CR-134161

EVALUATION OF POSSIBLE INTERACTION AMONG DRUGS CONTEMPLATED FOR USE DURING MANNED SPACE FLIGHTS

Progress Report For The Period
July 1972 To September 1973

prepared for

MANNED SPACECRAFT CENTER

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

HOUSTON, TEXAS

by

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TABLE OF CONTENTS

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Arthur D Little, Inc.

TABLE OF CONTENTS - CONTINUED

Arthur D Little Inc.

LIST OF TABLES

Arthur D Little Inc

Arthur D Little Inc

LIST OF FIGURES

Arthur D Little, Inc.

LIST OF FIGURES - CONTINUED

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FOREWORD

This program was undertaken to evaluate possible interactions among drugs contemplated for use during manned space flights. It was carried out during the period July 1, 1972 to September 30, 1973 and was the subject of our proposals dated March 10, 1972 and May 14, 1973, in response to RFP No. 9-13B32-79-2-185P and RFP No. 9-13B631-4-3177P. The program will continue until June 30, 1974.

Dr. Vernon Carter of the NASA Manned Spacecraft Center is Technical Officer for this agency. Dr. David W. Yesair, Dr. Francis J. Bullock (former Arthur D. Little, Inc. staff member), Dr. Philip S. Thayer, Mrs. Marianne Callahan, and Mr. James Braun comprise the Arthur D. Little, Inc., project team.

SUMMARY

Possible interactions among drugs contemplated for use during manned spaceflights have been studied in several animal species. The following seven drugs were investigated: nitrofurantoin, chloral hydrate, hexobarbital, phenobarbital, flurazepam, diphenoxylate, and phenazopyridine. Particular combinations included: chloral hydrate, hexobarbital or flurazepam with nitrofurantoin; phenobarbital or flurazepam with phenazopyridine; and diphenoxylate with two dose formulations of nitrofurantoin.

Specifically, rat liver microsomes were used to determine whether chronic dosage with nitrofurantoin would inhibit rates of oxidative drug metabolism. Rates of hexobarbital oxidation, N-demethylation of meperidine, and reduction of *p*-nitrobenzoic acid were studied in control and nitrofurantoin-treated rats. No difference in oxidative metabolic rates were found between control and nitrofurantoin-treated rats.

Pharmacological studies were carried out to determine whether premedication of rats with nitrofurantoin would result in potentiation of activity of hexobarbital, chloral hydrate, or flurazepam. This was determined for chloral hydrate and hexobarbital by studies of sleeping times determined by loss of the righting reflex. Flurazepam was investigated by use of the inclined screen test. No potentiation of activity was found between control and nitrofurantoin-treated animals.

Studies were carried out in several species to determine whether induction of liver microsomal enzymes would increase the tendency of phenazopyridine to produce methemoglobin *in vivo*. Animals were premedicated with phenobarbital, a known inducer of azoreductase, and in a separate experiment with flurazepam, before administration of phenazopyridine. Methemoglobin production was determined in each animal after receiving phenazopyridine. No evidence was found for increased production of methemoglobin in the rat, dog, or rabbit that could be attributed to increased amounts of microsomal enzymes.

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The final group of experiments was conducted to determine whether premedication of the dog with diphenoxylate would result in altered bioavailability of Furadantin® or Macrodantin®. The rate of drug excretion in the urine was determined and a plot of percent drug absorbed versus time was constructed for each dosage form, with and without diphenoxylate pretreatment. Drug in the urine was determined by both chemical and microbiological methods. Two doses of diphenoxylate were studied in four female beagles with each dog serving as its own pre-diphenoxylate control. Diphenoxylate was found to alter substantially the excretion pattern of both forms of nitrofurantoin, generally increasing total absorption.

On the basis of this work, the present contract was extended to include nitrofurantoin/diphenoxylate studies in man. The clinical study was carried out by Medical and Technical Research Associates, Inc., Needham, Massachusetts. Six subjects received both dosage forms of nitrofurantoin alone, and in combination with diphenoxylate. The results of these studies are inconclusive. The mechanism of action and an explanation of the interaction between diphenoxylate and nitrofurantoin still remains unclear. In man, the interaction does not appear to be significant, affecting only two subjects out of six and with only one dose formulation (Furadantin®).

I. INTRODUCTION

The general approach to the use of drugs by astronauts during space flights has been cautious, but in-flight medical problems requiring their use have occurred. With the advent of the Sky Lab program, the probability that astronauts may find it necessary to take one or more of the drugs carried in their medical kits greatly increases. As has been reviewed elsewhere,[1] results of epidemiological studies of adverse drug effects were of great consequence in bringing attention to drug interactions. Of particular importance, a direct relationship between the number of drugs used and the probability of adverse drug reactions clearly emerged from these studies. Unquestionably, use of drugs in combination carries with it an increased probability of an adverse drug effect.

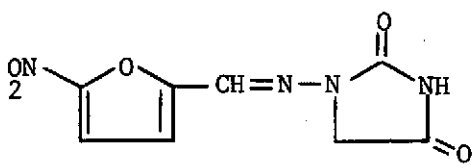
Adequate clinical data on risk/benefit ratios for particular drugs are just now beginning to emerge. Similar data for drugs used in combination are generally unavailable. The relative probabilities for therapeutic mishap are highest for antimicrobials, cardiac drugs, and hypnotics and sedatives. These classes are all heavily represented in the list of drugs now contemplated for possible use during manned space flight. With the Sky Lab program involving longer residence for astronauts in space, this project was concerned with the increased possibility that drugs would be taken in combination, with unknown effects.

II. BACKGROUND

A. Studies of Potential Interactions between Nitrofurantoin and Chloral Hydrate, Barbiturate, or Flurazepam *In Vitro* and *In Vivo*

Nitrofurantoin

Nitrofurantoin (I) is one of several nitrofuran derivatives possessing antibacterial activity. It is used exclusively as a urinary antiseptic.



I

Nitrofurantoin (Furadantin®, Macrodantin®), sulfisoxazole (Gantrisin®), and sulfamethoxazole (Gantanol®) are frequently considered to be therapeutic equivalents in the treatment of urinary tract infections.[2] A recent study determined specific reaction rates for adverse effects with these three drugs in hospitalized patients.[2] Where a urinary antiseptic was indicated, patients were treated with one of these three drugs on a random basis. For our purposes, it is significant that adverse reaction rates in this group were highest for nitrofurantoin - 5.1%. Incidence of adverse effects was dose dependent and dependent on the duration of treatment, suggesting a correlation with increased blood levels. Reaction frequently was manifest in nausea and vomiting. The higher incidence of reactions to nitrofurantoin, relative to sulfisoxazole suggests further study with it is appropriate.

A variety of enzymes are known to be inhibited by nitrofurans *in vitro*.[3,4] These enzymes are involved in redox reactions and include nitroreductase, glutathione reductase, and, of particular interest, aldehyde dehydrogenase.[5] Inhibition of such enzymic reactions is, in fact, thought to be intimately involved in the mechanism of action of these compounds.[5]

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Chloral Hydrate

The metabolism of chloral hydrate is reasonably well known in man.[6] The drug is rapidly converted, in a reaction catalyzed by alcohol dehydrogenase, to the believed active drug, trichloroethanol. Trichloroethanol is partially excreted in urine as its glucuronide, but is also slowly oxidized to trichloroacetic acid which slowly accumulates in plasma. This oxidation occurs largely in liver and kidney and is known to be NAD-dependent. Trichloroacetic acid is tightly bound to plasma protein and only slowly excreted.[7] The known metabolic fate of chloral hydrate is summarized in Figure 1. By analogy with the conversion of ethanol to acetate, the conversion of trichloroethanol to trichloroacetate likely involves both alcohol dehydrogenase and an aldehyde dehydrogenase.

In view of the above described inhibitory activity of nitrofurantoin on an aldehyde dehydrogenase, it seems to us not unreasonable that this drug may inhibit metabolism of trichloroethanol and thereby prolong its action.

Barbiturate

It has also been reported in a rather obscure paper[8] that nitrofurantoin (100 or 200 mg/kg) potentiates the sleeping time produced by hexobarbital in the rat. The mechanism of this potentiation is unknown, but it again seems not unreasonable that inhibition of oxidative metabolism of the barbiturate may be involved. As the average daily oral dose of nitrofurantoin in humans is 5-10 mg/kg, the dose used in the above study may be unrealistically high. Using dosage conversion factors suggested by Freireich *et al.*,[9] a dose for the rat, which is comparable to 5-10 mg/kg in the human, should be in the range 35-70 mg/kg, not the 100-200 mg/kg reportedly used. Freireich's conversion factors are used for estimating dosage equivalency on the basis of mg/m^2 of body surface area, rather than body weight.

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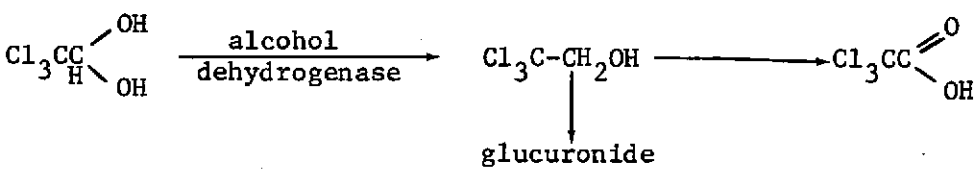
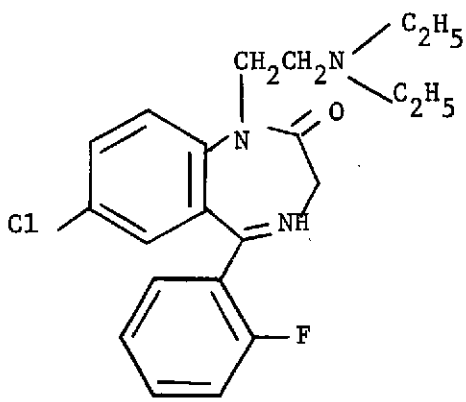


FIGURE 1

Metabolism of chloral hydrate

Flurazepam

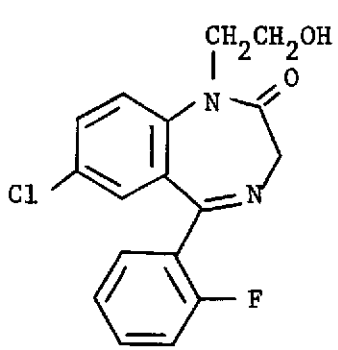
The rationale for including flurazepam (II) here is, in part, merely to complete this set of experiments with the third operational sedative drug. Flurazepam is a relatively new drug. These experiments



II

should help shed some light on the possible mechanisms involved in the drug's action and metabolism. The route of metabolism of nitrofurantoin is unknown so that the possibility of chronic dosing with nitrofurantoin, affecting the way rats responded to flurazepam, was speculative.

The metabolism of flurazepam has only recently been worked out in detail.[10] The major metabolite is III in man and IV in dog, and metabolism is very rapid.



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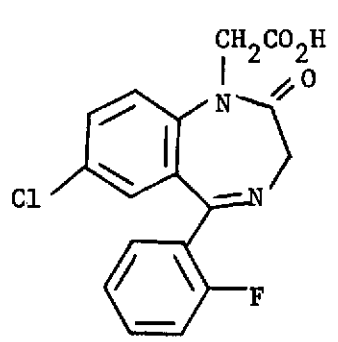

IV

A likely scheme accounting for these metabolites is shown in Figure 2.

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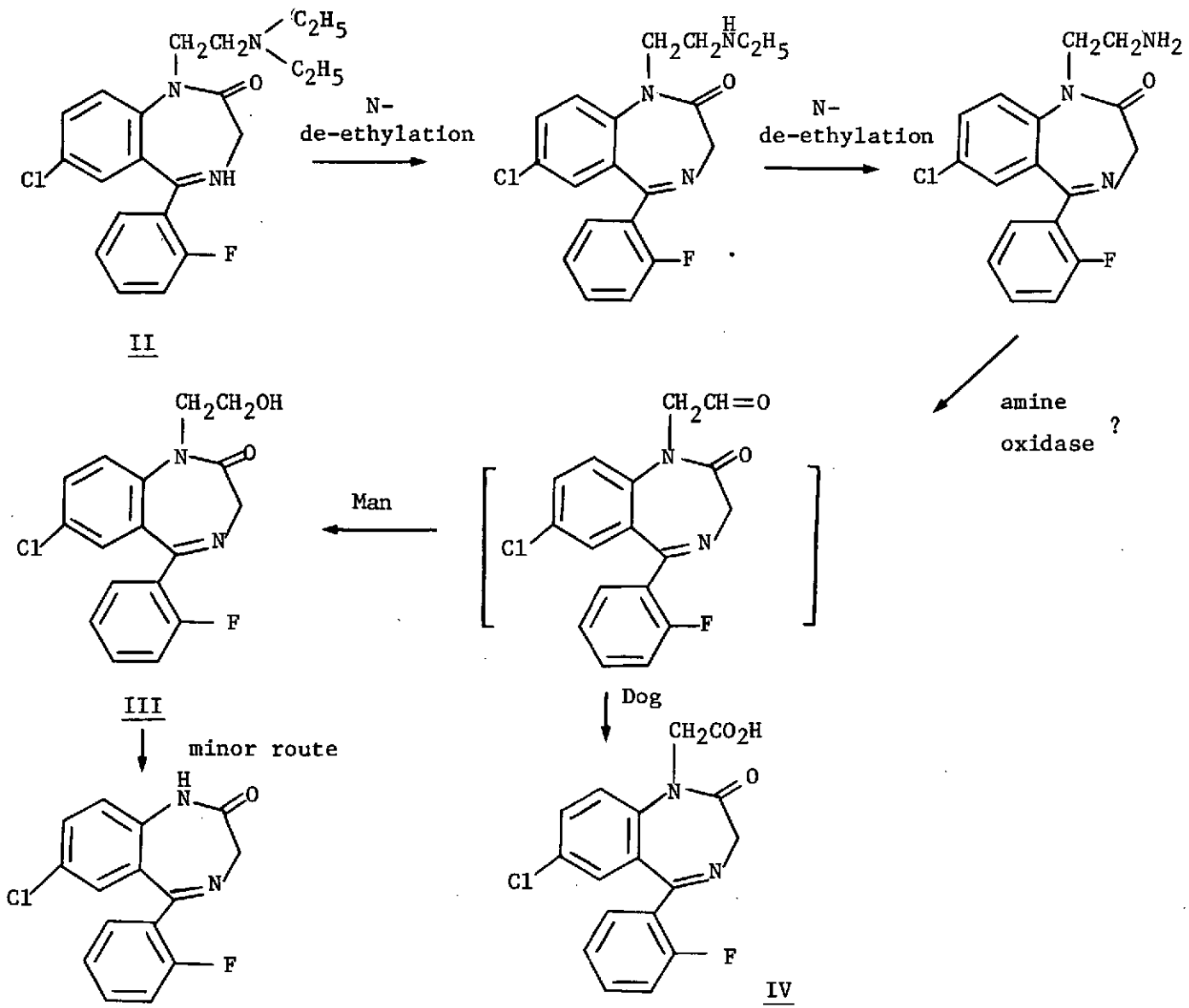

FIGURE 2

Proposed metabolism of flurazepam

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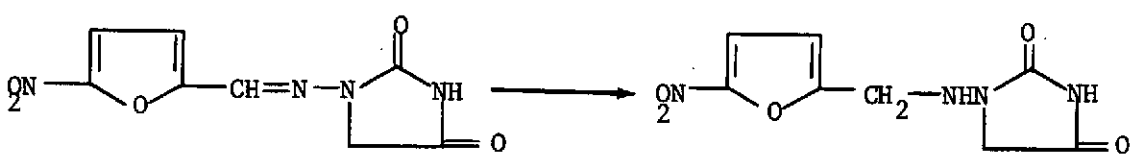
If one assumes an N-dealkylation, then invokes an amine oxidase catalyzed conversion to a hypothetical aldehyde, as shown, one can account for the major metabolites of flurazepam in both species.

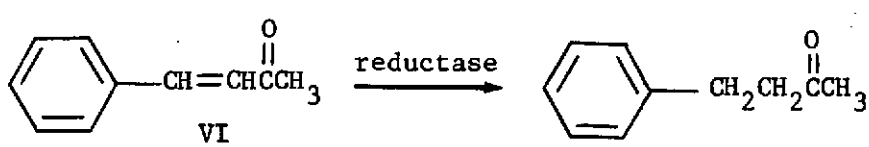
The route of metabolism of nitrofurantoin is unknown, but a variety of mammalian tissues are known to metabolize nitrofurans.[11,12] Metabolism appears to involve a reductive transformation, but the drug is not metabolized *in vitro* by nitro reductase from guinea pig microsomes.[4] If reduction of nitro is excluded, there are few alternative reductive transformations possible with this molecule.

We do not know a precedent for enzymic reduction of a Schiff base by microsomes. One alternative to nitro group reduction is indicated below.



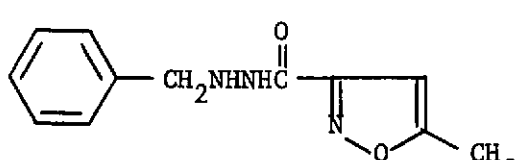
V

Remotely related may be the NADPH-dependent reduction of VI by a reductase known to be present in dog erythrocytes.[13]



VI

What would be of interest about the highly speculative transformation of nitrofurantoin to V is that the product (V) is closely related to an acyl hydrazide. Many acyl hydrazides, for example, isocarboxazid, (Marplan®, VII) are potent inhibitors of monoamine oxidase, an enzyme which may be involved in the metabolism of flurazepam.



VII

To determine whether nitrofurantoin can potentiate the action of chloral hydrate, to determine if the reported potentiation of barbiturate action by nitrofurantoin occurs at more reasonable doses, and to study the potential interaction between nitrofurantoin and flurazepam, we carried out the following.

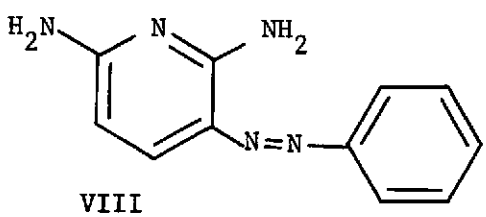
(a) Male rats (100-150 g) were given daily oral doses of nitrofurantoin (72 mg/kg) for periods of up to 7 days in three equally divided doses. Nitrofurantoin can be expected to be used on such a chronic dosage schedule. Sleeping times produced by i.p. dosage of chloral hydrate and hexobarbital were determined and compared with control groups not dosed with nitrofurantoin. Since flurazepam did not produce a "sleeping time" as it is usually defined, the inclined screen test was used to evaluate the potential interaction between nitrofurantoin and flurazepam.

