0 (1)	9.45 (8)
minimum	6.3 (16)	5.0 (28)	5.1 (18)	6.8 (22)
Leukocyte Counts				
Maximum (/mm.^{3})	10,450 (2)	9,800 (1)	6,250 (-3)	6,850 (-3)
Minimum	4,750 (23)	3,650 (15)	1,900 (25)	1,300 (22)
Serum Proteins	Decrease	Decrease	Decrease	Decrease
Maximum (gm. %)	5.4 (2)	5.0 (1)	4.8 (1)	6.2 (2)
Minimum	4.5 (23)	4.1 (17)	4.0 (15)	4.5 (22)
Diarrhea	No	No	No	Yes (10)
Death	Killed (28)	Killed (28)	Killed (28)	Died (27)
Gross Lesions	No	No	No	Yes[a]
Lymphoid Depletion	Yes	Yes	Yes	Yes

()Indicates day of occurrence of event.

[a]Lesions not thought to be due to the compound.

Diarrhea occurred only in calf # 8 which received the highest dosage level and it persisted until the death of the calf. This severe diarrhea could have produced an ionic imbalance.

The calf receiving 2.288 μmoles of HN2 per kg. per week was the only animal that died during the experimental period and the other calves were electrocuted. It is suggested that a severe ionic imbalance in addition to a secondary bacterial infection could have contributed to the cause of death.

At necropsy, gross lesions were observed only in the lungs of the calf receiving the highest dosage level. The pneumonia-like lesions were restricted to the anteroventral portions of the lungs. The lungs in these areas were dark red in color and had a firm consistency. Subsequent microscopic examination of the lesions showed that they were typical of a pneumonia. The alveoli in these areas were engorged with erythrocytes and had numerous septal cells, neutrophils and lymphocytes present.

Lymphoid depletion was a prominent finding in the thymus, spleen and lymph nodes of all four animals. There were indications of graded amounts of lymphoid depletion corresponding to the graded increases of the dosage levels. Detailed evaluation of these lymphoid tissues will be discussed in a later section.

The results of this graded multiple dose experiment with HN2 suggest that there were graded dose-responses in these calves treated with HN2. The responses observed in the leukocyte counts and especially in the lymphoid tissues were highly suggestive of a graded response to HN2.

Preliminary experiments with uracil mustard

A preliminary experiment was undertaken to establish a range of dosage levels for uracil mustard (UM). It had been previously shown that UM, on a molar basis, was approximately two times as toxic as HN2 to the calf (1). A dosage level for UM of 2.288 μmoles per kg. was selected. This level was equal to the highest dosage level in the multiple dose HN2 experiment and was one half of the highest HN2 dosage level used in the preliminary single dose HN2 experiments.

Dimethylacetamide (DMA) had been used in intravenous injections of UM to increase the solubility of the UM (1). An internal experiment was added to determine if the DMA could be used in the remaining experiments with UM. One calf was given the UM with sterile physiological saline (PSS) as the UM solvent and another calf was given the same dosage level of UM with DMA as the solvent. It was observed that both solutions were essentially suspensions and the DMA did not enhance the solubility of the UM. The results of this preliminary experiment are presented in Table 10.

Deaths occurred within 5 days in both calves and there was no real possibility for much weight change. The thrombocyte counts in both calves were similar and did not decrease to thrombocytopenic levels.

There was a wide variation in the leukocyte counts in these two calves. The calf which received UM with PSS as solvent developed a marked leukopenia following administration of the compound. The calf which received UM with DMA as the solvent developed a marked leukocytosis which was in direct contrast to the other calf. It appeared that the DMA

Table 10. Responses of calves to single doses of uracil mustard

Calf No.	10		11[a]	
Dosage Data				
Amt./kg./wk. (μg.)	575		575	
(μmoles)	2.288		2.288	
Total Compound Given (mg.)	25.1		27.7	
(μmoles)	99.0		110.2	
Number of Treatments	1		1	
Weight Data	Stable		Stable	
Initial (kg.)	43.6		48.1	
Maximum	43.6		48.1	
Final	43.6		48.1	
Thrombocyte Counts				
Maximum (X 10^5/mm.^{3})	7.7	(-5)	8.9	(-6)
Minimum	5.0	(1)	5.9	(4)
Leukocyte Counts				
Maximum (/mm.^{3})	6,200	(-8)	25,200	(4)
Minimum	1,700	(1)	7,150	(-9)
Serum Proteins	Stable		Stable	
Maximum (gm. %)	6.3	(1)	4.7	(-9)
Minimum	4.6	(-5)	4.5	(0)
Diarrhea	Yes	(1)	Yes	(1)
Death	Died	(4)	Died	(5)
Gross Lesions	Yes[b]		Yes[b]	
Lymphoid Depletion	Yes		Yes	

[a]Dimethylacetamide was used as the solvent for the UM.

()Indicates the day of occurrence of the event.

[b]Lesions not thought to be due to the compound.

could be the cause of the leukocytosis but this response of calves to DMA has not been reported. No explanation can be offered for this response.

The serum proteins did not show any apparent changes. Both the total proteins and the albumin-globulin ratios fluctuated wildly and did not show any definite trends.

Diarrhea occurred in both calves starting the day of administration of the UM. The diarrhea continued until the end of the experiment.

Death occurred in both calves in 4-5 days. The times of death were comparable to the time of occurrence of death with HN2 at 4.576 μmoles per kg. in the calf. This finding substantiates the reported finding that UM is approximately two times as toxic as HN2 to the calf.

The gross lesions which were observed in these two calves were similar to those observed in the calf which received 4.576 μmoles of HN2 per kg. These lesions were petechiation on the epicardium and patches of pneumonia in the lungs. These lesions were attributed to a possible secondary bacterial infection or a "toxemia" due to unknown causes. The UM was not thought to be the primary cause of the lesions.

The major microscopic lesions observed in these two calves were lymphoid depletion in the thymus, spleen and lymph nodes. There were no apparent differences in the magnitude of the lymphoid depletion in either calf. The amount of lymphoid depletion in these two calves appeared to be similar to that observed in the calf which received 4.576 μmoles of HN2 per kg., again suggesting a factor of 2 difference in the toxicity of UM over HN2. A detailed discussion and evaluation of these microscopic findings will be discussed in a later section.

The findings of this preliminary experiment with UM indicated that a dosage level of 2.288 μmoles per kg. was much too high for the proposed graded dosage level studies in calves. The time of occurrence of death and preliminary information from observing the magnitude of the lymphoid depletion suggested that UM was two times as toxic as HN2 to the calf.