(b) To determine whether chronic dosage of nitrofurantoin can result in inhibition of oxidative hepatic drug metabolism, the following experiments were carried out. On the seventh day of the above described dosage regimen, we characterized the barbiturate oxidase and meperidine N-demethylase of rat liver microsomes from the nitrofurantoin and control groups of rats. We proposed to use meperidine rather than flurazepam itself for probing N-dealkylation *in vitro* since acetaldehyde, one product of N-de-ethylation of flurazepam, is not as readily determined as formaldehyde. To complete the profile, we determined microsomal nitroreductase and cytochromes P-450 and b_5 after treatment of animals with nitrofurantoin.

While there is not now any evidence that nitrofurantoin or nitrofurans can act as enzyme inducers the design of this experiment additionally provided information on this point. The idea that an enzyme inhibitor can also be an inducer of biosynthesis of the enzyme is not new. 7,8-Benzoflavone is both an inducer and an inhibitor *in vitro* of benzpyrene hydroxylase in the mouse.[14]

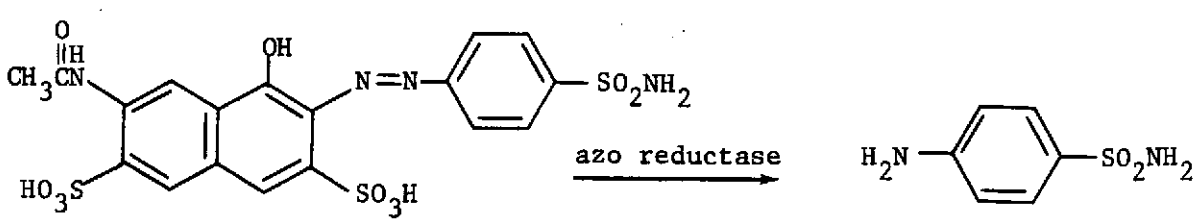
B. Studies of Potential Interactions of Enzyme Inducers with Phenazopyridine.

Phenazopyridine (VIII) is an old drug, developed in the 1930's as a urinary antiseptic. It is now used as a urinary tract anesthetic, either alone (Pyridium®, Warner-Chilcott Laboratories) or in combination with sulfonamides [as in Azo-Gantrisin®, Thiosylfil-A® (Lederle Laboratories), Azo Gantanol® (Roche Laboratories), Azo Kynex® (Lederle Laboratories)]. Methemoglobinemia is frequently produced by the drug;[15] more rarely, it may produce hemolytic anemia.[16,17] Accidental overdosage with phenazopyridine has led to production of large amounts of methemoglobin and cyanosis.[18] A dose of 200 mg/kg, admittedly quite high, produced anemia in the dog.[19]



VIII

The metabolism of phenazopyridine has apparently never been studied, but there is ample analogy for the suggestion that it will be metabolized by azoreductase. For example, the transformations of neoprontosil, salicylazosulfapyridine (Azulfidine®) and azobenzene indicated below are particularly well known.



Neoprontosil

Sulfanilamide

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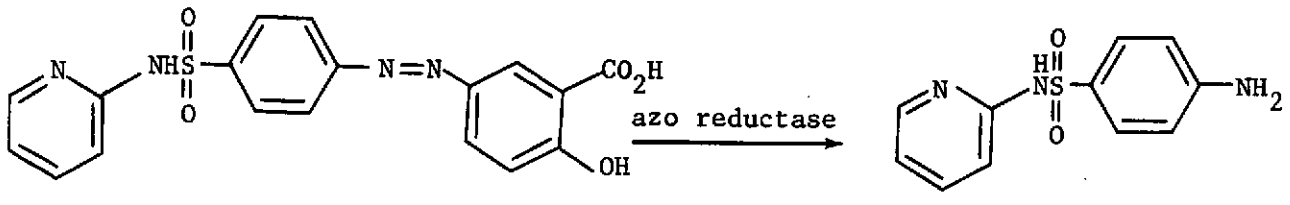
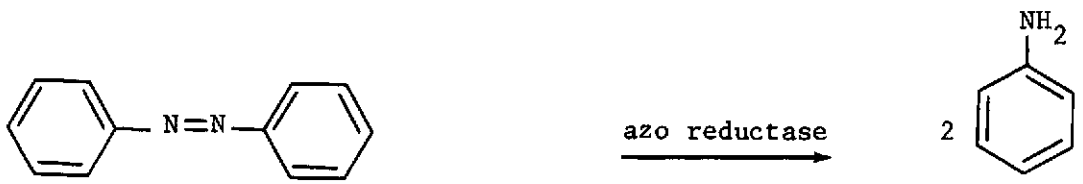


Salicylazosulfapyridine

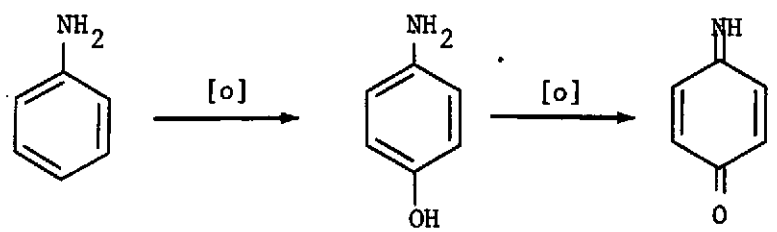
Sulfapyridine

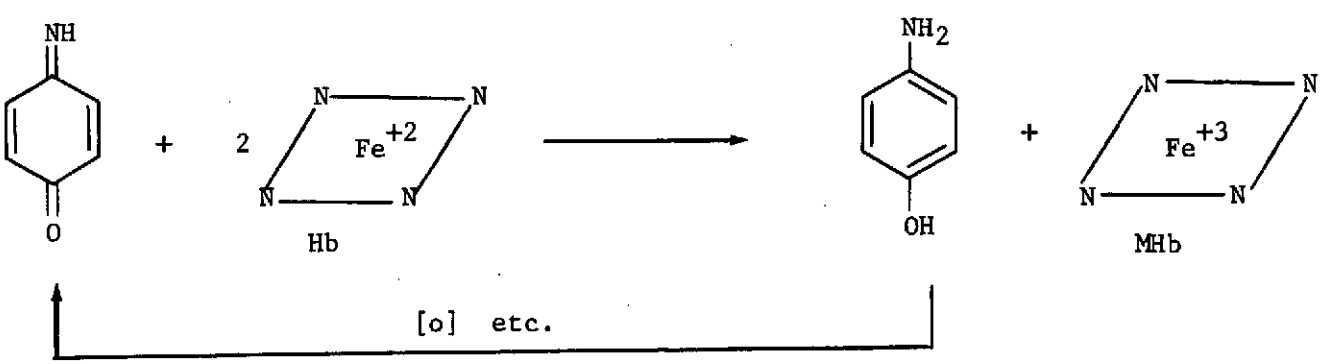


Azobenzene

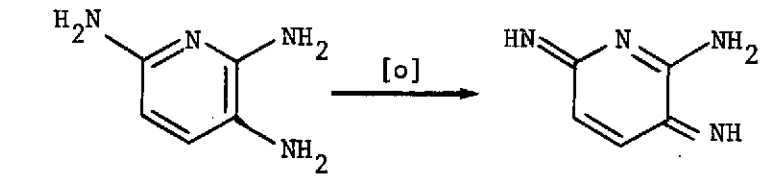
Aniline

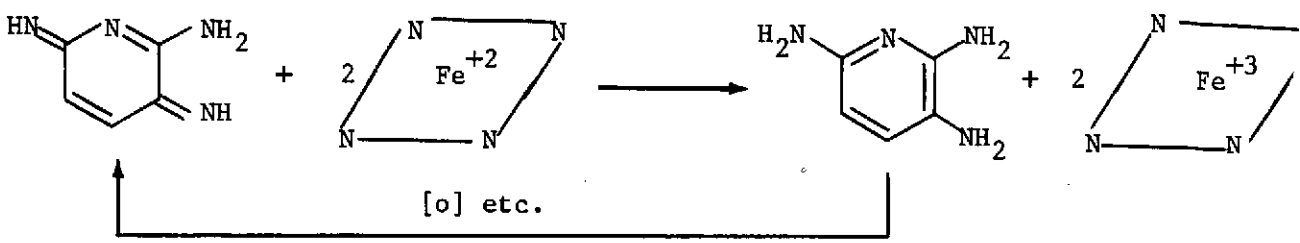
While phenazopyridine apparently can, by itself, produce methemoglobin *in vitro*[16] the products of reduction of the azo linkage should be much more potent in producing methemoglobin, as illustrated below. Aniline is one of the most potent methemoglobin producers known.[20] It requires metabolic activation for this action, the active agent apparently being the quinone imine.[20] The process is detailed below.





A similar cycle can be constructed for 2,3,6-triaminopyridine, the other product of reduction of phenazopyridine, as also shown.





Production of some methemoglobin by a drug is ordinarily not an important clinical problem, but impairment of any of the oxygen transport capacity of individuals subject to extreme physical exertion may be another matter. Azo reductase is an inducible enzyme and our interest here is whether the presence of enzyme inducers will tend to increase formation of methemoglobin by phenazopyridine. We proposed the following but due to lack of any interaction, we deleted several aspects of this program.

Phenobarbital

This drug is already known to induce azoreductase in the rat[21] and other species including humans. The older literature[20] suggests the cat for work with methemoglobin formers. In our experience, healthy cats are not easy to procure for experimental purposes and are a favorite species of the antivivisectionists. Accordingly, we proposed to use the rabbit and the rat for this study. Liver azoreductase has been demonstrated in both species.[22] Upon subsequent evaluation of these species as methemoglobin formers, we found they were poor and used the dog as the species of choice for this work.

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Azoreductase was induced by daily administration of phenobarbital for not less than three days. Phenazopyridine was then administered to control and phenobarbital-treated animals. Periodically, blood samples were taken and methemoglobin determined. As a measure of change in overt toxicity of phenazopyridine as a result of premedication with phenobarbital, LD_{50} for phenazopyridine in the rat were planned in control and barbiturate-treated animals, but not completed.

Flurazepam

Induction of drug metabolizing enzymes by flurazepam has not been reported, but flurazepam is still a relatively new drug. The closely related drugs, diazepam and chlordiazepoxide, will induce microsomal enzymes in the rat.[23] The particular enzymes shown to be inducible in this study[23] were an O-demethylase (substrate = *p*-nitroanisole), an aryl hydroxylase (substrate = aniline), and an N-demethylase (substrate = amidopyrine).

We proposed to carry out the experiment described above for phenobarbital with flurazepam. Possible increase of methemoglobin from phenazopyridine was studied after chronic administration of flurazepam to rats and guinea pigs but the latter study was not performed. Determination of LD_{50} for phenazopyridine in control and flurazepam-treated rats were also planned but not carried out. In view of the fact that nothing is known about the enzyme-inducing properties of flurazepam, we determined whether microsomal azoreductase is induced by this drug. In addition, we carried out determinations of cytochrome P-450 and b_5 in control and flurazepam-treated rats.

C. Study of the Effect of Diphenoxylate on the Bioavailability of Nitrofurantoin.

In our previous program for NASA, we have studied the effect of spacecraft environment on drug metabolism and on the pharmacological activity of drugs (Contract NAS 9-9806). Generally, we have concluded

that most drugs under spacecraft environment had no significant adverse effects. However, diphenoxylate does appear to alter the pharmacokinetics of at least two drugs.

In our first NASA-sponsored studies, diphenoxylate potentiated the secobarbital- and, to a lesser extent, hexobarbital-induced sleeping times in rats. Diphenoxylate had been given 16-18 hr prior to the barbiturates. Barbiturate metabolism by liver microsomes *in vitro* was unchanged in diphenoxylate-treated rats compared to control animals and the potentiation does not appear to involve inhibition of barbiturate metabolism by diphenoxylate.

Diphenoxylate has been shown to be extensively metabolized in rats, dogs, and man.[1,24,25] In bile-cannulated rats, 90% of the administered label was recovered in the bile.[25] The major excretory pathway is the feces and the rate of fecal excretion of the metabolites is slow. Thus, the extensive biliary secretion and slow fecal excretion is consistent with having an enterohepatic cycle for diphenoxylate and its metabolites. A metabolite of diphenoxylate, difenoxine (R 15403), has been shown to be responsible for the pharmacological activity associated with diphenoxylate[26-29] and the pharmacokinetics of difenoxine in man and animals[26-29] are similar.

The striking feature of the drug interaction between diphenoxylate and barbiturates in rats was the 16 hr between dosing the two drugs. If diphenoxylate enters an enterohepatic cycle, then one would expect a long biological half-life of the active component in both animals. Thus, any interaction between diphenoxylate and a second drug, many hours after the diphenoxylate treatment, could reasonably be expected.

Since diphenoxylate decreases G.I. motility, an increased absorption of secobarbital probably is due to the increased residence time of the drug in the G.I. tract. This was, in part, documented by finding an increased total Cxt of secobarbital in plasma of rats

(NASA Final Report, Contract NAS 9-9806), but other factors must also be important, especially since hexobarbital was given intraperitoneally in the hexobarbital/diphenoxylate interaction study.

The increased sleeping time with the hexobarbital/diphenoxylate drug combination was less than that for the secobarbital/diphenoxylate study. The rate of metabolism of hexobarbital is rapid compared to secobarbital in the rat. Diphenoxylate apparently did not grossly affect the overall rate of hexobarbital metabolism (NASA Final Report, Contract NAS 9-9806). Therefore, the rate that the drug is metabolized might also play an important role in the overall expression of the diphenoxylate interaction. Specifically, even if diphenoxylate results in an increased absorption of a drug, if such a drug were rapidly metabolized, one might not see an enhanced pharmacological effect. It is this possibility that might explain the absence of any diphenoxylate/chloral hydrate interaction in the rat (NASA Final Report, Contract NAS 9-9806).

Nitrofurantoin

Nitrofurantoin is marketed in two solid dosage forms, microcrystalline in a tablet (Furadantin®, Eaton Laboratories) and macrocrystalline in capsules (Macrodantin®, Eaton Laboratories). Oral absorption of these dosage forms has been studied by following urinary excretion of drug in the dog,[30] although detailed kinetics were not determined. It was established that the rate of absorption of these two crystalline forms of nitrofurantoin is different, the macrocrystalline form being more slowly absorbed. It has also been established[30] that with the macrocrystalline dosage form, the amount of antibiotic excreted in urine is less than that found with the microcrystalline form during the first 24 hr after the first dose. It was noted, however, that urinary antibiotic exceeded minimum effective antibacterial levels with both dosage forms. Use of diphenoxylate, which decreases intestinal motility, may further affect the rate of absorption of nitrofurantoin, especially the macrocrystalline form (Macrodantin®).

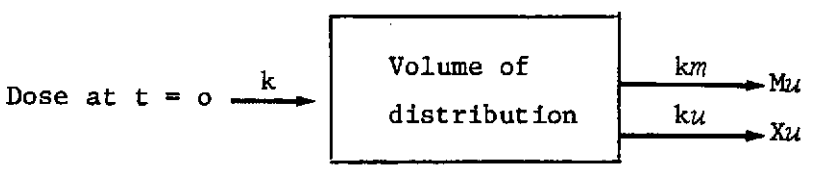
It is possible to determine the percent of drug absorbed with time by following the kinetics of urinary excretion. Before describing the experiments, it seems well to present the manner in which urinary excretion data can provide quantitative information on rate and amount of drug absorption.[31]

The time course of nitrofurantoin in plasma after intravenous injection suggests a simple one-compartment open model is adequate for describing the kinetics of this drug. That is, a model such as that in Figure 3 should be adequate for treating the kinetics of drug excreted in urine after an oral dose. After a single dose, the amount of unchanged drug appearing in urine with time typically follows a course like that of Figure 4.

It may be shown[31] that the fraction (percent) of an oral dose absorbed at a time T (fDo) follows the expression:

$$f\text{Do} = \frac{\left(1/_{K_e l}\right)\left[\left(dX_u/dt\right)\right] + X_u}{(X_u)_\infty} \times 100$$

X_{u_∞} is the amount of drug ultimately excreted in urine after a single dose. X_u is the amount eliminated up to a time T. Reliable estimation of X_{u_∞} requires that urine be collected for eight to ten times the biological half-life of the drug. The differential, $d(X_u/dt)$ is obtained from the slope of the plot of total unchanged drug excreted in urine versus time (Figure 4). $K_e l$ is the overall rate constant for elimination of drug. $K_e l$ may be evaluated by plotting $ln(X_{u_\infty} - X_{u_t})$ from the "tail end" of the plot in Figure 4 versus time. This gives all the elements needed for solution of the equation and, consequently, a plot of "Fraction of Oral Dose Absorbed vs. Time" may be constructed. This is a procedure commonly used to determine bioavailability of drugs. Blood levels may be similarly used, but in the case of nitrofurantoin, available analytical methods appear insufficiently sensitive for work at the dose involved.



M_u is amount of metabolite excreted in urine

X_u is amount of drug excreted in urine

FIGURE 3

Model for rate of appearance of unchanged drug in urine

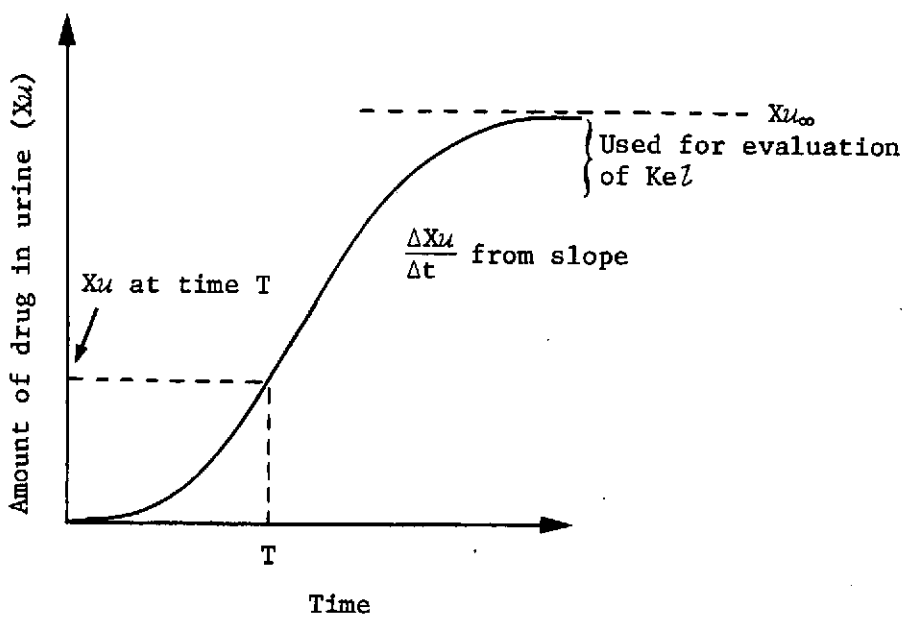


FIGURE 4

Typical cumulative excretion of drug in urine

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Studies to determine the effect of diphenoxylate on the bio-availability of nitrofurantoin have been carried out in dogs and man. In view of the purpose of this study, we used the commercial dosage forms, Furadantin® and Macrodantin®, rather than bulk drug. These studies were done using four female dogs and six male subjects. The rate of absorption of antibiotic and total antibiotic absorbed will be compared in each group and with each dosage form.

III. RESULTS

A. Studies of Potential Interaction between Nitrofurantoin and Chloral Hydrate, Barbiturates, or Flurazepam *In Vitro* and *In Vivo*.

1. Summary

Rat liver microsomes were used to determine whether chronic dosage with nitrofurantoin would inhibit rates of oxidative drug metabolism. Rates of hexobarbital oxidation, N-demethylation of meperidine, and reduction of *p*-nitrobenzoic acid were studied in control and nitrofurantoin-treated rats. No difference in oxidative metabolic rates was found between control and nitrofurantoin-treated rats.

Pharmacological studies were carried out to determine whether premedication of rats with nitrofurantoin would result in potentiation of activity of hexobarbital, chloral hydrate, or flurazepam. This was determined for chloral hydrate and hexobarbital by studies of sleeping times determined by loss of the righting reflex. Flurazepam was investigated by use of the inclined screen test. No potentiation of activity was found between control and nitrofurantoin-treated animals.

2. Introduction

Nitrofurantoin is one of several nitrofuran derivatives possessing antibacterial activity. It is used exclusively as a urinary antiseptic and is contained in the Sky Lab medical kits for that purpose. A variety of enzymes are known to be inhibited by nitrofurans *in vitro*.[3,4] These enzymes are involved in redox reactions and include nitroreductase, glutathione reductase, and aldehyde dehydrogenase. Inhibition of such enzymic reactions is, in fact, thought to be intimately involved in the mechanism of action of these compounds.[5]

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Three other drugs scheduled for use in the Sky Lab program, when the need necessitates, are chloral hydrate, barbiturate, and flurazepam. The purpose of the following work was to investigate the possible interaction of these sedative drugs with nitrofurantoin, which might result from its inhibition of a variety of enzymes. Specific studies have compared the metabolic capabilities of microsomes from nitrofurantoin-pretreated animals and controls, and the duration of sleep produced by the sedative drugs in the two groups of animals.

In vitro metabolic rates for several substrates were studied using liver microsomes from control rats and from rats chronically dosed with nitrofurantoin. The substrates were as follows: hexobarbital, which provides a measure of barbiturate oxidase activity; meperidine, which provides a measure of microsomal N-demethylase; and *p*-nitrobenzoic acid, which measures the enzyme nitroreductase. In addition to measuring gross body and liver weights in the two groups of animals, cytochrome P-450 and b_5 were determined in microsomal preparation of control and nitrofurantoin-treated animals.

Sleeping times produced after i.p. dosing with chloral hydrate and hexobarbital were determined in control and nitrofurantoin-pretreated animals. Since flurazepam did not produce a "sleeping time" as it is usually defined, the inclined screen test was used to evaluate the potential interaction between nitrofurantoin and flurazepam.

3. Materials and Methods

Male Sprague-Dawley rats were premedicated with nitrofurantoin (24 mg/kg, p.o.) three times daily for five days for a total daily dose of 72 mg/kg. The drug was given as a suspension in saline with 1% Tween 80. Control animals received only the vehicle (0.5 ml/100 g rat) on the same dose regimen as the treated animals. On the sixth day, the animals were sacrificed by decapitation and exsanguinated. Livers were excised from control and treated rats, immediately placed in cold 1.15% KCl,[32] and kept on ice. Each liver was weighed and homogenized in 20 ml 1.15%

KCl and the homogenate spun at 9000 x *g* for 30 min. The supernatant was poured through a double layer of gauze to remove floating lipids. It was then centrifuged in a refrigerated Beckman Model L3-50 ultracentrifuge (head 30) for 1.25 hr at 100,000 x *g*. The supernatant was decanted and the pellets representing the microsomal fraction were resuspended in cold 0.05M phosphate buffer, pH 7.4.

A protein determination was carried out on each microsomal preparation using the Folin Phenol procedure.[33] Each preparation was then diluted with buffer to 20 mg protein/ml for the hexobarbital oxidase, N-demethylase, and nitroreductase experiments, or to 2 mg protein/ml for measurement of cytochrome levels. Incubation mixtures to be used for *in vitro* metabolism studies were prepared immediately.

Secobarbital is the barbiturate being carried and used on the Sky Lab program. This barbiturate was not metabolized *in vitro* by rat liver microsomes at a rate sufficient to permit reliable kinetic studies (NASA Final Report, Contract No. 9-9806). Hexobarbital was known to be rapidly metabolized by rat liver microsomes *in vitro* [34] and was used in place of secobarbital as the substrate for the barbiturate oxidase experiments.

The kinetics of metabolism of hexobarbital was followed by observing the rate of disappearance of substrate using the analytical method of Brodie *et al.*[35] Incubation mixtures were prepared fresh for each experiment and contained 20 mg microsomal protein, 20 μmoles nicotinamide (Mann Research Labs.), 20 μmoles glucose-6-phosphate (Sigma Chemical Co.), 25 μmoles of magnesium chloride, 2.25 μmoles of NADP (Sigma), 2 units of glucose-6-phosphate dihydrogenase (Sigma) and 0.2, 0.3, 0.5, or 1.0 μmole of hexobarbital per 6 ml. Each mixture (12 ml) was dept at 0° on ice at all times prior to incubation. At zero time, a 5-ml aliquot of the mixture was taken and placed in 30 ml of heptane containing 1.5% of isoamyl alcohol with 5 ml of 0.05 molar phosphate buffer, pH 5.3, and saturated with NaCl. After removal of the zero time sample, the incubation mixture was placed in a 37°C shaking water

bath (New Brunswick Scientific Co., Model G-77) and incubated for 7 min. All incubations were carried out in air using a shaking rate of 150 oscillations per min. After 7 min, another 5-ml aliquot of the incubation mixture was taken and placed in the heptane/buffer mixture to stop the reaction. All samples were extracted by shaking in a reciprocal shaking apparatus for 30 min. After extraction, each tube was centrifuged at 700 x *g* for 10 min in an International Model PR-6000 centrifuge.

For the determination of the barbiturate, a 7-ml aliquot of the upper organic phase was added to 3 ml of 0.8M phosphate buffer, pH 11, and shaken for 10 min. Samples were centrifuged, the organic phase aspirated, and the optical density of the aqueous phase read at 240 and 280 nm. Using the optical density difference at 240 and 280 nm, the concentration of barbiturate was determined. The amount of hexobarbital that was metabolized was calculated from the difference between this value and that found for the zero time sample.

Meperidine rather than flurazepam itself, was used for probing N-dealkylation *in vitro* since acetaldehyde - one product of N-de-ethylation of flurazepam - is not so readily determined as formaldehyde.

The rate of formaldehyde production from meperidine was determined using the Nash procedure.[36] Incubation mixtures were prepared as described for the hexobarbital oxidase, but with the addition of 15 μmoles of semicarbazide hydrochloride/ml of incubation mixture. This traps the formaldehyde as its semicarbazone, preventing its further oxidation to carbon dioxide. Drug levels were 1, 2, 10, and 20 μmoles of meperidine per 6 ml of incubation mixture. The mixture was incubated under air at 37°C in the shaking water bath and at 7 min a 5-ml aliquot was taken. This was immediately added to 2 ml of 10% $ZnSO_4$ (W/V using anhydrous $ZnSO_4$) to stop the reaction. Each sample was agitated with a Vortex mixer and after 5 min, again agitated. A saturated barium hydroxide solution (4 ml) was added to each sample and the tube was agitated and centrifuged at 15,000-17,000 rpm for 10 min. If the supernatant was

cloudy, known amounts of the 10% $ZnSO_4$ solution or saturated barium hydroxide solution were added until it became clear.

To a 5-ml aliquot of the clear supernatant, 2 ml of freshly prepared double-strength Nash reagent[36] was added and the mixture incubated for 30 min at 60°C. The yellow color which developed was quantitated by reading optical density at 412 nm. The yellow color was caused by the formation of 3,5-diacetyl pyridine from 2,3-butanedione and formaldehyde in the presence of ammonium acetate in the Nash reagent.

Nitroreductase activity was determined by the method of Fouts and Brodie[37] as modified by Umar and Mitchard.[4] The supernatant fraction from the 100,000 x *g* spin was used instead of the isolated microsomes for investigating nitroreductase activity. Nitroreductase appears to be a soluble cytoplasmic enzyme and not microsomal, as it has been reported that the ability to reduce *p*-nitrobenzoic acid is associated only with the supernatant fraction.[4] The incubation mixture used in these studies had the following composition: 3.0 µmole NADPH (Sigma Chemical Co.), 0.094 µmole riboflavin (Sigma), 4.5 µmole nicotinamide (Mann Research Laboratories), and 17.6 µmole glucose-6-phosphate (Sigma) in 0.5 ml phosphate buffer, pH 7.4, 2 ml liver supernatant, and 1 ml of either 10^{-4}, 5 x 10^{-4}, 10^{-3}, or 5 x 10^{-3}M *p*-nitrobenzoic acid. Incubations were carried out anaerobically at 37°C as nitroreductase is inhibited by oxygen. The rate of appearance of *p*-aminobenzoic acid was shown to be linear with time for 90 min. At the end of 1 hr, 1 ml 5% trichloroacetic acid solution was added to the samples to stop the reaction and precipitate protein. The precipitate was removed by centrifugation and 3 ml aliquots of the clear supernatant were removed for analysis.

The amount of *p*-aminobenzoic acid formed in 1 hr was determined by the method of Bratton and Marshall.[38] One ml of 0.1% sodium nitrite was added to the 3 ml of clear supernatant and the samples vortexed. After standing at room temperature for 3 min, 1 ml of 0.5% ammonium sulfomate was added and the samples were again vortexed. One ml of 0.1%

N(1-naphyl)ethylenediamine was added after 2 min, the samples vortexed and, following a 3 min wait, the absorption determined at 550 nm.

Throughout the section dealing with metabolism *in vitro*, rates will be expressed in terms of millimicromoles of substrate metabolized (or millimicromoles of metabolite appearing)/mg microsomal protein/min. These units are commonly used by other workers.[39]

Cytochrome b_5 was determined by the method of Strittmatter and Velick.[40] Cytochrome P-450 was determined as described by Omura and Sato.[41] An aliquot of the microsomal protein suspension was diluted with 0.05M phosphate buffer, pH 7.6 (EDTA 10^{-3}M) to a concentration of 2 mg/ml. Three ml of this dilution were aliquoted into two matched cuvettes. A few milligrams of sodium hydrosulfite were added to the cell sample and, after mixing the contents, the difference spectrum was recorded (Figure 5). The sample cuvette was then placed in the reference beam and the reference cuvette treated as follows: carbon monoxide was gently bubbled through for approximately 20 sec, then a few milligrams of sodium hydrosulfite were added, followed by carbon monoxide being bubbled through for another 20 sec. This cuvette was then placed in the sample beam and the difference spectrum recorded (Figure 6).

Cytochrome b_5 levels are expressed as the optical density difference between 427 nm and 407 nm/10 mg microsomal protein/3 ml. Cytochrome P-450 levels are expressed as optical density differences between 450 nm and 490 nm/10 mg microsomal protein/3 ml in this standard procedure. This is adequate for the purposes of this study since we are interested in comparing groups rather than absolute amounts of cytochrome.

Pharmacological studies were set up to measure the effect of chronic dosing with nitrofurantoin on chloral hydrate-induced sleeping times. Preliminary studies established that a dose of 200 mg/kg i.p. of chloral hydrate and 80 mg/kg i.p. of hexobarbital produced a convenient length sleeping time in normal rats. Sleeping times were recorded

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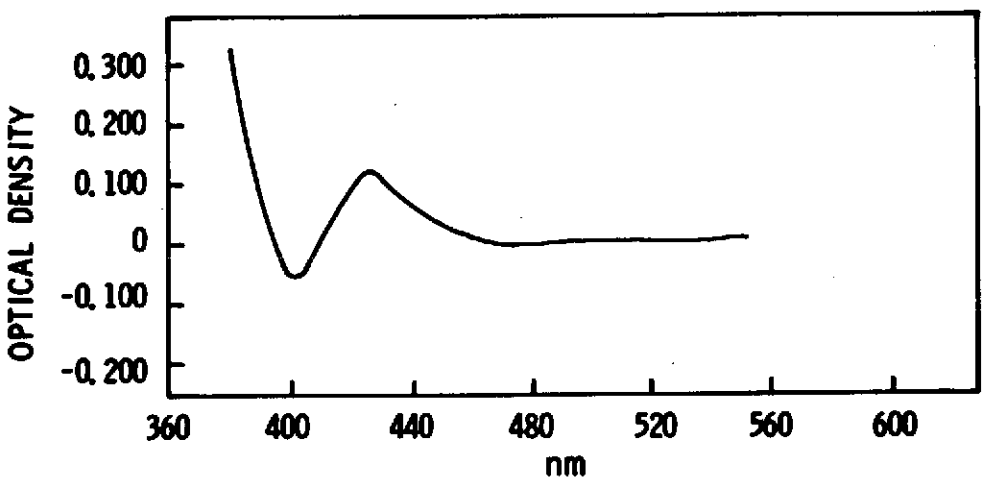

FIGURE 5

Difference spectra of cytochrome b_5

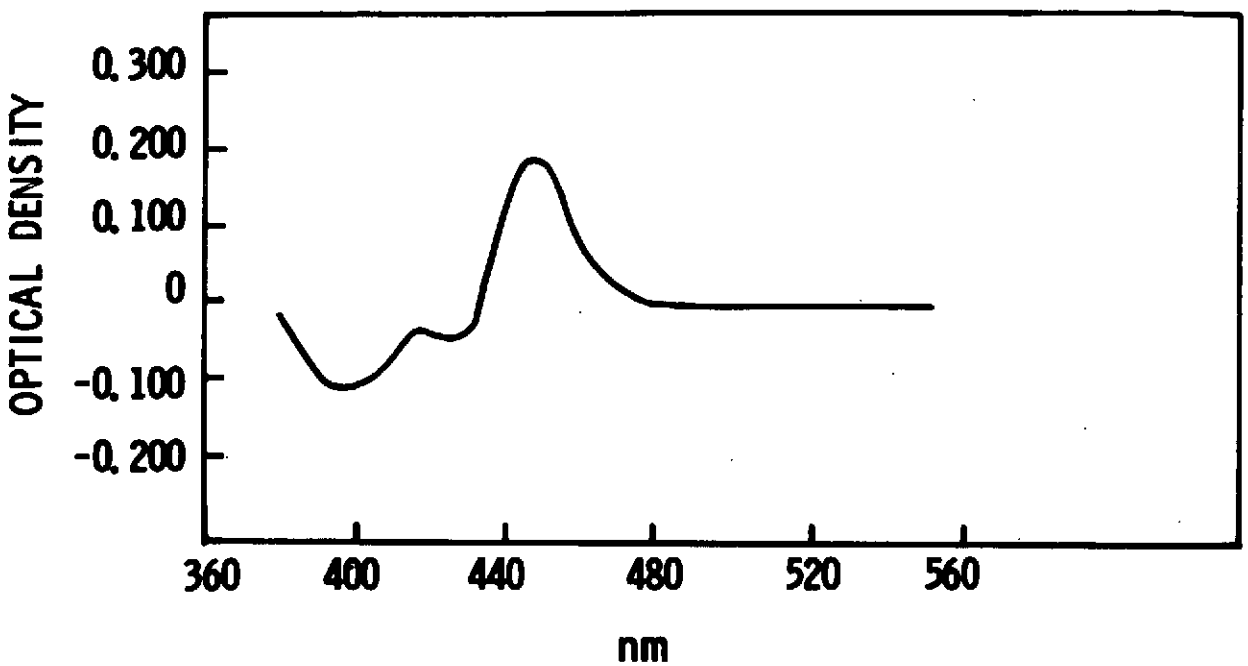

FIGURE 6

Difference spectra of cytochrome P-450

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as the interval between loss and return of the righting reflex. The criteria for the loss and return of the reflex were the inability and ability, respectively, of the animal to right itself within 30 sec when placed on its back.[42]

Since flurazepam did not appear to produce a "sleeping time" as it is usually defined by the righting reflex, the inclined screen test[43] was the method chosen to evaluate the potential interaction between nitrofurantoin and flurazepam. Control animals were dosed with different levels of flurazepam to establish an effective dose, defined as that dose which produced a paralyzing effect severe enough to allow a rat to slide off a 70° inclined screen within 1 min. At 100 mg/kg i.p., the length of time between administration of flurazepam and the paralyzing effect was approximately 10 min. It is this time which was used as a basis of comparison between control and nitrofurantoin-treated animals.

4. Results and Discussion

(a) Microsomal Studies. Chronic dosing of rats with nitrofurantoin produced no gross effects on total body weight, liver weight, or yield of microsomal protein (Table 1). Cytochrome P-450 and b_5 enzyme levels were equivalent from control and nitrofurantoin-treated animals (Table 2).

The rate of hexobarbital metabolism has been shown to be linear with time to 10 min and has good substrate dependence (Final Report to NASA, October 31, 1970, Contract NAS 9-9806). The metabolic rates for control and nitrofurantoin-treated animals are given in Table 3. The rate data plotted in Lineweaver-Burk form are presented in Figure 7. The modest trend towards an increase in hexobarbital metabolism is not significant since animals in subsequent pharmacological studies were not affected by pretreatment with nitrofurantoin.

The two major metabolic pathways for meperidine are N-demethylation and ester hydrolysis. Microsomal N-demethylase activity was studied

TABLE 1

EFFECT OF CHRONIC TREATMENT WITH NITROFURANTOIN ON TOTAL BODY AND LIVER WEIGHT AND YIELD OF MICROSOMAL PROTEIN

	Body Weight (g)[a]	Liver Weight (g)[a]	Ratio[b]	No. of Animals	Microsomal yield mg protein/g liver	Assayed for
Treated	146.0 ± 7.0	7.5±0.7	0.051	4	19.3	Nitroreductase/cytochrome
Control	145.3 ± 3.4	7.1±0.4	0.049	4	18.0	enzymes
Treated	161.8 ± 2.4	8.1±0.4	0.048	4	19.0	Nitroreductase/barbiturate
Control	161.0 ±6.5	8.1±1.4	0.051	4	18.6	oxidase
Treated	167.0 ±1.8	7.9±1.1	0.047	4	18.8	N-demethylase/cytochrome
Control	168.5 ± 5.2	8.5±0.5	0.050	4	19.1	enzymes
Treated	168.5 ± 5.2	9.1±0.6	0.054	4	18.5	N-demethylase/barbiturate
Control	169.3 ± 7.6	7.9±0.6	0.047	4	17.9	oxidase

[a] Mean ±S.D.

[b] Ratio of mean liver weight/mean body weight.

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TABLE 2

EFFECT OF CHRONIC TREATMENT WITH NITROFURANTOIN ON RAT LIVER CYTOCHROME[a]

	No. of animals	Cytochrome P-450[b]	Cytochrome b_5[b]
Treated	4	0.292±0.024	0.394±0.042
Control	4	0.263±0.060	0.423±0.077
Treated	4	0.287±0.036	0.381±0.053
Control	4	0.291±0.041	0.401±0.068

[a] Results from two experiments reported separately.
[b] Mean ±S.D.