Multiple dose studies with uracil mustard

The dosage levels chosen for the multiple dose studies with UM in calves were 0.143, 0.286, 0.572 and 1.144 μmoles per kg. per week. These dosage levels corresponded to one half the molarity of the dosage levels used in the multiple dosage studies with HN2. PSS was used as the UM solvent. The results of the multiple dose studies with UM appear in Table 11.

The only animal that gained weight was calf # 17 which received the lowest dosage level of UM. The calves receiving the two intermediate dosage levels of UM lost weight and calf # 16 which received the highest dosage level of UM had a stable weight throughout the 28 day period. These findings indicated that the compound was having an effect on the growth of the animals.

The thrombocyte counts of the calves receiving the two higher dosage levels of UM became low in the latter periods of the experiment and were at thrombocytopenic levels. The other two calves had decreases in the thrombocyte counts but the counts did not reach thrombocytopenic porportions. These findings are in contrast to the results of the HN2 experiments and could indicate that UM is more thrombocytopenic in its action than HN2.

Table 11. Responses of calves to graded multiple doses of uracil mustard

Calf No.	17	19	15	16
Dosage Data				
Amt./kg./wk. (µg.)	36	72	144	288
(µmoles)	0.143	0.286	0.572	1.144
Total Cpd. Given (mg.)	6.6	17.1	24.7	54.9
(µmoles)	26.26	67.86	98.54	218. 8
No. of Treatments	4	4	4	4
Weight Data	Gain	Loss	Loss	Stable
Initial (kg.)	44.5	60.4	44.0	47.7
Maximum	52.2 (27)	60.8 (13)	45.9 (20)	49.0 (6)
Final	52.2	47.7	37.7	47.7
Thrombocyte Counts				
Maximum (X 10^5/mm.^{3})	10.2 (-2)	12.1 (-3)	7.65 (4)	7.45 (9)
Minimum	6.0 (28)	5.65 (28)	1.35 (28)	2.20 (23)
Leukocyte Counts				
Maximum (/mm.^{3})	9,550 (0)	11,800 (-8)	9,650 (-5)	7,250 (-6)
Minimum	4,800 (19)	5,000 (7)	1,350 (24)	300 (23)
Serum Proteins	Stable	Stable	Decrease	Decrease
Maximum (gm. %)	5.5 (-5)	7.0 (21)	5.0 (-3)	5.9 (-6)
Minimum	4.8 (0)	5.2 (28)	4.1 (21)	4.2 (23)
Diarrhea	No	No	No	No
Death	Killed (28)	Killed (28)	Killed (28)	Died (28)
Gross Lesions	No	No	Yes[a]	Yes[a]
Lymphoid Depletion	Yes	Yes	Yes	Yes

()Indicates day of occurrence of event.

[a]Lesions not thought to be due to the compound.

The leukocyte counts in the calves treated with UM appeared to show a graded degree of leukopenia corresponding with the increasing dosage levels. The leukocyte counts were lower with the two higher dosage levels of UM than with the comparable higher dosage levels of HN2. A relative lymphocytosis of over 85 percent was observed during the leukopenic stages in both animals receiving the two higher levels of UM. The two lower dosage levels of UM did not produce as low leukocyte counts as did the comparable two lower levels of HN2. While the responses of the leukocytes appeared to be graded, it would be more appropriate to conclude that the responses of leukocytes are better qualitative criteria than quantitative.

The serum proteins and the albumin-globulin ratios fluctuated appreciably. The total proteins showed a decline but did not get below 4.0 gm. per 100 ml. of serum. There were no definite trends observed in the albumin-globulin ratios.

Diarrhea was not a problem in any calves treated with UM including the calf which received the highest dosage level. This finding was in contrast to the calf which received the highest level of HN2.

Death occurred only in calf # 16 which received the highest dosage level of UM. The other three calves were electrocuted at the end of the experiment. The time of occurrence of death in the calf receiving the highest dosage level of UM was comparable to the time of death of the calf receiving the 2.288 μmoles of HN2. This finding again suggests that UM is two times as toxic as HN2 to the calf.

Gross lesions were observed in the calves which received the two higher levels of UM. The calf which received 1.144 μmoles per kg. per week had multifocal areas of pneumonia in all lobes of the lungs and petechial hemorrhages on the epicardium. The calf which received 0.572 μmoles per kg. per week had pneumonia-like areas in a few lobes of the lungs and petechial hemorrhages on the serosal surfaces of the spleen and rumen. All of the lesions could be attributed to a "toxemia" due to a secondary bacterial infection and the UM did not appear to be the primary cause of the lesions. The lung lesions were confirmed as pneumonia by microscopic examination of the tissues.

Lymphoid depletion in the thymus, spleen and lymph nodes was the main microscopic finding. There appeared to be a graded amount of lymphoid depletion corresponding with the graded dosage levels of UM. Detailed discussion and examination of these lymphoid tissues will be presented in a later section.

The findings of this experiment with UM suggested quantitative responses as indicated by the degree of leukopenia and the graded occurrence of lymphoid depletion. These findings also appear to confirm previously reported results that on a molar basis UM is two times as toxic as HN2 to the calf.

Quantitative Dose-Response Relationships

The responses of the experimental calves which appeared to be quantitatively related to the graded dosage levels of HN2 and UM were the time of occurrence of death and the appearance of graded amounts of lymphoid

depletion. The clinical and hematologic findings in calves which received HN2 or UM gave suggestions of quantitative responses, but did not appear suitable for use in a biological assay system.

Time of death

Single injection studies with HN2 at 4.576 μmoles per kg. and UM at 2.288 μmoles per kg. showed that the time of occurrence of death in calves was the same in both instances. Figure 3 illustrates these findings. It is of interest to note that the dosage level of HN2 at 2.288 μmoles per kg. did not produce death in a calf during the experimental period. There was no apparent difference in the time of deaths of calves given UM with the two different solvents.

Figure 4 illustrates the times of occurrence of deaths of calves which received four treatments of HN2 or UM. Deaths occurred only with the dosage levels of 2.288 μmoles of HN2 per kg. per week and 1.143 μmoles of UM per kg. per week. The times of occurrence of the deaths in both calves were 27-28 days.

The times of occurrence of death in calves given single or multiple injections of HN2 or UM indicate that UM is twice as toxic as HN2 to the calf. These findings confirm previous reports with these compounds given to calves in graded daily dosage levels (1). The findings with the single dose, multiple weekly dose and daily dose regimens indicate that the time of occurrence of death in calves with HN2 and UM is quantitative and is a good assay criterion.

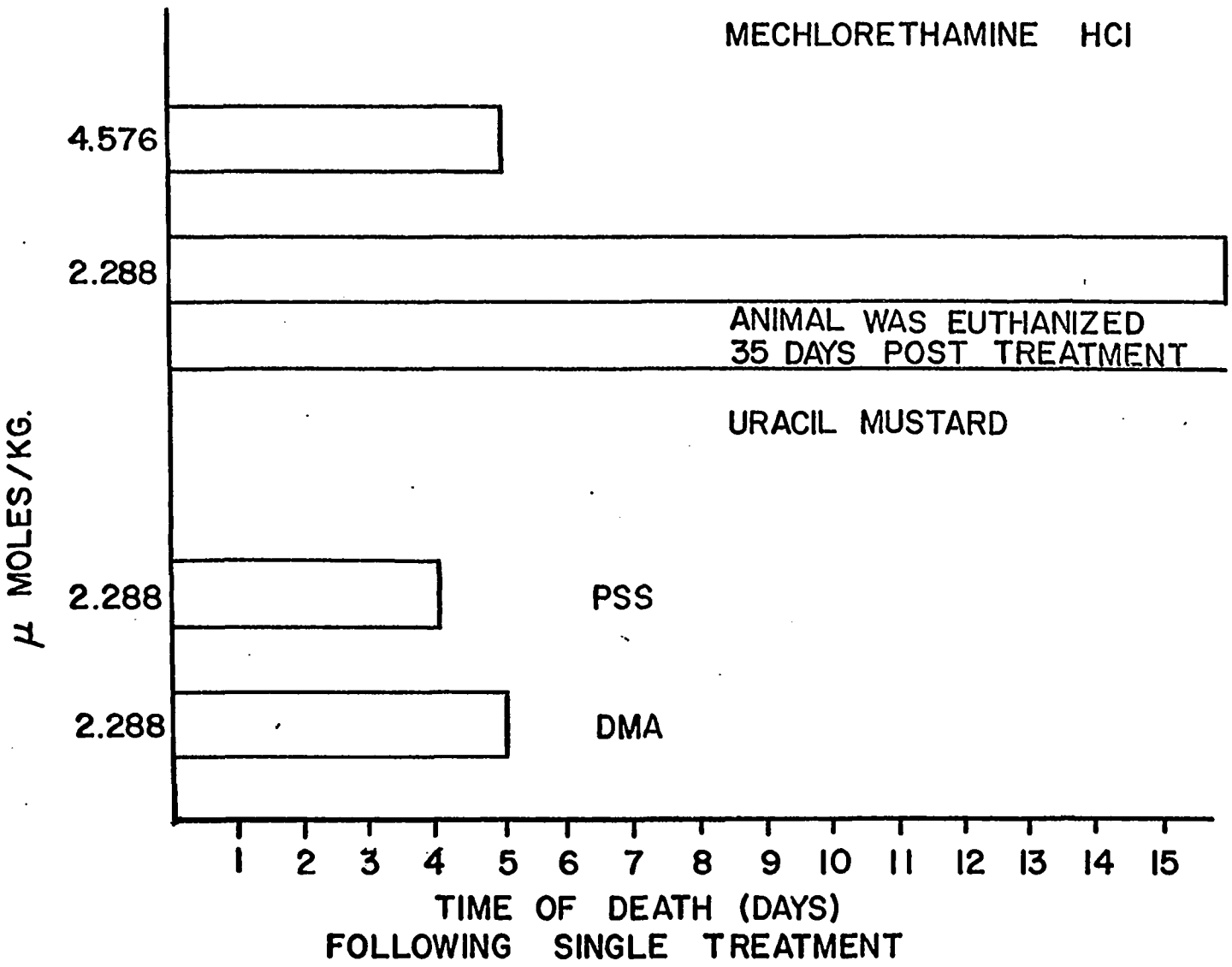


Figure 3. Time of death of calves following single treatments of HN2 or UM

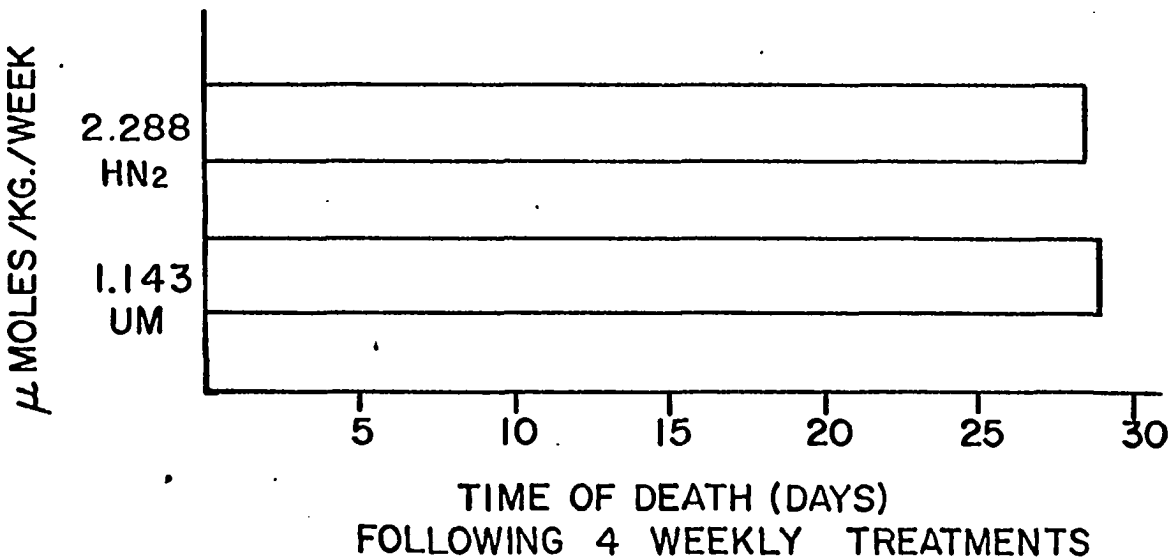


Figure 4. Time of deaths of calves following multiple treatments with HN2 or UM

Lymphoid depletion

The microscopic lesions in the thymus, lymph nodes and spleen of calves treated with HN2 or UM gave definite indications of a graded response. The changes in these tissues were lymphoid depletion with a reticulum-type cell infiltration. These changes varied from a decrease in lymphoid germinal centers to a loss of a clear cortico-medullary junction to an almost complete absence of any lymphoid tissue.