TABLE 3

MICROSOMAL HEXOBARBITAL OXIDASE ACTIVITY AFTER CHRONIC NITROFURANTOIN PREMEDICATION

Substrate (micromoles/6 ml of incubation mixture)	Rate (mμmoles hexobarbital metabolized/mg protein/minute)[a]	
	EXPERIMENT 1	
	Control	Treated
0.32	0.254±0.089	0.402±0.085
0.50	0.489±0.087	0.566±0.176
0.92	0.730±0.182	0.789±0.209
1.86	1.150±0.297	1.572±0.233
	EXPERIMENT 2	
0.32	0.216±0.078	0.350±0.072
0.50	0.426±0.073	0.484±0.149
0.92	0.625±0.156	0.679±0.183
1.86	0.976±0.248	1.342±0.213

[a] Mean ±S.D. for 4 animals

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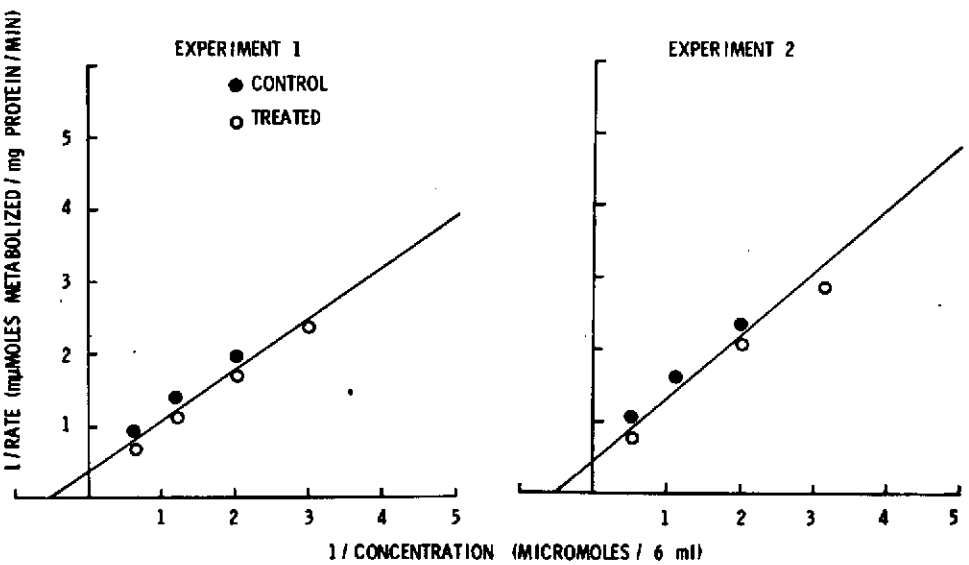

FIGURE 7

Lineweaver-Burk plot of hexobarbital oxidase activity in control and nitrofurantoin-treated rats.

by following the rate of appearance of formaldehyde. The rate of formaldehyde production has been shown to be linear with time to about 10 min and has good substrate dependence (see ref. for hexobarbital). The rate data obtained using liver microsomes from control and nitrofurantoin-treated rats are given in Table 4. The rate data plotted in Lineweaver-Burk form are presented in Figure 8. It is apparent that there was no significant effect on N-demethylase due to chronic dosing of rats with nitrofurantoin.

Furadantin® and some related nitrofuran analogues, added directly to guinea pig liver microsomal preparations, have been reported to inhibit completely the nitroreductase reduction of *p*-nitrobenzoic acid.[5] To complete the metabolic profile of rats chronically dosed with nitrofurantoin, nitroreductase was determined by using *p*-nitrobenzoic acid as the substrate and following the rate of appearance of *p*-aminobenzoic acid. Figure 9 depicts the linearity and substrate dependency of the reaction. Metabolic rates are presented in Table 5. Lineweaver-Burk graphs of the data are shown in Figure 10.

These studies, like the previous ones, demonstrated the absence of drug effect of nitrofurantoin on the nitroreductase activity in microsomal preparation from livers.

Each parameter - body and liver weights, microsomal protein concentrations, liver cytochrome concentrations, hexobarbital oxidase activity, N-demethylase activity, and nitroreductase activity - has been examined in two separate experiments. Each experiment involved the highest proposed dose level of nitrofurantoin (24 mg/kg t.i.d. or 72 mg/kg/day for 5 days). No significant effect was observed, except possibly on hexobarbital oxidase. However, even this possibility was not demonstrated at the pharmacological level.

TABLE 4

METABOLIC RATES OF MEPERIDINE *IN VITRO* USING LIVER MICROSOMES FROM CONTROL AND NITROFURANTOIN-TREATED RATS

Substrate Concentration (μM)	Rate ($m\mu moles\ H_2CO/mg\ protein/min$)[a]	
	Experiment 1	
	Control	Treated
1	0.39 ± 0.01	0.52 ± 0.12
2	0.49 ± 0.05	0.54 ± 0.12
10	0.61 ± 0.06	0.61 ± 0.12
20	0.75 ± 0.12	0.74 ± 0.18
	Experiment 2	
	Control	Treated
1	0.57 ± 0.08	0.52 ± 0.10
2	0.73 ± 0.07	0.61 ± 0.11
10	0.86 ± 0.09	0.78 ± 0.09
20	0.89 ± 0.10	0.75 ± 0.10

[a] Mean ±S.D. of 4 animals

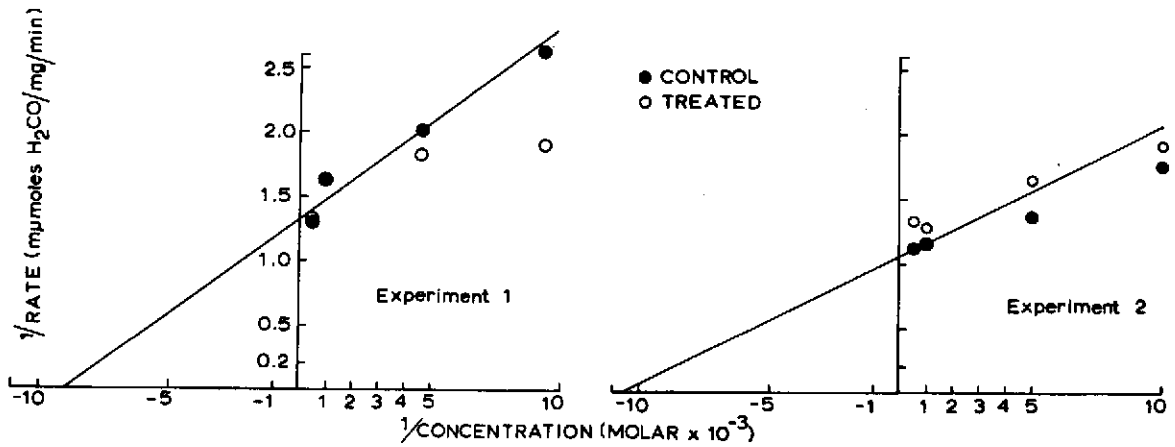

FIGURE 8

Lineweaver-Burk plot of N-demethylase activity in control and nitrofurantoin-treated rats.

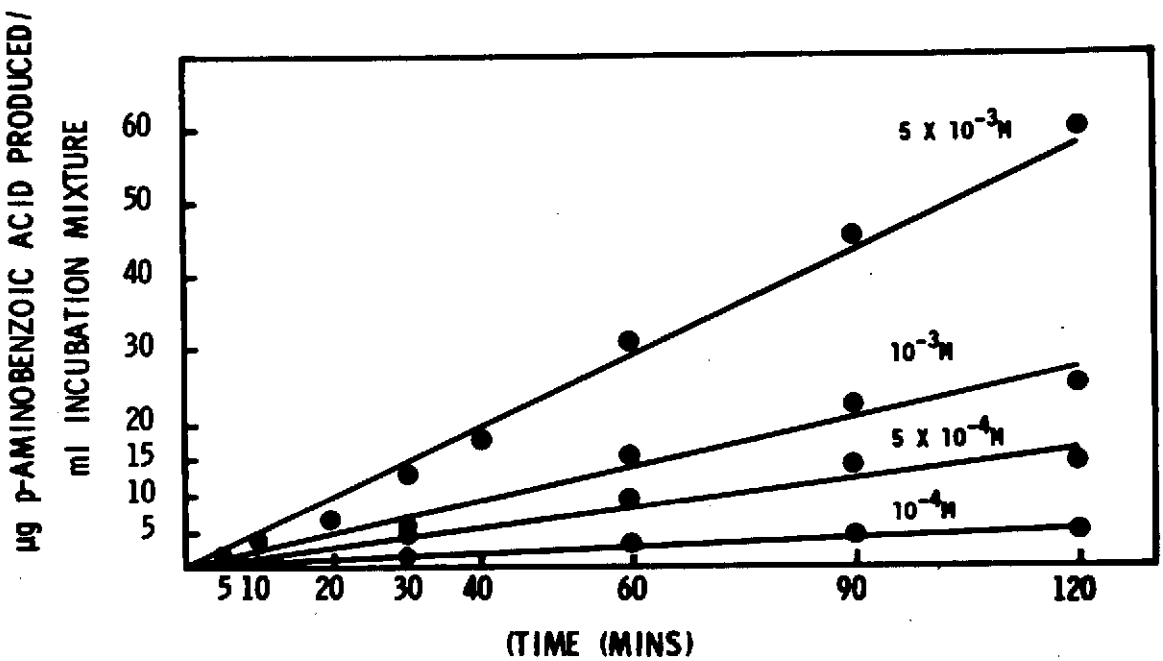

FIGURE 9

Rate of production of *p*-aminobenzoic acid from *p*-nitrobenzoic acid at several substrate concentrations.

TABLE 5

MICROSOMAL NITROREDUCTASE ACTIVITY AFTER CHRONIC NITROFURANTOIN PREMEDICATION

Substrate Concentration (Molar)	Rate (mμmoles *p*-aminobenzoic acid/mg protein/hr)[a]	
	Experiment 1	
	Control	Treated
5×10^{-3}	5.33 ± 1.78	5.33 ± 1.65
10^{-3}	3.32 ± 0.81	2.90 ± 0.46
5×10^{-4}	1.76 ± 0.41	2.01 ± 0.31
10^{-4}	0.77 ± 0.13	0.93 ± 0.08
	Experiment 2	
5×10^{-3}	3.75 ± 0.77	4.61 ± 1.40
10^{-3}	1.87 ± 0.27	1.83 ± 0.13
5×10^{-4}	1.00 ± 0.07	0.93 ± 0.08
10^{-4}	0.50 ± 0.13	0.59 ± 0.03

[a] Mean ±S.D. for 4 animals

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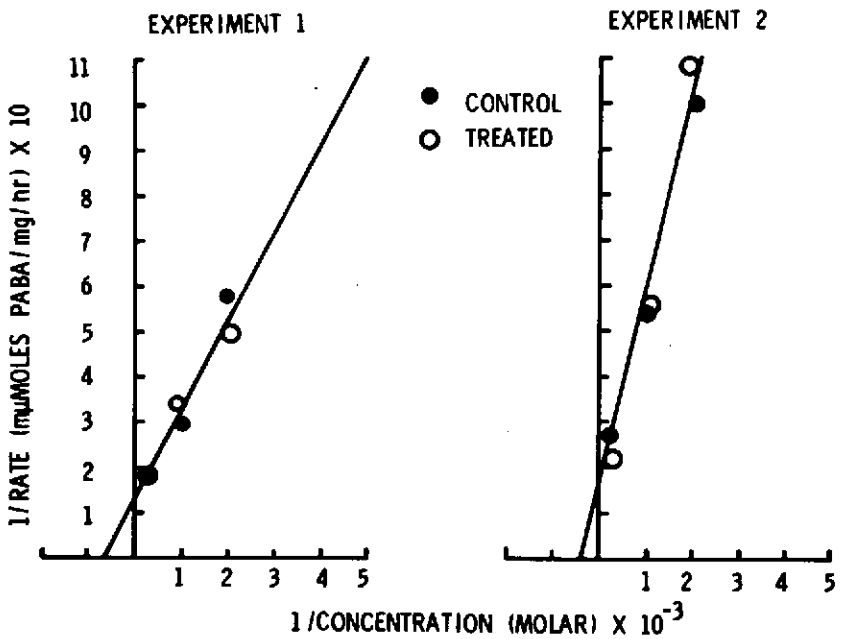

FIGURE 10

Lineweaver-Burk plot of nitroreducrase activity in control and nitrofurantoin-treated rats.

(b) Pharmacological Studies. It has been reported in a rather obscure paper[8] that nitrofurantoin (100 or 200 mg/kg) potentiates the sleeping time produced by hexobarbital in the rat. The mechanism of this potentiation is unknown, but it again seemed not unreasonable that inhibition of oxidative metabolism of the barbiturate may be involved. As the average daily oral dose of nitrofurantoin in humans is 5-10 mg/kg, the dose of 100 or 200 mg/kg used in the above study may be unrealistically high. Using dosage conversion factors suggested by Freireich *et al.*[9] and recognizing their approximate nature, a dose for the rat which is comparable to 5-10 mg/kg in the human, should be in the range of 35-70 mg/kg. Freireich's conversion factors are used for estimating dosage equivalency on the basis of mg/m^2 of body surface area, rather than body weight.

Pharmacological studies were set up to measure the effect of chronic dosing with nitrofurantoin on hexobarbital-induced sleeping times. Rats were previously dosed with nitrofurantoin (72 mg/kg) or the control vehicle for 5 consecutive days. On the sixth day, all animals were dosed with hexobarbital. Preliminary studies established that a dose of 80 mg/kg i.p. of hexobarbital produced a convenient length sleeping time in normal rats. The results, compiled from two separate experiments, are reported in Table 6. It is readily apparent that nitrofurantoin pretreatment did not have any effect on the duration of sleep produced by hexobarbital. A similar conclusion is also reached when chloral hydrate (200 mg/kg i.p.) was the sedative (Table 6).

The rationale for including flurazepam in these studies was merely to complete this set of experiments with the third operational sedative drug. The route of metabolism of nitrofurantoin is unknown so that the possibility of chronic dosing with nitrofurantoin affecting the way rats responded to flurazepam was speculative.

TABLE 6

EFFECT OF CHRONIC DOSING WITH NITROFURANTOIN ON HEXOBARBITAL- AND CHLORAL HYDRATE-INDUCED SLEEPING TIMES IN RATS

Group	No. of Animals	Mean Body Weight (g) ± S.D.	Chronic Treatment	Hexobarbital/Chloral Hydrate Dose	Sleeping Times (min ± S.D.)
1	15	165.5 ± 10.3	1% Tween 80/saline, 0.5 ml/100 g rat p.o., t.i.d. for 5 days	80 mg/kg i.p. hexobarbital, Day 6	18.6 ± 5.2
2	20	159.9 ± 12.2	Nitrofurantoin in 1% Tween 80/saline, 24 mg/kg p.o., t.i.d. for 5 days	80 mg/kg i.p. hexobarbital, Day 6	19.9 ± 8.9
3	15	169.7 ± 16.1	1% Tween 80/saline, 0.5 ml/100 g rat p.o., t.i.d. for 5 days	200 mg/kg i.p. chloral hydrate, Day 6	17.8 ± 9.0
4	20	162.4 ± 13.7	Nitrofurantoin in 1% Tween 80/saline, 24 mg/kg p.o., t.i.d. for 5 days	200 mg/kg i.p. chloral hydrate, Day 6	15.6 ± 5.6

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As explained in the methods section, the inclined screen test was chosen to evaluate the potential interaction between nitrofurantoin and flurazepam since the latter drug did not appear to produce a measurable sleeping time. Rats were predosed with either nitrofurantoin or control vehicle as described above, and on Day 6, received 100 mg/kg flurazepam i.p. The mean time in minutes between dosing with flurazepam and the paralyzing effect which caused the animal to slide off the 70° inclined screen was essentially the same for control and nitrofurantoin-treated animals, 10.89 ± 5.49 and 10.89 ± 4.88, respectively. Based on our experience, we feel that the inclined screen test used to measure the flurazepam interaction does not appear to be a very sensitive mechanism for noting small differences between groups of animals. Since there was no difference between the two groups, we must assume that there was no significant potentiation or inhibition of flurazepam action by nitrofurantoin. Any subtleties of drug interaction, however, would not have been detected by this method.

The significance of this work has been defined as test results that differ from control values by a "p" value of less than 0.05, as measured by the "t" Test. In other words, the probability (p) that the values obtained with treated animals and values obtained with control animals are different by chance alone is less than 5%. Based on this definition, neither the *in vivo* nor the *in vitro* experiments produced any significant differences between control and nitrofurantoin-treated animals. The only possible exception was the hexobarbital oxidase experiments which did show an increase in the rate of hexobarbital metabolized by microsomes from treated animals but significance at the defined level was found only at the lowest (0.32 μmoles/6 ml incubation mixture) substrate level. Complementary experiments measuring the *in vivo* effect of nitrofurantoin on hexobarbital-induced "sleeping times" did not indicate any differences between control and treated animals.

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B. Studies of Potential Interactions of Enzyme Inducers with Phenazopyridine.

1. Summary

Studies were carried out in several species to determine whether induction of liver microsomal enzymes would increase the tendency of phenazopyridine to produce methemoglobin *in vivo*. Animals were premedicated with phenobarbital, a known inducer of azoreductase, and in a separate experiment with flurazepam, before administration of phenazopyridine. Methemoglobin production was determined in each animal after receiving phenazopyridine. No evidence was found for increased production of methemoglobin in the rat, dog, or rabbit that could be attributed to increased amounts of microsomal enzymes.

2. Introduction

Phenazopyridine is an old drug developed originally as a urinary antiseptic but used now principally as a urinary tract anesthetic, either alone or in combination with sulfonamides. The clinical structure of phenazopyridine is related to the aniline compounds whose most commonly described toxic effect is methemoglobinemia. In fact, methemoglobinemia, and more rarely, hemolytic anemia, has been reported to be produced by this drug.[15-17] Accidental overdoses with phenazopyridine have led to production of large amounts of methemoglobin and cyanosis[18] in humans, while a dose of 200 mg/kg, admittedly quite high, produced anemia in the dog.[19] The large doses required to produce this effect suggests that this is not a complication to be expected in the usual clinical administration.

The metabolism of phenazopyridine has apparently never been studied, but there is ample analogy for the suggestion that it will be metabolized by azoreductase. While phenazopyridine apparently can, by itself, produce methemoglobin *in vitro*[16] the products of reduction of the azo linkage should be much more potent in producing methemoglobin.

Production of some methemoglobin by a drug is ordinarily not an important clinical problem, but impairment of any of the oxygen transport capacity of individuals subject to extreme physical exertion may be another matter. Azoreductase is an inducible enzyme and our interest here is whether the presence of enzyme inducers will tend to increase formation of methemoglobin by phenazopyridine. As pointed out above, methemoglobin production by this drug has only been reported with excessively large doses and accidental overdoses. The standard dose used for our studies will be roughly equivalent to the dose for man computed as mg/m^2 of body surface area.

Two drugs, phenobarbital and flurazepam, were investigated as to their ability to induce azoreductase and the subsequent effect of an increased production of enzyme on the amount of methemoglobin produced by phenazopyridine. These studies utilized several animal species.

3. Materials and Methods

Phenobarbital is already known to induce azoreductase in the rat[21] and other species including humans. Before beginning the drug combination studies, the ability of phenobarbital to induce azoreductase in the rat was re-demonstrated under our laboratory conditions.

Rats were dosed with phenobarbital (80 mg/kg, i.p.) for 3, 4, or 5 consecutive days. Control animals received saline (0.5 ml/100 g rat, i.p.) at the same time that the experimental group received phenobarbital. The animals were sacrificed by decapitation and exsanguinated. The livers were excised immediately and placed in cold 1.15% KCl. The method of Smith and VanLoom[44] utilizing 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (CPA) as a substrate for measuring azoreductase activity was chosen over the method of Fouts *et al.*[22] which used Prontosil (4'-sulfamyl-2,4-diaminoazobenzene), as the latter is not commercially available. *p*-Aminobenzoic acid is a product of CPA cleavage and, therefore, the standards and procedures for the colorimetric determination of nitroreductase can be utilized.

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Crude enzyme preparations were obtained by homogenizing the livers at 4°C in 20 ml 1.15% KCl and centrifuging at 9000 x *g* for 30 min. The supernatant was poured through a double layer of gauze to remove floating lipids. Incubation mixtures consisted of: 1.43 ml 0.1M sodium phosphate buffer, pH 7.4; 0.5 ml of a mixture containing 25 µmoles glucose-6-phosphate, 12.5 µmoles $MgCl_2$, and 25 µmoles nicotinamide; 0.12 ml NADP; 0.2 ml buffer containing 1.2 enzyme units of yeast glucose-6-phosphate dehydrogenase; and 0.25 ml 9000 x *g* supernatant fraction. Individual incubation flasks were flushed with nitrogen for 5 min before the addition of 0.2 ml CPA (0.01M solution in ethanol/0.5M Na_2HPO_4, 1:1;V:V). Incubations were continued under nitrogen for 30 min in a 37°C shaking water bath (150 oscillations/min). The reaction was stopped by the addition of 2.5 ml 10% trichloroacetic acid (TCA). The mixtures were then centrifuged at 4°C and 2.5 ml clear supernatant was removed for analysis. 0.5 ml of freshly prepared 0.1% (W/V) sodium nitrite was added to each sample, followed after 5 min by 0.5 ml 0.5% (W/V) ammonium sulfonate. Approximately 5 min later, 0.5 ml 0.1% (W/V) N-(1-naphthyl)ethylenediamine was added and the mixture allowed to react for 15 min. Solutions were swirled rigorously after each addition. Absorbence was measured at 540 nm.

The amount of nonenzymic cleavage was determined by the simultaneous incubation of the NADPH-generating system with CPA for the incubation period. Aliquots of the 9000 x *g* supernatant fraction (0.25 ml) were added after the addition of TCA and the samples were treated as above. The amount of enzyme present was expressed as µg *p*-aminobenzoic acid/mg protein/30 min incubation.

The experiment described above in detail for phenobarbital was carried out with flurazepam. Rats were dosed i.p. consecutively for 3, 4, or 5 days with flurazepam at either 100 mg/kg or 200 mg/kg and the livers were analyzed for flurazepam.

Once the ability of phenobarbital to induce azoreductase had been demonstrated in the rat, an experiment was run with a known methemoglobin producer, aniline, as a check on the experimental design and methods to be used in subsequent drug interaction studies with phenazopyridine. Rats were predosed with phenobarbital (80 mg/kg i.p.) or saline (0.5 ml/100 g rat) for 3 days. On day 4, half of the phenobarbital-treated rats and half of the control rats received aniline at 1 g/kg orally while the other half of the animals received a dose of 500 mg/kg p.o. Blood was analyzed at 30 min, 1, 3, 6, 12, and 24 hr for MHb concentration.

The original procedure used to determine the level of methemoglobin (MHb) in blood was the method of Evelyn and Mallory.[45] Fresh whole blood (0.1 ml) was pipetted into 10 ml of M/60 phosphate buffer, pH 6.6, and the optical density measured at 635 nm (A_1). One drop of neutralized sodium cyanide was then added to convert any methemoglobin present to cyanmethemoglobin, and a second reading was taken at 635 nm (A_2). The optical density difference (A_1-A_2) should be proportional to the concentration of methemoglobin in the original blood sample. Problems were encountered with this method when blood samples contained little or no methemoglobin. In this instance, the lysed cells caused light scattering and thus interfered with the optical density measurement at 635 nm. This problem was solved by centrifuging the samples before optical density readings were taken.[46] This eliminated the turbidity due to the lysed cells and allowed for a more accurate determination. Standards have been analyzed and have demonstrated the reliability of the procedure (Figure 11).

4. Results and Discussion

(a) Induction of Azoreductase. Phenobarbital is already known to induce azoreductase in the rat[21] and other species including humans. As seen in Table 7, azoreductase in rat liver was found to be induced within three days of dosing with phenobarbital but was not

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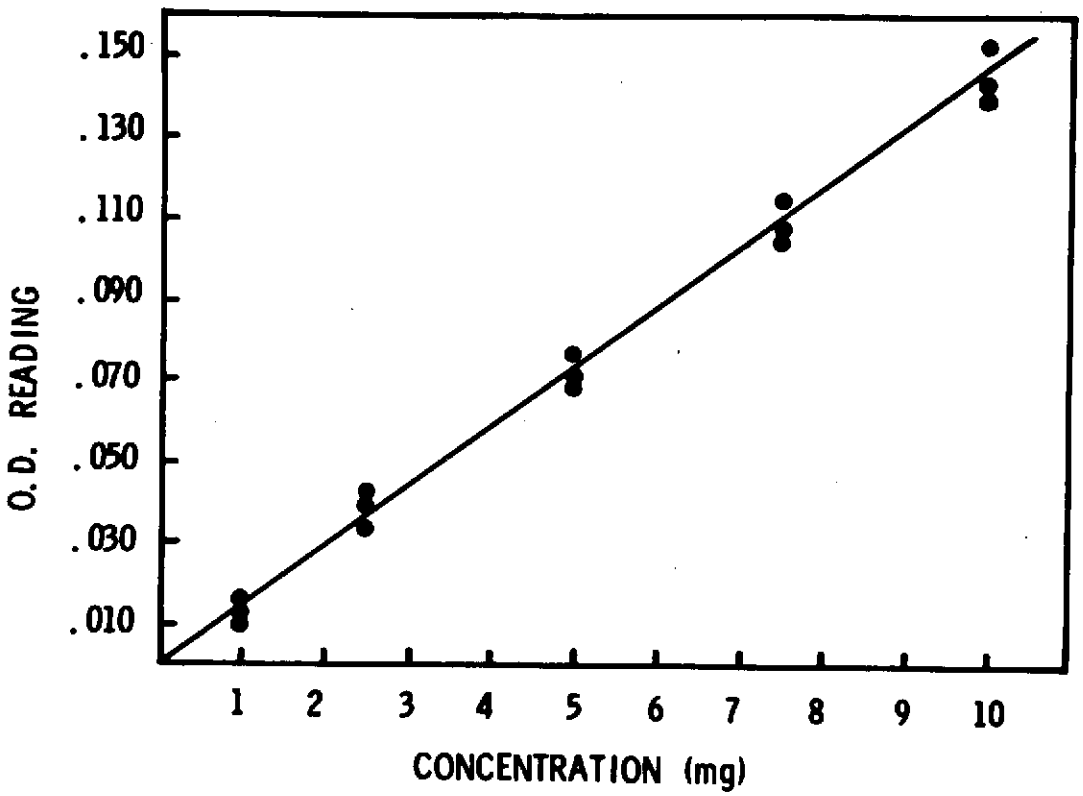

FIGURE 11

Standard curve for methemoglobin determination

TABLE 7

MEASUREMENT OF AZOREDUCTASE INDUCTION IN CONTROL AND PHENOBARBITAL-TREATED RATS

Number of days of phenobarbital pretreatment	μg p-aminobenzoic acid/mg protein/30 min incubation	
	Phenobarbital-treated rats[a]	Control rats[a]
3 days	0.72 ± 0.06	0.41 ± 0.05
3 days	0.77 ± 0.06	0.41 ± 0.07
4 days	0.86 ± 0.19	0.49 ± 0.09
5 days	0.94 ± 0.10	0.48 ± 0.08

[a] Mean of 5 animals ± S.D.

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significantly higher after 4 or 5 days of pretreatment. Changes in other parameters, e.g., liver weight, protein concentration, were not apparently affected by the phenobarbital treatment (Table 8).

Induction of drug-metabolizing enzymes by flurazepam has not been reported, but flurazepam is still a relatively new drug. The closely related drugs, diazepam and chlordiazepoxide, will induce microsomal enzymes in the rat.[23] The particular enzymes shown to be inducible were an O-demethylase (substrate = *p*-nitroanisole), an aryl hydroxylase (substrate = aniline), and an N-demethylase (substrate = amidopyrine). Table 9 shows that pretreatment of rats with flurazepam did not significantly induce azoreductase concentrations. Although the amounts of azoreductase were slightly high in the flurazepam-treated animals, the results did not show any significant difference between the two drug levels and only slight differences in enzyme levels over control animals. Changes in other parameters, e.g., liver weights, protein concentration, were not apparently affected by the flurazepam treatment (Table 10). Table 11 shows that flurazepam only slightly increases cytochrome P-450 concentrations and has no effect on cytochrome b_5 levels.

(b) Methemoglobin Production by Phenazopyridine. Rats normally have very low levels of methemoglobin in blood, 1.3 ± 0.3 mg/ml, as reported by Heubner *et al.*[47] The combination study with aniline and phenazopyridine showed both dose levels of aniline increased the amount of MHb found in the blood, while phenobarbital-treated animals had concentrations of MHb significantly greater than saline-treated rats (Figures 12 and 13). Since phenobarbital can enhance methemoglobin production by aniline, the metabolic activation of aniline must also be enhanced by phenobarbital. The induction of both enzyme systems, azoreductase and metabolic activation of aniline and possibly 2,3,6-triamino pyridine by phenobarbital have been demonstrated in the rat and, thus, an interaction between phenobarbital and phenazopyridine seemed plausible.

TABLE 8

EFFECT OF PHENOBARBITAL PRETREATMENT ON LIVER WEIGHTS AND PROTEIN LEVELS[a] OF RATS

No. of days of phenobarbital	Mean body weight (g)[b]		Liver weight (g)[b]		Gm wt. liver wt./ 100 g body wt.		Mg protein/g liver[b]	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
3 days	143.20± 6.16	160.20±7.80	9.48±0.52	7.64±0.78	6.62	4.77	4.41±0.21	4.25±0.22
3 days	120.30± 5.08	122.10±5.28	8.99±0.68	7.12±0.59	7.50	5.84	3.70±0.34	3.65±0.14
4 days	132.00±10.93	133.50±9.26	9.54±0.93	8.64±1.24	7.23	6.47	3.36±0.24	3.02±0.19
5 days	141.89±18.15	142.50±8.61	9.85±1.94	9.72±0.88	6.94	6.83	3.18±0.24	2.83±0.34

[a] Protein determination carried out on 9000x *g* supernatant fraction.

[b] Mean ±S.D. for 4 animals.

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TABLE 9

MEASUREMENT OF AZOREDUCTASE INDUCTION IN CONTROL AND FLURAZEPAM-TREATED RATS

Number of Days of Pretreatment	μg *p*-aminobenzoic acid/mg protein/30 min incubation		
	Flurazepam-treated rats[a]		Control Rats[a]
	100 mg/kg	200 mg/kg	
3 days	0.52 ± 0.07	0.55 ± 0.10	0.41 ± 0.05
4 days	0.53 ± 0.09	0.55 ± 0.18	0.49 ± 0.09
5 days	0.53 ± 0.11	0.63 ± 0.10	0.48 ± 0.08

[a] Mean of 4 animals ±S.D.

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TABLE 10

EFFECT OF FLURAZEPAM TREATMENT IN RATS ON LIVER WEIGHTS AND PROTEIN LEVELS

Number of Days of Flurazepam Treatment	Mean Body Weight		Mean Liver Weight		Ratio[c]		mg Protein/g Liver	
	Dose I[a]	Dose II[b]	Dose I[a]	Dose II[b]	Dose I[a]	Dose II[b]	Dose I[a]	Dose II[b]
3	145.0±5.2	135.8±7.4	6.92±0.59	7.19±0.36	0.048	0.053	22.4	27.3
4	145.0±8.1	133.0±5.4	7.07±0.82	6.99±1.00	0.049	0.050	23.2	22.3
5	146.8±3.9	145.5±4.5	7.00±0.65	7.37±0.72	0.048	0.048	25.6	26.0
	Saline	Dose II	Saline	Dose II	Saline	Dose II	Saline	Dose II
Saline[d] vs. Flurazepam for 5 days	166.0±10.4	141.0±8.2	8.13±0.32	7.56±0.42	0.049	0.054	17.5	19.3

[a] Dose of flurazepam - 100 mg/kg i.p.

[b] Dose of flurazepam - 200 mg/kg i.p.

[c] Ratio of mean liver weight/mean body weight

[d] Control vehicle - 0.5 ml saline/100 g rat

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TABLE 11

EFFECT OF CHRONIC DOSING WITH FLURAZEPAM[a] ON CYTOCHROME ENZYME LEVELS IN RAT LIVER MICROSOMES

Cytochrome[b] P-450		Cytochrome[b] b_5	
Treated	Control	Treated	Control
0.484	0.300	0.434	0.400
0.467	0.344	0.434	0.408
0.467	0.292	0.450	0.392
0.434	0.320	0.400	0.430
0.463±0.021[c]	0.314±0.023[c]	0.430±0.021[c]	0.408±0.016[c]

[a] Rats dosed with flurazepam at 200 mg/kg i.p. for 5 days. Control animals received comparable doses of saline i.p. for 5 days.

[b] Results expressed as O.D. difference/2 mg protein/10 ml.

[c] Mean ±S.D. for 4 animals.

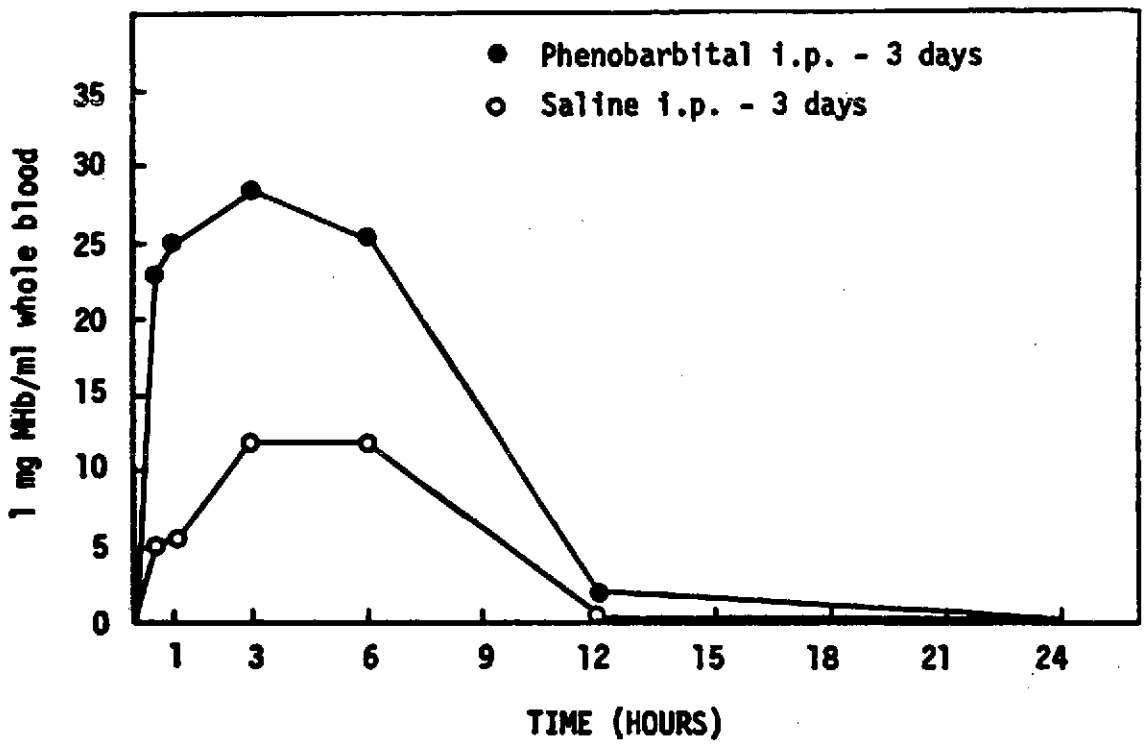

FIGURE 12

Concentration of methemoglobin following a single dose of aniline (500 mg/kg) in control and phenobarbital-treated rats.

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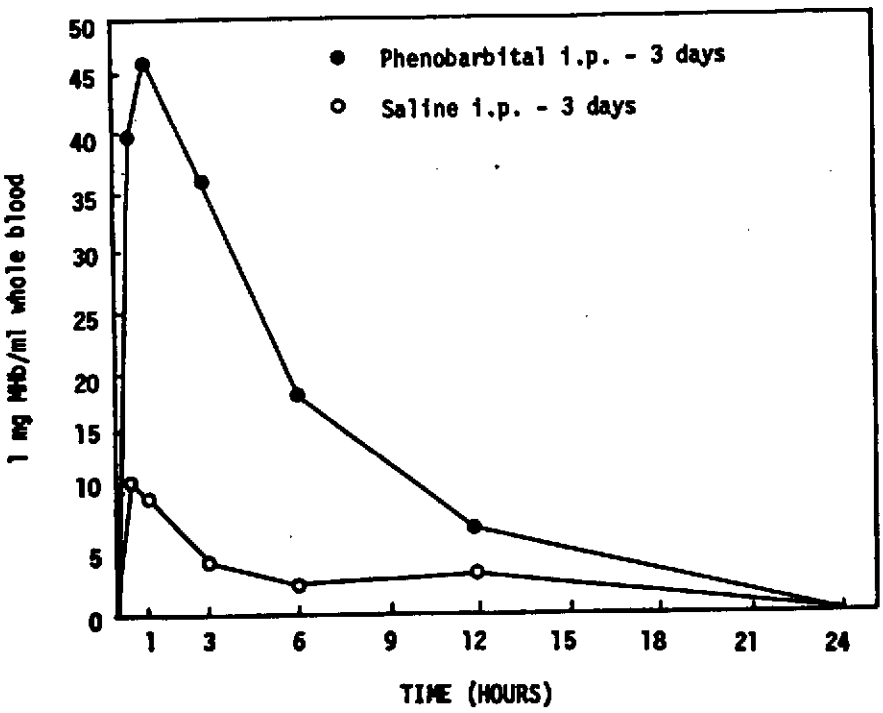

FIGURE 13

Concentration of methemoglobin following a single dose of aniline (1 g/kg) in control and phenobarbital-treated rats.