Figure 5 shows a photomicrograph of the right popliteal lymph node from calf # 2 which received a single injection of 4.576 μmoles of HN2 per kg. and Figure 6 shows a photomicrograph of a similar lymph node taken from calf # 10 which received a single injection of 2.288 μmoles of UM per kg. The sections show that lymphoid depletion of almost equal intensity occurred in both animals. The tissue changes in the lymph nodes consisted of almost complete loss of all germinal centers with reticulum-type cell infiltration. A similar lymph node from calf # 11 (not shown) which also received 2.288 μmoles of UM had a similar amount of lymphoid damage. The lymph node from calf # 3 which received 2.288 μmoles of HN2 per kg. (not shown) did not show the marked lymphoid depletion of the other calves.

The tissue changes observed in the spleens of the calves appeared to be much more severe than those observed in the lymph nodes. Figures 7 and 8 show photomicrographs of spleen sections from calves # 2 and # 10. These sections show that the germinal centers are absent or inactive. These sections also show a marked loss of cellular structures which is not observed in the lymph node tissue sections. This marked loss of

Figure 5. Right popliteal lymph node from calf # 2 which received a single injection of HN2 at 4.576 μmoles per kg. Marked lymphoid depletion is present with no active germinal centers and loss of the cortico-medullary junctions. The sinusoids are highly congested. (X 50)

Figure 6. Right popliteal lymph node from calf # 10 which received a single injection of UM in PSS at 2.288 μmoles per kg. Marked lymphoid depletion is present with no active germinal centers. Lymphocytes are present but decreased in number. The sinusoids are highly congested. (X 50)

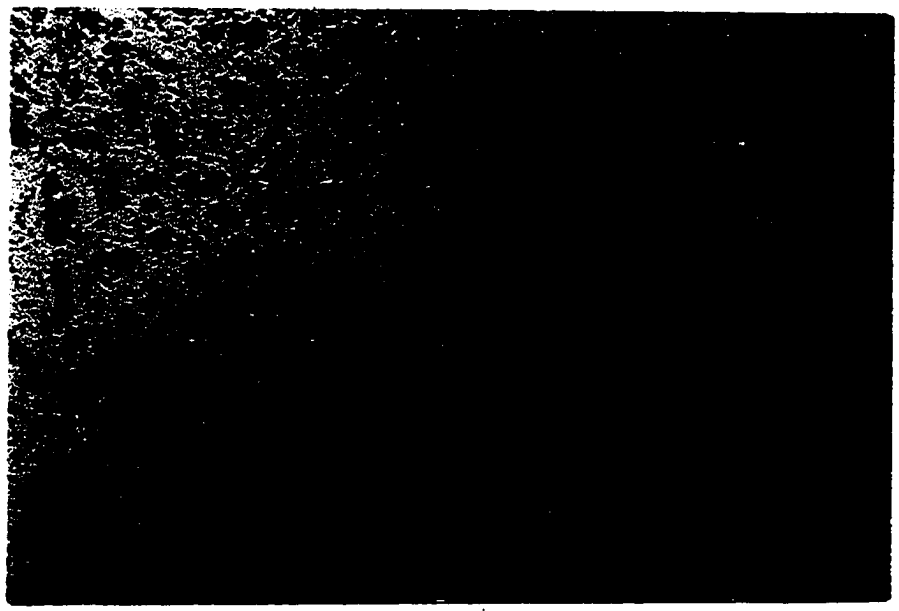
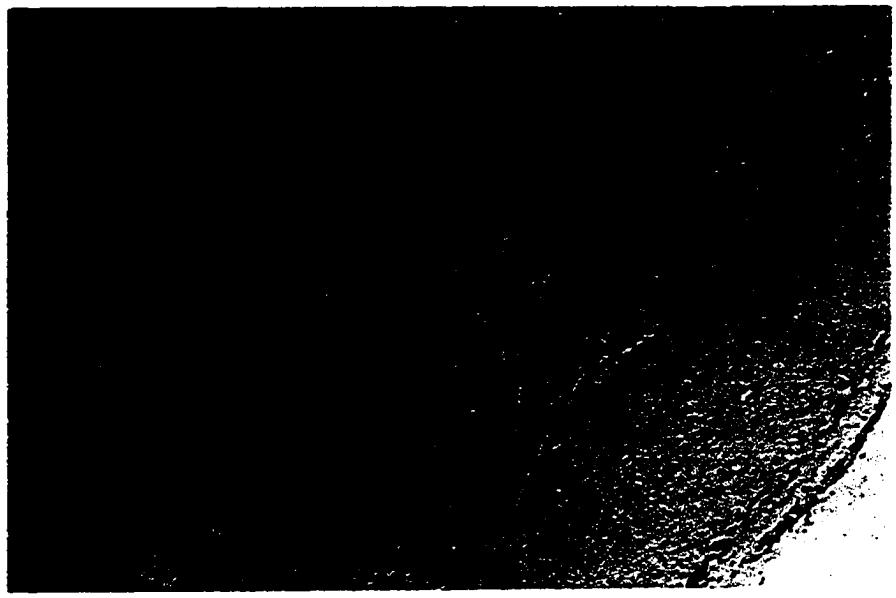
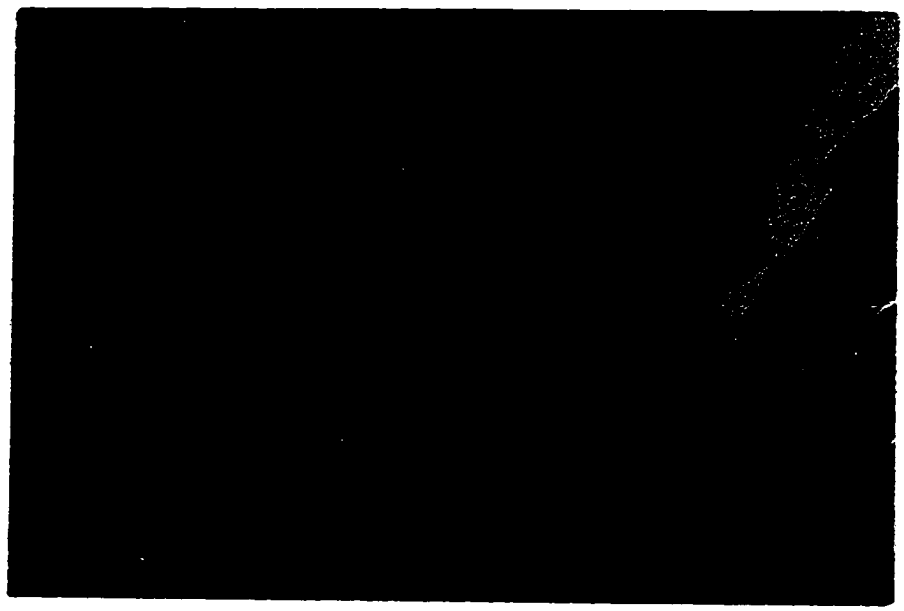