Since phenazopyridine is a drug that would normally be given in multiple doses, over extended periods of time, an experiment was run using repetitive doses of phenobarbital and phenazopyridine. The results for this experimental design are presented in Table 12 and show that even after 6 and 7 days of consecutive doses with phenobarbital and phenazopyridine, the concentration of methemoglobin in the blood of treated and control rats was not affected. The methemoglobin concentration approximated that reported by Heubner *et al.*[47] previously quoted as 1.3 ± 0.3 mg methemoglobin/ml whole blood.

A final experiment investigating the potential interaction of phenobarbital and phenazopyridine in the rat was set up involving high levels of phenazopyridine (500 mg/kg and 1 g/kg), doses equivalent to the test experiment run with aniline. All the previous experiments had been run with the dose of phenazopyridine set at 20 mg/kg, which is equivalent, on a mg/m^2 basis, to the dose for man. Two experimental designs were chosen: one group of animals received multiple doses of phenobarbital followed by a single dose of phenazopyridine (i.e., like the aniline experiment), while the other group received multiple doses of both phenobarbital and phenazopyridine. Appropriate control animals were run with each design.

The results from this experiment are presented in Tables 13 and 14. Table 13 reports the results of the single dose study and shows that no differences could be detected between the various groups. In these experiments, azoreductase had been induced in the phenobarbital-treated rats.

The results from multiple dosing with phenazopyridine (Table 14) indicate an increase in methemoglobin content in all phenazopyridine-treated animals (Groups 6-9) vs. control animals (Group 10). At most of the time periods, however, no differences could be seen between those animals treated with phenobarbital or saline, or those animals that received 1 g/kg or 500 mg/kg phenazopyridine. The possible exception

TABLE 12

LEVELS OF METHEMOGLOBIN IN CONTROL AND PHENOBARBITAL-TREATED RATS FOLLOWING MULTIPLE DOSES OF PHENAZOPYRIDINE

Treatment (dose)	Sample time (hr)	Concentration of MHb mg/ml whole blood - mean±S.D.[a](range) Six days[b]	Seven days[b]
Group 1			
Phenobarbital (80 mg/kg i.p.)	0.5	3.6 ± 3.9 (8.3 - <1.0)	<1.0
plus	1	1.5 ± 1.4 (2.7 - <1.0)	1.2 ± 1.2 (2.7 - <1.0)
Phenazopyridine (20 mg/kg p.o.)	3	2.3 ± 0.5 (2.7 - 1.5)	<1.0
	6	<1.0	<1.0
	24	<1.0	<1.0
Group 2			
Phenobarbital (80 mg/kg i.p.)	0.5		2.1 ± 1.4 (3.5 - <1.0)
plus	1		1.0 ± 0.9 (2.0 - <1.0)
1% Tween 80/saline (0.5 ml/	3		<1.0
100 g rat p.o.)	6		1.0 ± 0.9 (2.0 - <1.0)
	24		<1.0
Group 3			
Saline (0.5 ml/100 g rat i.p.)	0.5	2.0 ± 2.1 (4.2 - >1.0)	
plus	1	<1.0	
Phenazopyridine (20 mg/kg p.o.)	3	<1.0	
	6	<1.0	
	24	<1.0	

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TABLE 12 (CONTINUED)

Treatment (dose)	Sample time (hr)	Concentration of MHb mg/ml whole blood - mean±S.D.[a] (range) Six days	Seven days
Group 4			
Saline (0.5 ml/100 g rat i.p.)	0.5	2.5 ± 2.6 (6.2 - >1.0)	
plus	1	<1.0	
1% Tween 80/saline (0.5 ml/	3	<1.0	
100 g rat p.o.)	6	2.5 ± 2.5 (5.5 - >1.0)	
	24	<1.0	

[a] Mean of 5 animals

[b] Animal received both drugs or saline for 6 or 7 consecutive days

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TABLE 13

LEVELS OF METHEMOGLOBIN[a] IN CONTROL AND PHENOBARBITAL-TREATED RATS FOLLOWING A SINGLE DOSE OF PHENAZOPYRIDINE

Treatment →	(3 days Pb Phen. 1 g/kg Day 4)	(3 days Pb Phen. 500 mg/kg Day 4)	(3 days Saline Phen. 1g/kg Day 4)	(3 days Saline Phen. 500 mg/kg Day 4)	(3 days Saline 1% Tween 80/Saline)
30 min					
	7.5	5.5	1.5	4.0	4.0
	3.5	1.5	>0.5	>0.5	1.5
	0.7(4.8±2.8)[b]	>0.5(2.9±3.1)	2.7(1.0±1.1)	2.7(2.0±1.6)	4.0(2.0±1.9)
	5.5	7.0	0.7	2.7	0.7
	7.0	0.7	>0.5	0.7	>0.5
1 hour					
	6.7	2.0	2.0	2.0	>0.5
	1.5	3.5	7.0	5.5	1.5
	1.5(3.6±2.8)	1.5(1.9±1.0)	3.5(3.8±1.9)	4.0(2.7±2.1)	5.0(2.1±2.1)
	6.7	0.7	4.0	2.0	3.5
	1.5	2.0	2.7	>0.5	0.7
3 hours					
	4.0	4.0	2.7	15.0	4.0
	0.7	2.0	>0.5	11.0	5.5
	7.0(2.5±3.0)	>0.5(2.5±1.7)	>0.5(2.3±2.3)	7.0(8.4±5.5)	6.7(4.6±1.5)
	>0.5	2.7	4.0	>0.5	2.7
	0.7	4.0	5.0	9.0	4.0

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TABLE 13

Treatment →	(3 days Pb Phen. 1 g/kg Day 4)	(3 days Pb Phen. 500 mg/kg Day 4)	(3 days Saline Phen. 1 g/kg Day 4)	(3 days Saline Phen. 500 mg/kg Day 4)	(3 days Saline 1% Tween 80/Saline
	6 hours				
	>0.5	7.0	>0.5	1.5	2.7
	4.0	5.0	6.7	5.5	>0.5
	5.0(3.3±2.1)	2.0(4.3±2.0)	4.0(4.2±3.0)	13.0(6.4±4.2)	3.5(3.6±3.3)
	2.7	5.0	7.5	7.0	2.7
	5.0	2.7	2.7	5.0	9.0
	24 hours				
	9.0	7.5	5.0	7.0	0.7
	3.5(5.6±3.1)	5.5(4.9±1.8)		5.0	7.0(2.1±2.8)
	1.5	5.0	>0.5(6.0±4.8)	11.0(7.6±2.2)	0.7
	6.7	2.7	11.5	7.5	>0.5
	7.5	4.0	7.5	7.5	2.0

a Concentration of MHb expressed as mg/ml whole blood

b Mean±S.D.

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TABLE 14

LEVEL OF METHEMOGLOBIN[a] IN CONTROL AND PHENOBARBITAL-TREATED RATS FOLLOWING MULTIPLE DOSES OF PHENAZOPYRIDINE

Treatment →	Group 6 (6 days Pb 3 days Phen. 1 g/kg)	Group 7 (6 days Pb 3 days Phen. 500 mg/kg)	Group 8 (6 days Saline 3 days Phen. 1 g/kg)	Group 9 (6 days Saline 3 days Phen. 500 mg/kg)	Group 10 (6 days Saline 3 days 1% Tween 80/Saline)
30 min					
	5.5	10.0	13.0	24.0	0.7
	9.5	1.5	8.2	20.5	5.5(2.5±2.5)
	1.5(5.6±3.9)[b]	11.0(8.2±4.1)	11.5(10.1±2.1)	4.0(14.4±8.2)	>0.5
	2.0	7.0	8.2	14.5	1.5
	9.5	11.5	9.5	9.0	5.0
1 hour					
	11.0	14.5	17.5	8.2	>0.5
	7.5	4.0	8.2(9.0±5.7)	7.5(10.9±4.7)	>0.5(0.5±1.2)
	10.0(8.0±2.7)	11.0(9.4±6.4)	4.0	7.0	>0.5
	4.0	15.0	11.5	17.0	>0.5
	7.5	0.7	4.0	15.0	2.7
3 hours					
	7.5	0.7	>0.5	2.0	>0.5
	>0.5	7.0	>0.5	10.0	1.5
	5.5(3.7±3.2)	>0.5(5.3±4.7)	8.2(3.0±3.7)	4.0(5.4±4.9)	>0.5(0.3±0.7)
	5.0	9.5	1.5	11.0	>0.5
	0.7	9.5	5.5	>0.5	>0.5

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TABLE 14 CONTINUED

Treatment →	Group 6 (6 days Pb 3 days Phen. 1 g/kg)	Group 7 (6 days Pb 3 days Phen. 500 mg/kg)	Group 8 (6 days Saline 3 days Phen. 1 g/kg)	Group 9 (6 days Saline 3 days Phen. 500 mg/kg)	Group 10 (6 days Saline 3 days 1% Tween 80/Saline)
6 hours					
	7.0	5.0	49	2.7	1.5
	Dead	6.5	23	16.5	>0.5(1.0±0.9)
	12.5(9.5±7.9)	0.7(5.1±2.6)	11.5(24.0±15.6)	1.5(9.8±7.4)	2.0
	18.5	6.5	10.2	17.0	>0.5
	>0.5	7.0	26.5	11.5	1.5
24 hours					
	32.5	7.0	7.5	4.2	>0.5
	Dead	5.5	4.4	13.0	2.7(1.6 2.5)
	19.0(22.7±8.6)	0.7(7.7±6.5)	15.0(8.7±6.1)	7.0(6.2±4.2)	5.5
	Dead	18.5	15.0	2.0	>0.5
	16.5	1.0	1.5	5.0	>0.5

[a] Concentration of MHb expressed as mg/ml whole blood

[b] Mean ±S.D.

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is Group 8, where two out of five rats which were dosed with 1 g/kg phenazopyridine were dead 24 hr after the last dosing. Methemoglobin levels in the remaining three animals were significantly higher than the other groups.

The pattern of methemoglobin production seen in Table 14 is similar to that found by Herken[48] in cats, following the administration of 5 mg/kg nitrophenylhydroxylamine. There is an initial rise in the methemoglobin content of the blood, a marked decrease at 3 hr, followed by another rise at 6 hr. Herken referred to this increase/decrease/increase of methemoglobin formation as the "two-phase" formation which might be based upon the velocity of the reconversion of MHb to hemoglobin.

Preliminary experiments in our laboratory confirmed the fact that rabbits are poor experimental animals to use when investigating methemoglobin formation. The rabbit normally has very little methemoglobin present in its blood (0.4 mg/ml) as reported by Heubner *et al.*[47] An initial experiment investigating the potential interaction of multiple doses of phenobarbital and phenazopyridine in rabbits was set up along the lines of the rat experiments described above. Blood samples were analyzed for MHb and no differences were found between control and treated animals. Although the method of Evelyn and Mallory[45] is not sensitive enough to determine accurately MHb in the small concentrations normally present in rabbit blood, it would detect any prominent increases.

An experiment was run giving large doses of aniline to control and phenobarbital-treated animals. Rabbits were predosed for 3 days with either 50 mg/kg, i.p. phenobarbital or a comparable amount of the control vehicle, saline. Although the level of MHb in the rabbit's blood increased, the amount found was still very low when compared to the results of the rat experiment with aniline.

Different species of animals have shown varying susceptibilities to MHb formation by a particular compound. Using as a criterion of sensitivity the reciprocal of the dose required to produce a stated concentration of methemoglobin, Lester[49] found that the cat was the most sensitive species. If this species is listed as 100, the sensitivities of the other species tested are as follows for the drug, acetanilid: man, 56; dog, 29; rat, 5; rabbit, 0; monkey, 0. Cats are an extremely difficult species to work with and healthy cats are not easy to procure for experimental purposes.

As the rabbit was unacceptable, we investigated the possibility of using the dog as the second species for this work. A single experiment run with aniline at 200 mg/kg, p.o., without predosing with phenobarbital was run to test the ease with which this species produces MHb. Figure 14 shows the rate of methemoglobin production over the 24-hr sampling period. The dose of aniline was equivalent on a mg/m^2 basis to the dose given to rats but the amount of MHb produced was greater in the dog.

Methemoglobin formation was then investigated in dogs pretreated with phenobarbital and dosed with phenazopyridine. Two dogs received phenobarbital at 50 mg/kg, i.p., and two received a comparable volume of saline. Four hours later, all four dogs received an oral dose of phenanopyridine (50 mg/kg). Blood samples were tested for MHb content at 30 min, 1, 3, and 6 hr after the animals received phenazopyridine. This schedule of dosing and sampling was repeated in the same four dogs for a total of four days. The results, although somewhat erratic, indicate a trend towards increased production of MHb following multiple dosing with phenazopyridine. Phenobarbital, however, did not appear to potentiate this effect.

Flurazepam was the second drug investigated for possible potentiation of methemoglobin production by phenazopyridine. From the above results involving phenobarbital and phenazopyridine, it appeared

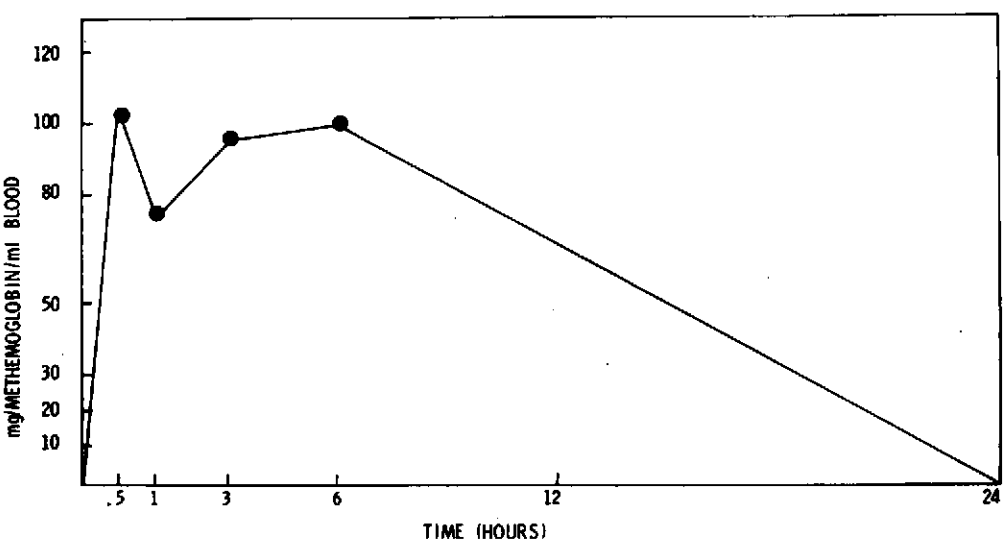

FIGURE 14

Level of MHb found in the blood of a dog following a single dose (200 mg/kg) of aniline.

that even if flurazepam did induce production of azoreductase, the interaction between flurazepam and phenazopyridine in all likelihood would not increase the production of MHb. This supposition was further supported by the fact that flurazepam did not significantly induce azoreductase and did not affect the cytochrome concentrations. Nevertheless, several experiments were carried out in rats.

Rats received both drugs for 6 or 7 days and, as seen in Table 15, no significant differences in MHb concentration were noted among any of the four groups of treated animals. A final experiment involving high levels of phenazopyridine (500 mg/kg and 1 g/kg) was set up along the lines described in the section on phenobarbital.

The results of this latter experimental design are presented in Tables 16 and 17. Table 16 shows that a single dose of phenazopyridine had no effect on methemoglobin concentrations, with the possible exception at 24 hr after dosing which does show a slight increase in methemoglobin in the plasma of phenazopyridine-treated rats.

The results from multiple dosing with phenazopyridine and flurazepam (Table 17) indicate an increase in methemoglobin content in phenazopyridine-treated animals (Groups 6-9) over control animals (Group 10). No significant differences were noted between the two dose levels of phenazopyridine or between flurazepam-treated animals over saline-dosed animals. One interesting point is that three out of five animals which received flurazepam and the highest dose of phenazopyridine were found dead at 24 hr. This is similar to the results with phenobarbital. However, methemoglobin levels in the two remaining animals were not greatly increased over the other groups. The increase/decrease/increase pattern of MHb production noted in the phenobarbital experiment was seen in this experiment but at a much lower level.

Rats pretreated with either phenobarbital or flurazepam followed by phenazopyridine given as a single dose or a series of daily

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TABLE 15

LEVELS OF METHEMOGLOBIN IN CONTROL AND FLURAZEPAM-TREATED RATS FOLLOWING MULTIPLE DOSES OF PHENAZOPYRIDINE

Treatment (dose)	Sample time (hr)	Concentration of MHb mg/ml whole blood - mean ±S.D.[a] (range) Six days[b]	Seven days[b]
Flurazepam (200 mg/kg i.p.) plus Phenazopyridine (20 mg/kg p.o.)	0.5	3.9 ± 2.4 (7.0 - 1.5)	1.9 ± 2.4 (5.0 - <0.5)
	1	3.2 ± 1.1 (4.2 - 2.0)	0.8 ± 0.9 (1.5 - <0.5)
	3	1.2 ± 0.7 (2.0 - 0.7)	6.2 ± 7.4 (17.0- 0.7)
	6	1.5 ± 0.9 (2.7 - 0.7)	5.5 ± 4.4 (9.5 - 0.7)
	24	1.2 ± 0.6 (1.5 - <0.5)	1.8 ± 0.3 (2.0 - 1.5)
Group 2			
Flurazepam (200 mg/kg i.p.) plus 1% Tween 80/saline (0.5 ml/100 100 g rat p.o.)	0.5	2.0 ± 1.3 (4.2 - 0.7)	
	1	2.1 ± 0.8 (3.5 - 1.5)	
	3	0.7 ± 1.6 (3.5 - <0.5)	
	6	1.1 ± 0.6 (2.0 - 0.7)	
	24	2.1 ± 1.9 (5.5 - 0.7)	
Group 3			
Saline (0.5 ml/100 g rat i.p.) plus Phenazopyridine (20 mg/kg p.o.)	0.5	1.7 ± 1.1 (2.7 - 1.5)	1.1 ± 1.6 (3.5 - <0.5)
	1	1.0 ± 0.7 (1.5 - <0.5)	1.0 ± 1.1 (2.7 - <0.5)
	3	0.6 ± 0.6 (1.5 - <0.5)	4.5 ± 3.4 (9.5 - <0.5)
	6	0.3 ± 0.4 (0.7 - <0.5)	2.2 ± 3.0 (7.0 - <0.5)
	24	0.7 ± 0.6 (1.5 - <0.5)	1.1 ± 1.0 (2.7 - 0.7)

CONTINUED . . .