Figure 7. Spleen from calf # 2. There is complete loss of splenic tissue which accentuates the trabecula. The tissue is congested. (X 50)

Figure 8. Spleen from calf # 10. Marked lymphoid depletion is present with inactive germinal centers and a heavy infiltration of reticulum-type cells. The tissue is congested. (X 50)





cellular structures accentuates the splenic trabeculae and leaves congested sinusoids in a majority of each section. A similar amount of lymphoid depletion was observed in the spleen from calf # 11. The amount of lymphoid depletion in the spleen of calf # 3 was much less than in the other three animals.

The results indicate that HN2 and UM readily acted upon the lymphoid tissues. It was thought that the greater amount of lymphoid depletion observed in the spleen could have been due to the greater blood supply of the spleen as compared to the popliteal lymph node and hence would allow more compound to bathe the splenic tissues.

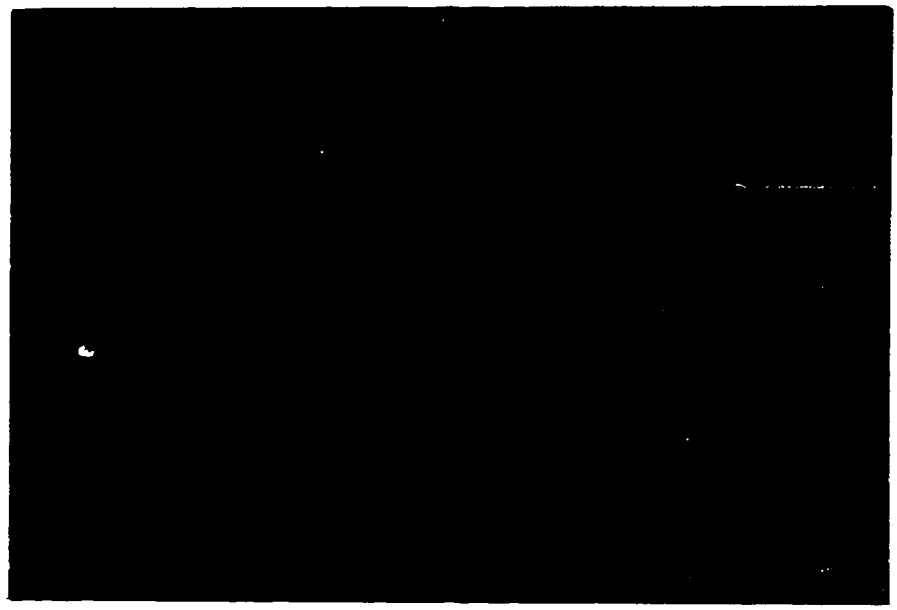
The appearance of almost equal amounts of lymphoid depletion in the respective tissues with these two compounds given in single injections at dosage levels differing by a factor of two, suggests that UM is approximately two times more toxic than HN2 to the calf. These findings are also suggestive of a quantitative response in lymphoid tissues. The results also show that lymphoid tissue is very labile and can respond in a short period of time to rather high dosage levels of these toxic agents.

The multiple administration of graded dosage levels of HN2 and corresponding one half molar dosage levels of UM produced graded amounts of lymphoid depletion in calves. The magnitude of the lymphoid depletion observed in the calves with corresponding dosage levels of HN2 and UM appeared similar.

Figures 9-14 show photomicrographs of histologic sections of the right popliteal lymph nodes of calves given the three higher graded dosage levels of HN2 or UM. The lymph node sections from the two calves which

Figure 9. Right popliteal lymph node from calf # 4 which received 0.572 μmoles of HN2 per kg. for 4 treatments. The tissue has lymphoid depletion but germinal centers are present. A cortico-medullary junction is present. (X 50)

Figure 10. Right popliteal lymph node from calf # 19 which received 0.286 μmoles of UM for 4 treatments. The tissue has large numbers of infiltrating reticulum-type cells but a cortico-medullary junction is visible. (X 50)



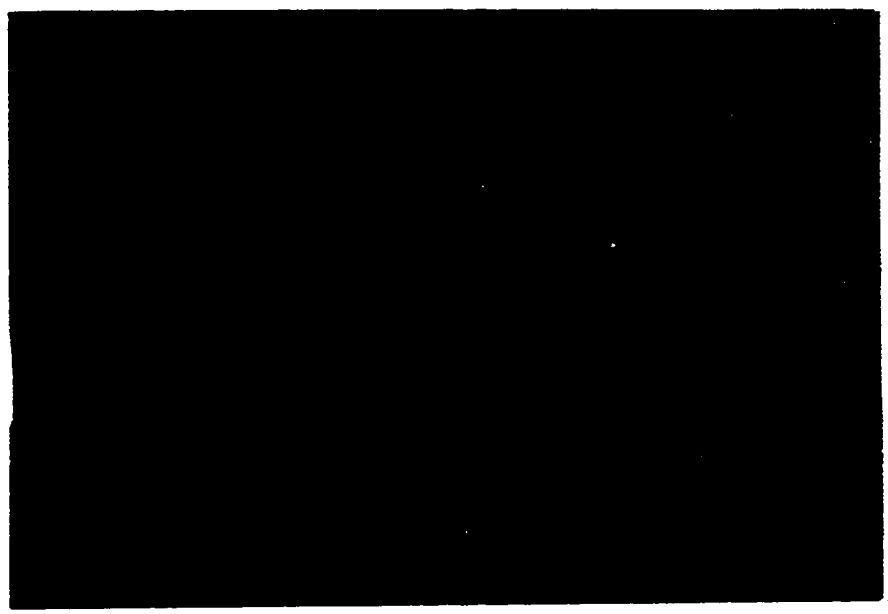
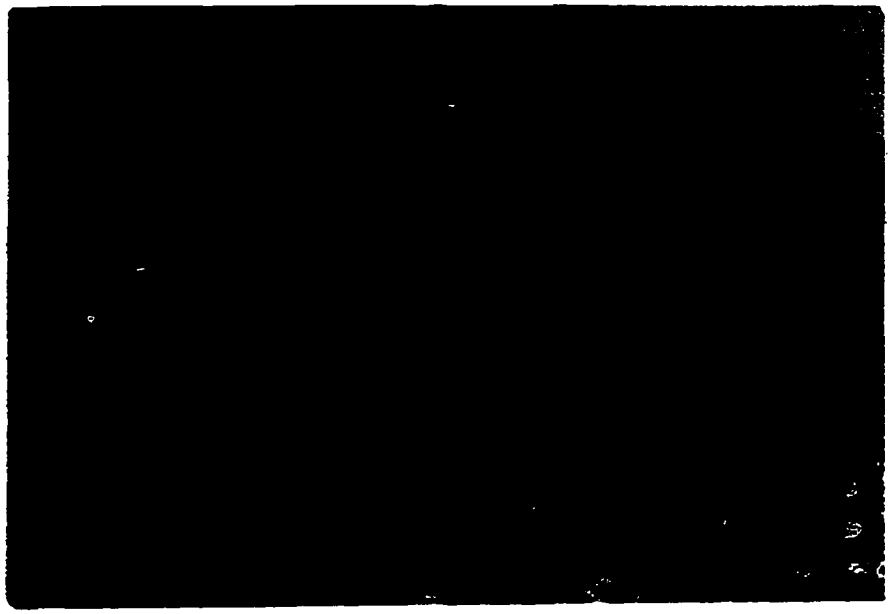