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TABLE 15 (CONTINUED)

Treatment (dose)	Sample time (hr)	Concentration of MHb mg/ml whole blood - mean±S.D.[a] (range) Six days[b]	Seven days[b]
Group 4			
Saline (0.5 ml/100 g rat i.p.) plus 1% Tween 80/saline (0.5 ml/100 g rat p.o.)	0.5		1.7 ± 1.7 (4.2 - <0.5)
	1		2.7 ± 1.4 (5.0 - 1.5)
	3		2.3 ± 3.0 (7.5 - <0.5)
	6		4.8 ± 3.7 (8.2 - 0.7)
	24		1.3 ± 0.4 (1.5 - 0.7)

[a] Mean of 5 animals

[b] Animal received both drugs or saline for 6 or 7 consecutive days

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TABLE 16

LEVELS OF METHEMOGLOBIN[a] IN CONTROL AND FLURAZEPAM-TREATED RATS FOLLOWING A SINGLE DOSE OF PHENAZOPYRIDINE

Treatment →	Group 1 (3 days Flur. Phen. 1 g/kg Day 4)	Group 2 (3 days Flur. Phen. 500 mg/kg Day 4)	Group 3 (3 days Saline Phen. 1 g/kg Day 4)	Group 4 (3 days Saline Phen. 500 mg/kg Day 4)	Group 5 (3 days Saline 1% Tween 80/Saline
30 min					
	7.0	0.7	1.5	4.0	4.0
	6.7	5.5	>0.5	>0.5	1.5
	4.0(5.1±1.6)[b]	3.5(3.3±2.2)	2.7(1.0±1.1)	2.7(2.0±1.6)	4.0(2.0±1.9)
	4.0	1.5	0.7	2.7	0.7
	4.0	5.5	>0.5	0.7	>0.5
1 hour					
	3.5	1.5	2.0	2.0	>0.5
	7.0	2.7	7.0	5.5	1.5
	3.5(3.8±2.6)	3.5(2.7±1.0)	3.5(3.8±1.9)	4.0(2.7±2.1)	5.0(2.1±2.1)
	>0.5	2.0	4.0	2.0	3.5
	5.0	4.0	2.7	>0.5	0.7
3 hours					
	6.7	>0.5	2.7	15.0	4.0
	3.5	2.7	>0.5	11.0	5.5
	2.7(4.5±2.2)	>0.5(2.4±4.1)	>0.5(2.3±2.3)	7.0(8.4±5.5)	6.7(4.6±1.5)
	7.0	>0.5	4.0	>0.5	2.7
	2.7	9.5	5.0	9.0	4.0

CONTINUED. . .

TABLE 16 CONTINUED

Treatment →	Group 1 (3 days Flur. Phen. 1 g/kg Day 4)	Group 2 (3 days Flur. Phen. 500 mg/kg Day 4)	Group 3 (3 days Saline Phen. 1 g/kg Day 4)	Group 4 (3 days Saline Phen. 500 mg/kg Day 4)	Group 5 (3 days Saline 1% Tween 80/ Saline)
	6 hours				
	6.7	2.0	>0.5	1.5	2.7
	5.5	4.0	6.7	5.5	>0.5
	1.5(5.8±4.3)	10.0(4.3±3.8)	4.0(4.2±3.0)	13.0(6.4±4.2)	3.5(3.6±3.3)
	12.5	5.5	7.5	7.0	2.7
	2.7	>0.5	2.7	5.0	9.0
	24 hours				
	7.0	10.0	5.0	7.0	0.7
	8.2	9.5		5.0	7.0(2.1 2.8)
	11.0(10.7±3.2)	5.5(8.0±3.1)	>0.5(6.0±4.8)	11.0(7.6±2.2)	0.7
	12.5	11.0	11.5	7.5	>0.5
	15.0	4.0	7.5	7.5	2.0

[a] Concentration of MHb expressed as mg/ml whole blood

[b] Mean ±S.D.

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TABLE 17

LEVELS OF METHEMOGLOBIN[a] IN CONTROL AND FLURAZEPAM-TREATED RATS FOLLOWING MULTIPLE DOSES OF PHENAZOPYRIDINE

Treatment →	Group 6 (6 days Flur. 3 days Phen. 1 g/kg)	Group 7 (6 days Flur. 3 days Phen. 500 mg/kg)	Group 8 (6 days Saline 3 days Phen. 1 g/kg)	Group 9 (6 days Saline 3 days Phen. 500 mg/kg)	Group 10 (6 days Saline 3 days 1% Tween 80/Saline
30 min					
	9.0	8.2	13.0	24.0	0.7
	9.0	19.0	8.2	20.5	5.5(2.5±2.5)
	10.0(9.7±2.4)[b]	7.0(9.9±6.2)	11.5(10.1±2.1)	4.0(14.4±8.2)	>0.5
	13.5	2.7	8.2	14.5	1.5
	7.0	12.5	9.5	9.0	5.0
1 hour					
	11.5	11.0	17.5	8.2	>0.5
	(10.4±1.9)	19.5(11.4±5.6)	8.2(9.0±5.7)	7.5(10.9±4.7)	>0.5(0.5±1.2)
	12.5	13.0	4.0	7.0	>0.5
	8.2	4.0	11.5	17.0	>0.5
	9.5	9.5	4.0	15.0	2.7
3 hours					
	>0.5	7.0	>0.5	2.0	>0.5
	7.5	13.5	>0.5	10.0	1.5
	7.0(4.5±4.2)	1.5(5.2±5.1)	8.2(3.0±3.7)	4.0(5.4±4.9)	>0.5(0.3±0.7)
	8.2	1.5	1.5	11.0	>0.5
	>0.5	2.7	5.5	>0.5	>0.5

CONTINUED . . .

TABLE 17 CONTINUED

Treatment →	Group 6 (6 days Flur. 3 days Phen. 1 g/kg)	Group 7 (6 days Flur. 3 days Phen. 500 mg/kg)	Group 8 (6 days Saline 3 days Phen. 1 g/kg)	Group 9 (6 days Saline 3 days Phen. 500 mg/kg)	Group 10 (6 days Saline 3 days 1% Tween 80/Saline)
	6 hours				
	-	8.2	49	2.7	1.5
	15.5	19.0	23	16.5	>0.5(1.0±0.9)
	11.0(10.9±3.4)	6.5(8.6±6.2)	11.5(24.0±15.6)	1.5(9.8±7.4)	2.0
	8.2	6.5	10.2	17.0	>0.5
	9.0	6.5	26.5	11.5	1.5
	24 hours				
	9.0	1.5	7.5	4.2	>0.5
	Dead	5.5	4.4	13.0	2.7(1.6±2.5)
	Dead(8.4±0.8)	0.7(4.4±3.1)	15.0(8.7±6.1)	7.0(6.2±4.2)	5.5
	Dead	7.0	15.0	2.0	>0.5
	7.8	7.5	1.5	5.0	>0.5

a Concentration of MHb expressed as mg/ml whole blood

b Mean ±S.D.

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doses did not greatly affect the level of methemoglobin found in the blood. Control rats dosed with a known methemoglobin producer, aniline, showed increased levels of MHb over normal animals, while those animals pretreated with phenobarbital showed an even greater level of MHb in the blood. This experiment clearly demonstrated the soundness of the experimental design and adequateness of the methods employed. This fact suggests that the potential interaction of phenobarbital and flurazepam with phenazopyridine is not affecting methemoglobin production.

Experiments involving high doses of phenazopyridine suggest that it is not the interaction with enzyme inducers that is important but the dose of phenazopyridine itself. Production of methemoglobin by phenazopyridine has been reported in the dog and in humans but only in overdose situations. Increased levels of methemoglobin were found in the rat after multiple doses of 1 g/kg and 500 mg/kg phenazopyridine in control-, phenobarbital-, and flurazepam-treated rats. The trend toward increased levels of MHb was also noted in the dog experiment following 50 mg/kg phenazopyridine. The chance that phenobarbital and flurazepam are reacting in some way with phenazopyridine is still a possibility and is suggested by the fact that phenobarbital- and flurazepam-treated animals died if they received high levels of phenazopyridine, whereas control animals did not.

C. Studies on the Effects of Diphenoxylate on the Bioavailability of Nitrofurantoin

1. Summary

Experiments were conducted in the dog and in man to determine whether premedication with diphenoxylate would result in altered bioavailability of Furadantin® or Macrodantin®. The rate of drug excretion in the urine was determined and a plot of percent drug absorbed vs. time was constructed for each dosage form, with and without diphenoxylate pretreatment. Drug in the urine was determined by both a chemical and microbiological method.

In the dog, two doses of diphenoxylate were studied with each dog serving as its own pre-diphenoxylate control. Diphenoxylate was found to alter substantially the excretion pattern of both forms of nitrofurantoin, generally increasing total absorption.

On the basis of the work originally done in dogs, the present contract was extended to include similar nitrofurantoin/diphenoxylate studies in man. Six subjects received both dosage forms of nitrofurantoin alone and in combination with diphenoxylate. The results of these studies are inconclusive. The mechanism of action and an explanation of the interaction between diphenoxylate and nitrofurantoin still remains unclear. In man, the interaction does not appear to be significant, affecting only two subjects out of six and with only one dose formulation (Furadantin®).

2. Introduction

Diphenoxylate is considered to be a drug of choice for the management of diarrhea[50] and has been used by astronauts for control of the bowel. It is clinically related to meperidine. A common side-effect of morphine and, less so, meperidine, is constipation.[51] With diphenoxylate, action on intestinal motility is exaggerated while control effects are minimal.

Quantitative understanding of the process of drug absorption has not been achieved but several factors are known to be involved. These include the rate of dissolution of the dosage form, the particle size of the drug, pH of the intestinal content, and the presence of parasympothetic blocking drugs and other drugs which may decrease intestinal motility.

Nitrofurantoin is marketed in two solid dosage forms, microcrystalline in a tablet (Furadantin®, Eaton Laboratories), and macrocrystalline in a capsule (Macrodantin®, Eaton Laboratories). Oral absorption of these dosage forms have been studied by following urinary excretion of drug in the dog[30] although detailed kinetics were not presented. It was established that the rate of absorption of these two

crystalline forms of nitrofurantoin is different, the macrocrystalline form being more slowly absorbed. It has also been established[30] that with the macrocrystalline dosage form, the amount of antibiotic excreted in urine is less than that found with the microcrystalline form during the first 24 hr after the first dose. It was noted, however, that urinary antibiotics exceeded minimum effective antibacterial levels with both dosage forms.

The following work was done to investigate the possibility that metabolism by intestinal micro-organisms and/or increased residence time of the drug in the gut may play a role in determining how much parent drug is ultimately absorbed. It should be noted that drug metabolism by intestinal micro-organisms can account for a wide variety of transformation.[52] The overall metabolic profile of a drug may be changed by metabolism in the gut which, in turn, may be altered by increasing residence time in the gut.

Studies to determine the effect of diphenoxylate on the bioavailability of nitrofurantoin have been carried out in dog and man. In view of the purpose of the study, the commercial dosage forms of Furadantin ® and Macrodantin ® were used, rather than bulk drug. These studies were done using four female beagle dogs and six male subjects. The rate of absorption of antibiotic and total antibiotic absorbed was compared in each group and with each dosage form.

3. Materials and Methods

Control experiments determining the rate of ^{14}C-inulin clearance were run on each dog as a check on kidney function before the dog was used for experimentation. ^{14}C-inulin (5 mg/ml in 0.9% NaCl, specific activity 4 mCi/mg) was infused into the right paw at approximately 1 ml/min. Blood samples were collected at 15, 30, 60, 90, 120 min and hourly thereafter for 8 hr. Each dog was catheterized and urine was collected hourly during the inulin infusion.

One hour after the start of the inulin each dog was dosed orally with either 100 mg of Furadantin® or, in a separate experiment, 100 mg of Macrodantin®. The level of antibiotic in the urine was measured in samples collected hourly to establish control levels for both dosage forms in each dog. ^{14}C-inulin clearance studies were also run on each dog at the end of the study to make sure kidney functioning had remained satisfactory throughout the series of experiments.

Nitrofurantoin in urine was analyzed using the chemical method of Conklin and Hollifield[53] and also by a modified microbiological method of Jones *et al.*[54] In the Conklin and Hollifield procedure, drug is extracted from urine with nitromethane, alkaline reagent is added to produce a visible color and the concentration of the drug determined by spectrophotometry. The method has a sensitivity of 10 µg/ml and a standard curve that is linear to 100 µg/ml (Figure 15).

One ml urine plus 4 ml 0.1N HCl was extracted with 10 ml nitromethane by mixing for 2 min and the biphase was centrifuged for 10 min. An aliquot (4 ml) of the nitromethane layer was removed to a clean test tube and 0.5 ml of 0.04M Hyamine solution (Packard) was added. Each sample was mixed well and allowed to stand at least 1 min before reading the optical density on a Zeiss spectrophotometer at 400 nm. Pure nitromethane was used to set the instrument to zero absorbence. Samples were read within 30 min after the addition of the Hyamine solution.

Urine samples were also assayed for growth inhibitory activity against *Escherichia coli* by a modified version of the tube method of Jones *et al.*[54]

Stock cultures and assays were grown in nutrient broth (Difco) with pH adjusted to 5.5 and incubation at 37°C. Incubation was for 16-18 hr and growth was measured nephelometrically at 550 nm in a Lumetron colorimeter.

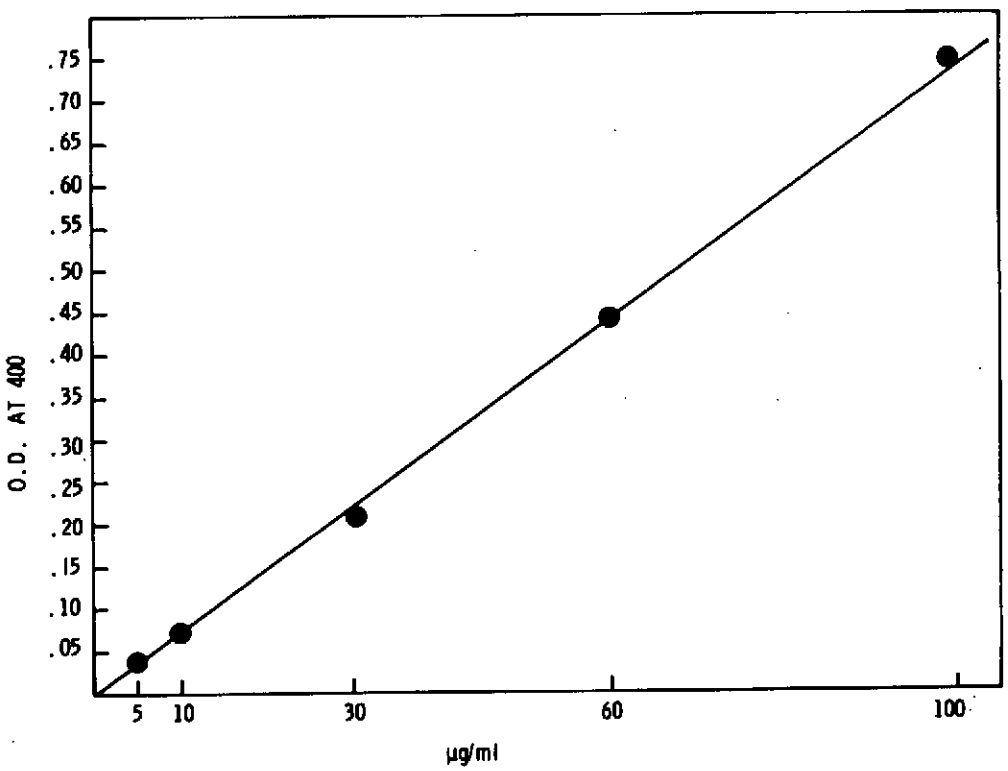

FIGURE 15

Standard curve of nitrofurantoin in urine

For each assay, a dose-response assay to nitrofurantoin (at 0, 1, 2, 4, and 8 μg/ml) was determined in 5 ml of culture median which contained 0, 0.25, 0.5, or 1 ml of zero time urine from one of the test dogs. Each test urine sample was assayed at 0.25, 0.5, and 1 ml for 5 ml culture.

Most control urines showed progressively greater inhibition ("protection") of nitrofurantoin activity as the volume was increased and there were occasionally indications of similar effects in the test urines. For this reason, the nitrofurantoin equivalent for each test volume of any given sample was calculated from the dose-response curve for the same control urine volume. The final nitrofurantoin value for a sample was calculated from the average of all the test volumes showing meaningful inhibition. Most values in the final results were thus based on 2 or 3 points except where low activity produced inhibition only in the 1 ml assay tube.

Drug equivalents (μg/ml) as calculated by this method compare with values obtained by analyzing the urine according to the clinical method of Conklin and Hollifield. The latter method is reported as specific for the parent drug. As there has been some speculation[55] that the metabolites of the nitrofurans, presumably reduced compounds, may be more active antibiotics than the parent drug, it was hoped that the microbiological method would pick up other biologically active forms of the drug. No other active species were found as the level of antibiotic determined by both methods was similar. This being the case, the results from only one method, the chemical method, are presented.

In the dog studies, the effect of two dose levels of diphenoxylate (2.5 mg/kg and 0.5 mg/kg) on the bioavailability of both the macrocrystalline (Macrodantin®) and the microcrystalline (Furadantin®) dose forms of nitrofurantoin were investigated. The animals were dosed orally with diphenoxylate 18 hr before receiving either Macrodantin® or Furadantin® p.o. To assure accurately timed urine collections,

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each dog was catheterized during the first 6 hr after receiving the nitrofurantoin. A slow i.v. infusion (1 ml/min) of physiological saline was given during the period to hydrate the animal and insure a good flow of urine. The animals were fasted at least 4 hr prior to dosing with diphenoxylate and food was withheld until the catheter was removed 6 hr following dosing with either dose form of nitrofurantoin. Water was supplied *ad libitum*.

The clinical study in man was carried out by Medical and Technical Research Associates, Inc., Needham, Massachusetts (FDA approved, registered as IND 9806). Six subjects were used in these studies and all received both dosage forms of nitrofurantoin (Macrodantin® and Furadantin®) alone and in combination with diphenoxylate.

Subjects were selected who were without a history of significant past illness and were found to have a normal medical examination immediately prior to initiation of the study.

Subjects selected for the study were between twenty-one and forty years of age and weighed between 135 and 185 pounds. Laboratory safety tests were undertaken prior to the study and consisted of hemoglobin, hemaocrit, WBC with differential, glucose-6-dehydrogenase, and an SMA 12/60 series of clinical tests. All laboratory parameters were found to be normal before the subjects were admitted to the study. Subjects were particularly screened and excluded if found to possess:

(1) Sensitivity to nitrofurantoin or related drugs
(2) Sensitivity to diphenoxylate or related drugs
(3) Any history of gastrointestinal complaints
(4) Sensitivity to atropine or atropic type drugs
(5) Impaired renal or hepatic function
(6) Abnormal glucose-6-phosphate dehydrogenase.

All volunteers were off any type of medication for two weeks prior to the study and throughout the duration of the study. The schedule is outlined in Table 18.