Figure 11. Right popliteal lymph node from calf # 7 which received 1.144 μmoles of HN2 per kg. for 4 treatments. Marked lymphoid depletion with loss of the cortico-medullary junction has occurred. Infiltration with reticulum-type cells has occurred. (X 50)

Figure 12. Right popliteal lymph node from calf # 15 which received 0.572 μmoles of UM per kg. for 4 treatments. Small amounts of lymphoid tissue are near the sub-capsular sinus and surrounding the trabecula but the majority of the section consists of reticulum-type cells. (X 50)



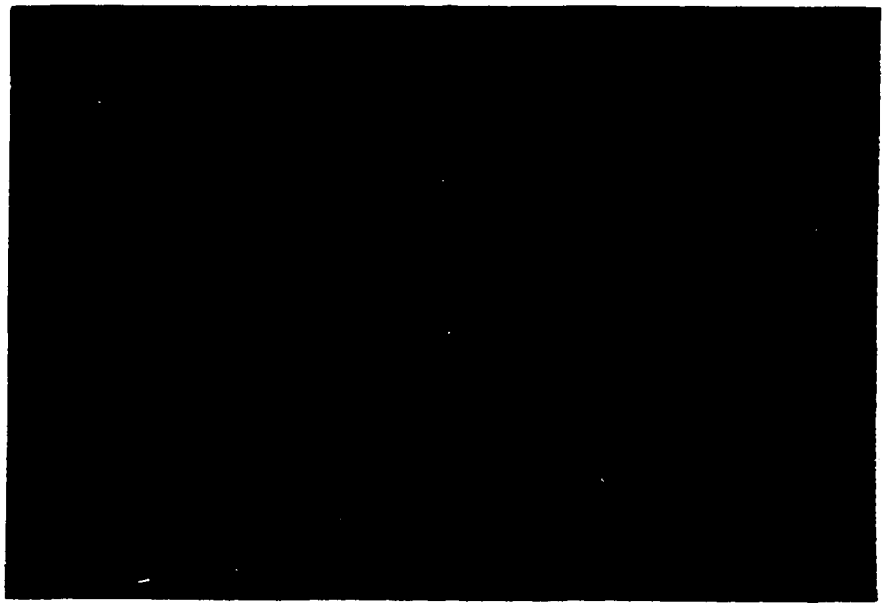
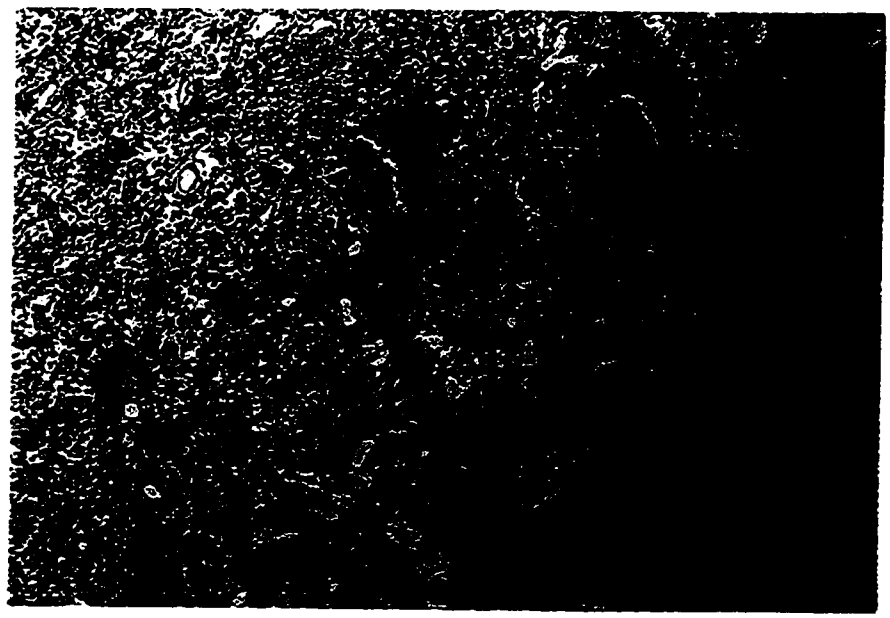
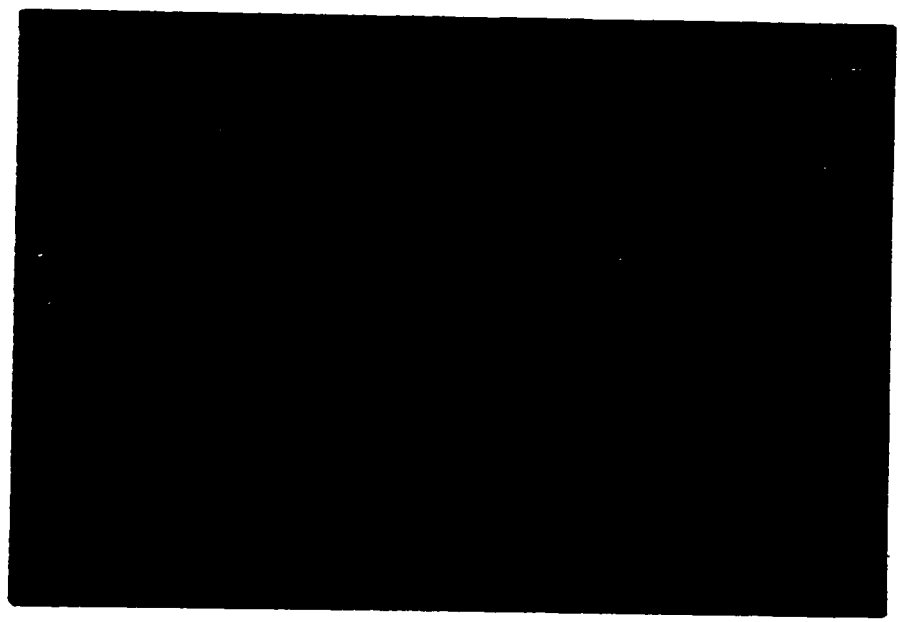


Figure 13. Right popliteal lymph node from calf # 8 which received 2.288 μmoles of HN2 per kg. for 4 treatments. A dense area of lymphocytes is adjacent to the sub-capsular sinus which gives the appearance of a cortico-medullary junction. There is engorgement of the sinusoids. (X 50)

Figure 14. Right popliteal lymph node from calf # 16 which received 1.144 μmoles of UM per kg. for 4 treatments. The tissue shows few lymphocytes dispersed in sheets of reticulum-type cells. The sinusoids are dilated and some congestion is present. (X 50)





received the lowest dosage levels of HN2 and UM, calves # 5 and # 17 (not shown) had small amounts of lymphoid depletion. The lymph node sections from the calves given the higher dosage levels of HN2 and UM show gradations of lymphoid depletion from a decrease in the number of germinal centers to a loss of the cortico-medullary junction to almost complete loss of the germinal centers.

The lymphoid depletion observed in the splenic tissues was more severe than that observed in the lymph nodes and was also of a graded nature. Figures 15-20 show photomicrographs of spleens from the experimental calves which received the three higher dosage levels of HN2 and UM. Calves # 5 and # 17 had very little tissue damage due to the lowest dosage levels of HN2 and UM. Here, as in the lymph nodes, there was a gradation of lymphoid depletion corresponding with the increasing dosage levels of HN2 and UM. The spleens from calves receiving the corresponding dosage levels of HN2 and UM appeared to show lymphoid depletion of an equal magnitude.

The tissue changes observed in the lymphoid tissues of calves given graded dosage levels of HN2 and UM indicated that this tissue was capable of showing a graded response to these two compounds. Graded tissue changes are difficult to classify, but the presence of graded amounts of lymphoid depletion in response to graded dosage levels of these two alkylating agents indicated that it does occur and can be detected.

The limited numbers of lymphoid tissues sectioned and examined in these studies indicated that more lymphoid tissues should be investigated.

Figure 15. Spleen from calf # 4 which received 0.572 μmoles of HN2 per kg. for 4 treatments. Some lymphoid depletion has occurred but several germinal centers are present. The parenchyma is composed of reticulum-type cells interspersed with lymphoid cells. The tissue is congested. (X 50)

Figure 16. Spleen from calf # 19 which received 0.286 μmoles of UM per kg. for 4 treatments. Few germinal centers are present. The majority of the section is composed of reticulum-type cells with a few interspersed lymphocytes. The tissue is congested. (X 50)

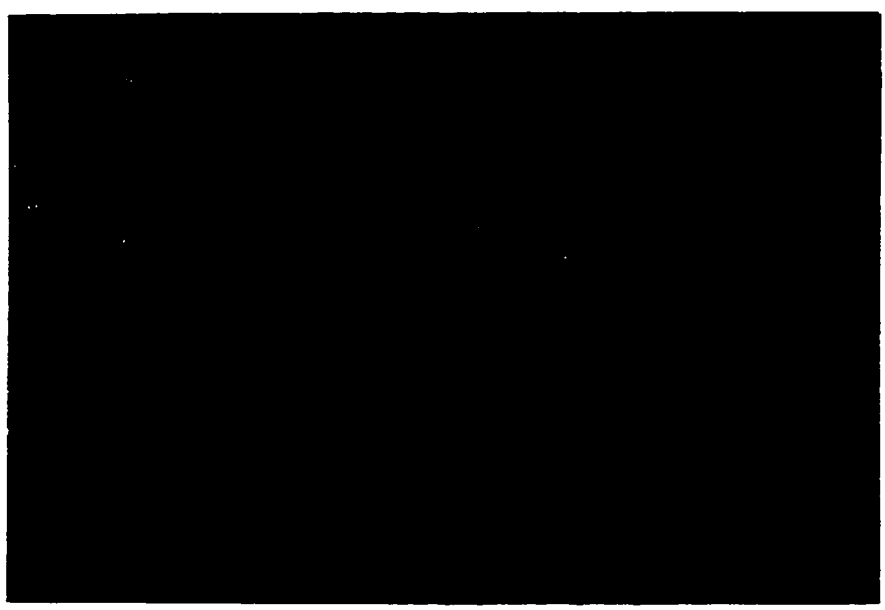


Figure 17. Spleen from calf # 7 which received 1.144 μmoles of HN2 per kg. for 4 treatments. Marked lymphoid depletion is present with inactive germinal centers surrounded by sheets of reticulum-type cells. The sinusoids are congested. (X 50)

Figure 18. Spleen from calf # 15 which received 0.572 μmoles of UM per kg. for 4 treatments. There is marked lymphoid depletion and the inactive germinal centers are surrounded by sheets of reticulum-type cells. The sinusoids are congested. (X 50)

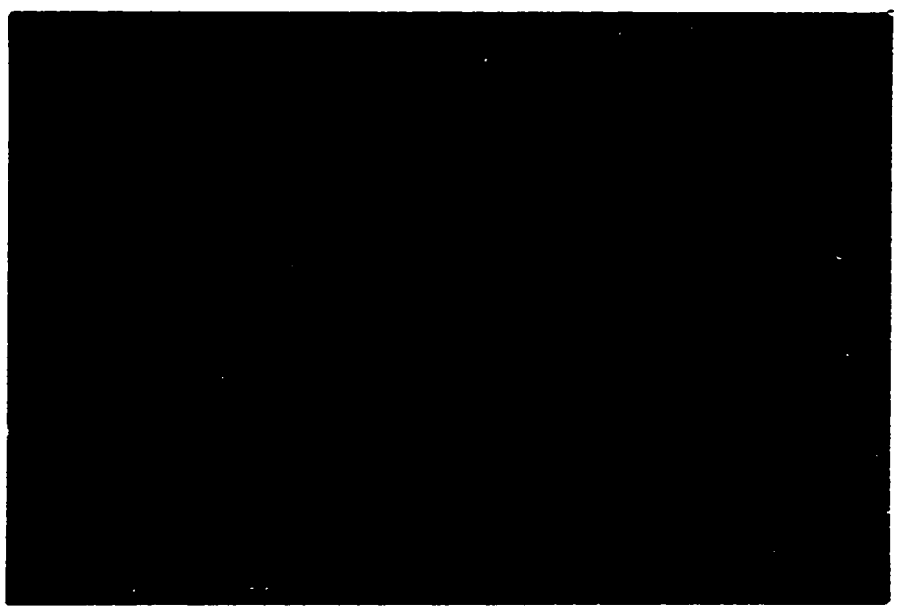


Figure 19. Spleen from calf # 8 which received 2.288 μmoles of HN2 per kg. for 4 treatments. Almost complete lymphoid depletion with loss of germinal centers has occurred. Few reticulum-type cells are present. The section is congested and the trabecula are accentuated. (X 50)

Figure 20. Spleen from calf # 16 which received 1.144 μmoles of UM per kg. for 4 treatments. Almost complete lymphoid depletion has occurred and no germinal centers are present. There are a few reticulum-type cells and lymphocytes interspersed among the engorged sinusoids. (X 50)

Examination of all of the regional lymph nodes and some of the thoracic and abdominal lymph nodes should give sufficient evidence to confirm these graded responses of calves to graded dosage levels of alkylating agents.

DISCUSSION

The ubiquity of the toxic properties of cancer chemotherapeutic agents has established a requirement for a more sensitive toxicity assay for these compounds. The undesirable activities of anticancer compounds have created the problem of treading the thin line which exists between death of the patient due to the chemotherapy or death due to the neoplastic growth.

A more sensitive assay system for the toxic properties of cancer chemotherapeutic agents could make it possible to engineer away from these toxic properties by the use of different isomers or analogues of presently used anticancer compounds. Conceivably, exploitation of this approach could lead to the detection and subsequent elimination of the toxic structures with retention of the anticancer properties. The use of this approach in conjunction with the elaborate assay systems for detecting antitumor activity could help to formulate better and safer cancer chemotherapeutic compounds.

The calf, for many years, has not been in the forefront as an experimental animal because of its size and the associated management problems. Recently, investigators in cardiovascular research have observed that the young calf, essentially a monogastric animal, is approximately the size of an average human and have begun to utilize this animal in surgical research in the cardiovascular area.

The calf assay system has been shown to be the most sensitive assay animal for the toxic properties of two alkylating agents, mechloreth-

amine·HCl and uracil mustard. This fact suggests that it could possibly be a more desirable assay animal than some of the more commonly used assay animals such as the rat, the mouse, the dog and the monkey.

The appearance and detection of graded amounts of lymphoid depletion in calves in response to graded dosage levels of the two alkylating agents in addition to the quantitative times of death of calves treated with these two agents, enhance the stature of the calf as a bioassay animal for these types of toxic compounds. Certainly, if the calf is a better assay animal for these toxic compounds, the management problems of the calf which are mysterious to many investigators, would become of little consequence to these uninitiated.

It is possible that the calf would not be accepted as a biological assay animal for the toxic properties of cancer chemotherapeutic agents. However, the fact that in the calf there was an apparent occurrence of graded amounts of lymphoid depletion due to graded amounts of the alkylating agents should suggest to investigators that they might re-evaluate the rat or mouse assay systems and study the lymphoid tissues more closely.

The graded responses of lymphoid tissues of calves suggest that an appropriate chemical assay method which resembles in principle the ovarian ascorbic acid depletion analytical procedure for lutenizing hormone could be developed for detecting the amounts of lymphoid tissue change. This chemical assay method might be developed in the highly sensitive calf and then could be incorporated into the rat or mouse assay systems for the advantages of greater numbers and economy.

It is readily apparent that further experimentation is necessary to delineate the modes of action of the toxic properties and the anti-cancer activities of cancer chemotherapeutic compounds in addition to the actual etiology(s) of neoplastic growths. Until adequate prophylactic measures are established against cancer, the use of the highly sensitive calf assay system could aid in the development of cancer chemotherapeutic agents with greater degrees of efficacy.

SUMMARY

1. An experimental study was designed to determine the nature of the responses of calves to graded dosage levels of the cancer chemotherapeutic agents, mechlorethamine·HCl and uracil mustard. Various clinical, hematologic and tissue responses were observed and measured in order to attempt to delineate the natures of these responses. Single injection and graded multiple dosage studies were undertaken for each compound. The dosage levels of uracil mustard corresponded to one half of the mechlorethamine·HCl dosage levels used, on a molar basis.

2. The qualitative responses observed in calves due to the effects of mechlorethamine.HCl and uracil mustard were decreases in the rate of weight gain, varying degrees of leukopenia, death and lymphoid depletion in several lymphoid tissues.

3. The quantitative responses observed in calves due to graded dosage levels of these alkylating agents were the length of the time intervals following the initiation of treatments until death and the graded increases of lymphoid depletion corresponding with the graded increasing dosage levels.

4. The responses observed in calves due to each dosage level of mechlorethamine.HCl were also observed with the corresponding one half molar dosage levels of uracil mustard indicating a factor of two difference in the toxicity of uracil mustard and mechlorethamine·HCl on a molar basis. These facts confirmed previously reported data.

5. Thrombocytopenia was observed only in the calves receiving the two highest dosage levels of uracil mustard in the multiple dosage studies.

6. A modified technique for determining alkylating agents using gamma-(4-nitrobenzyl)pyridine was developed for use in bovine plasma. The technique was applicable only for mechlorethamine·HCl.

7. Plasma levels of mechlorethamine·HCl were monitored in all calves which received treatments with this alkylating agent. No relationships were established between graded dosage levels of mechlorethamine·HCl administered to calves and the detected plasma levels of this compound.

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