TABLE 18

THE SEQUENCE OF ADMINISTERING DIPHENOXYLATE AND NITROFURANTOIN IN SIX HUMAN SUBJECTS

Weeks of Study:	1	2	3	4
Drug administered:	SUBJECT NUMBER			
Macrodantin®	1, 2, 3	4, 5, 6		
Furadantin®	4, 5, 6	1, 2, 3		
Diphenoxylate plus Macrodantin®			1, 2, 3	4, 5, 6
Diphenoxylate plus Furadantin®			4, 5, 6	1, 2, 3

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A 15-mg dose of diphenoxylate (two 2.5-mg tablets administered three times daily) was given for three consecutive days prior to dosing with nitrofurantoin to allow for a buildup of drug and metabolites in the enterohepatic pool. This expectation was based on the pharmacokinetics of diphenoxylate in man.[24,25] It was presumed that the plasma levels of diphenoxylate in man after three consecutive doses of 15 mg would approximate those obtained with the smaller dose (0.5 mg/kg) used in the dog study. The nitrofurantoin (200 mg) was given orally approximately 24 hr after the last dose of diphenoxylate. This dose appears appropriate based on the pharmacokinetics of nitrofurantoin in man.[56] The subjects were given water prior to and for the first 6 hr after administering the nitrofurantoin. Urine was collected at 2, 4, 6, 8, 12, and 24 hr after the nitrofurantoin dosing. The subjects were fasted, except for water, from the previous evening until the noon meal.

4. Results and Discussion

(a) Studies in Dogs. Results from control experiments utilizing both dosage forms of nitrofurantoin, Furadantin® tablets and Macrodantin® capsules, in all the dogs are illustrated in Figures 16 and 17. Using the clinical method of assay for nitrofurantoin, 26-45% of the oral dose of Furadantin® (Figure 16) was excreted in the first 12 hr, compared to 10-16% of the oral dose of Macrodantin® (Figure 17) excreted over the same period. As seen in Figures 18, 19, and 20, dogs 100, 101, and 102 exhibited similar excretion patterns; the macrocrystalline dosage (Macrodantin®) appeared to be more slowly absorbed than the microcrystalline form (Furadantin®) and the extent of absorption of the macrocrystalline form is less than the microcrystalline, as indicated by a smaller amount excreted during the 48-hr collection period. These results agree favorably with the work of Conklin *et al.*[30] They observed differences in urinary drug recoveries and in urinary drug excretion patterns between the two dosage forms suggesting a slower rate of absorption and a lower amount of antibiotic absorbed for the macrocrystals as opposed to the microcrystals. Dog 103, on the other hand, appeared

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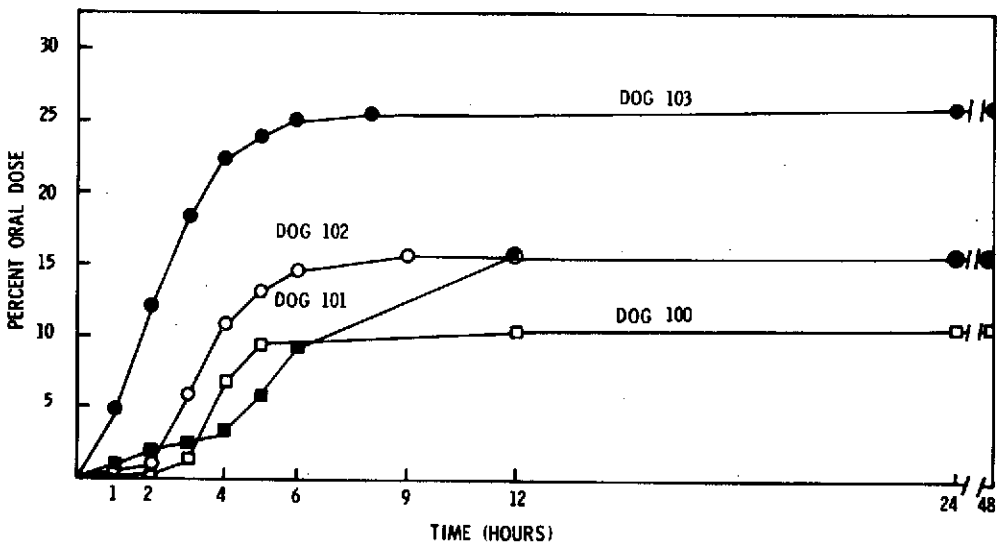

FIGURE 16

Cumulative excretion of Macrodantin® in the urine of all the dogs. Chemical method used to analyze urine.

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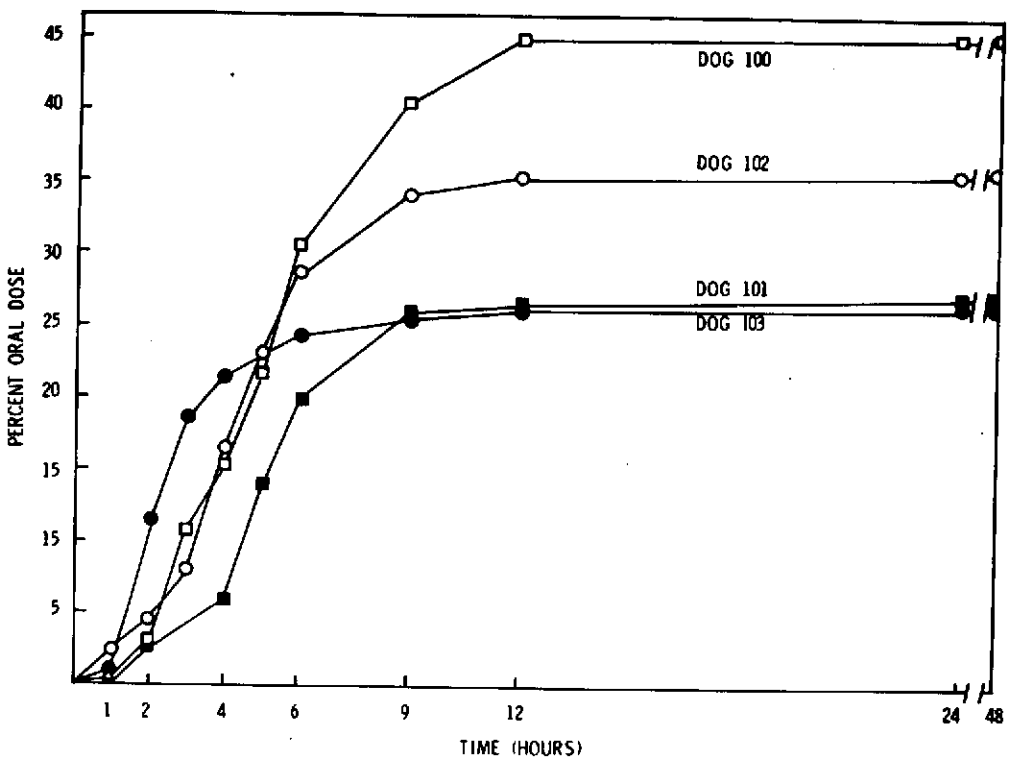

FIGURE 17

Cumulative excretion of Furadantin® in the urine of all the dogs. Chemical mehtod used to analyze urine.

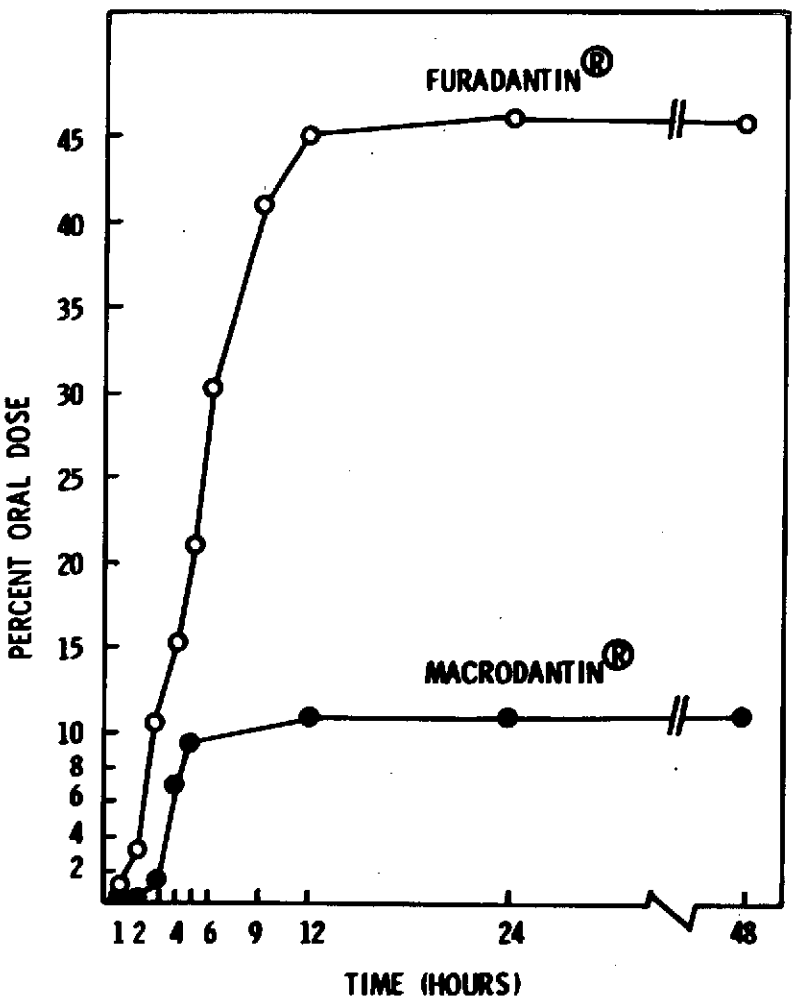

FIGURE 18

Cumulative excretion of Macrodantin® and Furadantin® in the urine of Dog 100. Chemical method used to analyze urine.

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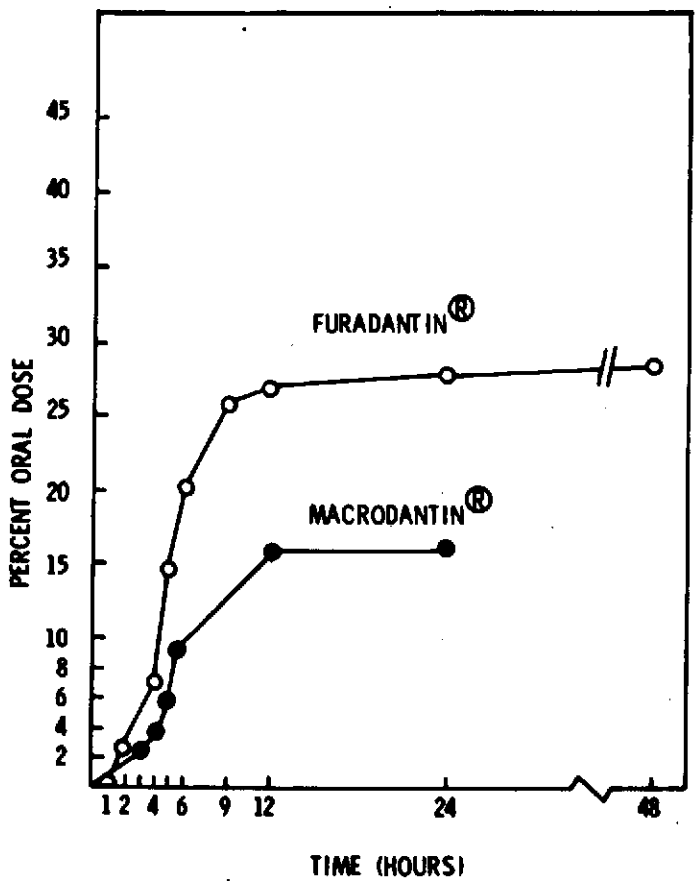

FIGURE 19

Cumulative excretion of Macrodantin® and Furadantin® in the urine of Dog 101. Chemical method used to analyze urine.

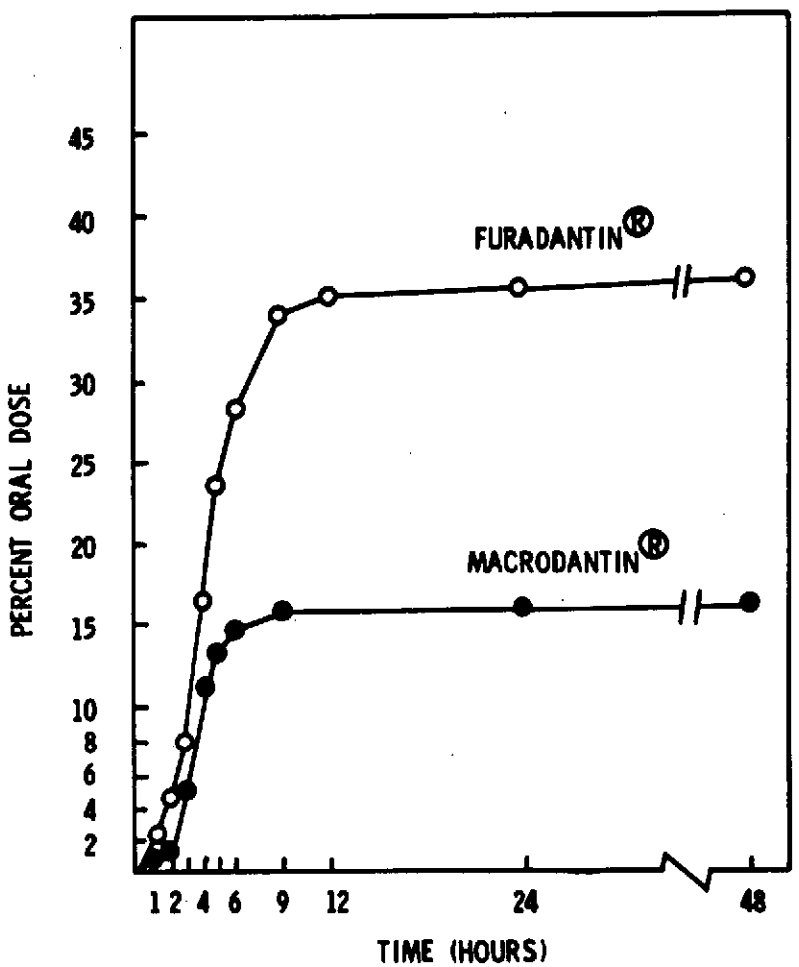

FIGURE 20

Cumulative excretion of Macrodantin® and Furadantin® in the urine of Dog 102. Chemical method used to analyze urine.

to handle both dosage forms similarly (Figure 21). The total amount of drug excreted (26-27%) and the pattern of excretion were similar for each dose form.

Results from the combination studies involving two dose levels of diphenoxylate (0.5 and 2.5 mg/kg) and both dosage forms of nitrofurantoin are presented in Figures 22-29. With two exceptions, which will be noted below in detail, diphenoxylate appeared to enhance the absorption of both forms of nitrofurantoin. There did not, however, appear to be a clear relationship between the amount of diphenoxylate given and the increased amount of nitrofurantoin absorbed, the data varying with each dog. In some animals, the lower dose of diphenoxylate (0.5 mg/kg) yielded a greater percentage of drug absorbed than the higher dose of 2.5 mg/kg.

Two dogs with different dosage forms showed a reversal of the general trend toward increased absorption. At both diphenoxylate levels, Dog 102 absorbed less Macrodantin® and Dog 100 absorbed less Furadantin® than they absorbed in control experiments without diphenoxylate (Figures 24 and 26).

The experiments investigating the interaction of diphenoxylate and Macrodantin® are illustrated in Figures 22-25. Dog 100 (Figure 23) showed increased drug absorption following the higher dose of diphenoxylate (2.5 mg/kg) but no statistical increase following the lower (0.5 mg/kg) dose. The pattern of excretion remained unchanged. Dog 101 (Figure 23) showed an increase with both dose levels but a greater increase occurred with the lower dose of diphenoxylate than with the higher dose, the largest increase occurring after the 2-hr sample at Dose II (0.5 mg/kg).

Dog 102 (Figure 24), on the other hand, showed a decrease in the amount of Macrodantin® absorbed following both dose levels of diphenoxylate. There was also a change in the pattern of drug excretion. In the diphenoxylate studies, there appeared to be an initial immediate increase in drug absorption which leveled off after 3 hr. In control

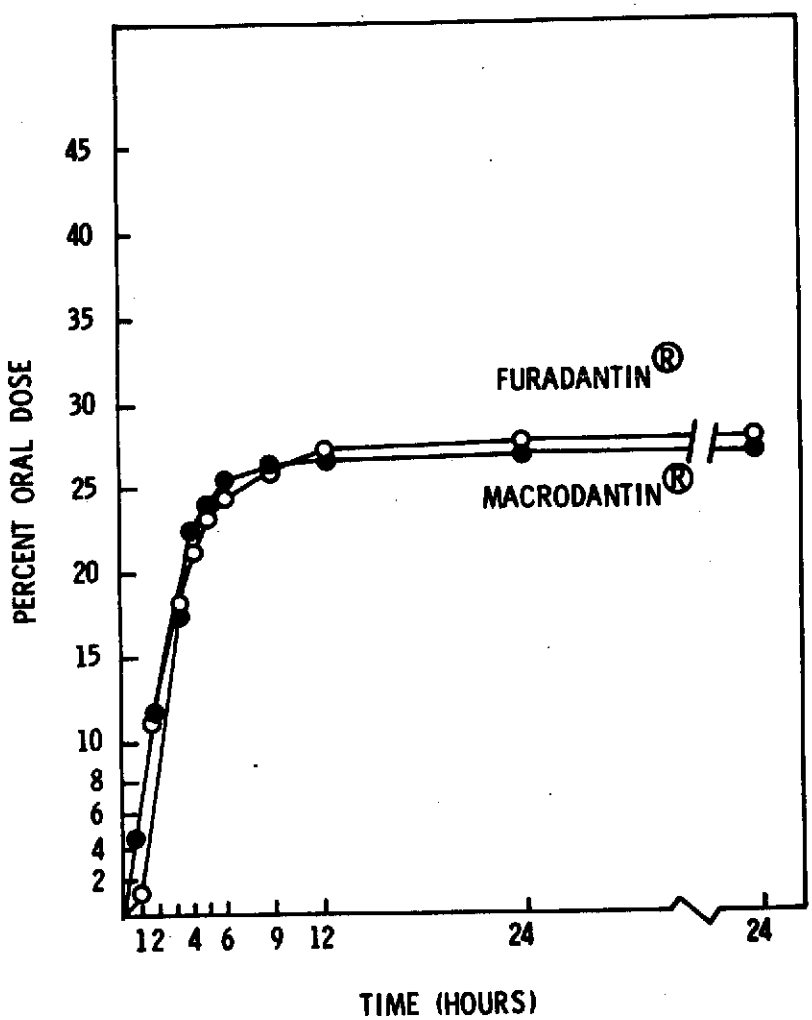

FIGURE 21

Cumulative excretion of Macrodantin® and Furadantin® in the urine of Dog 103. Chemical method used to analyze urine.

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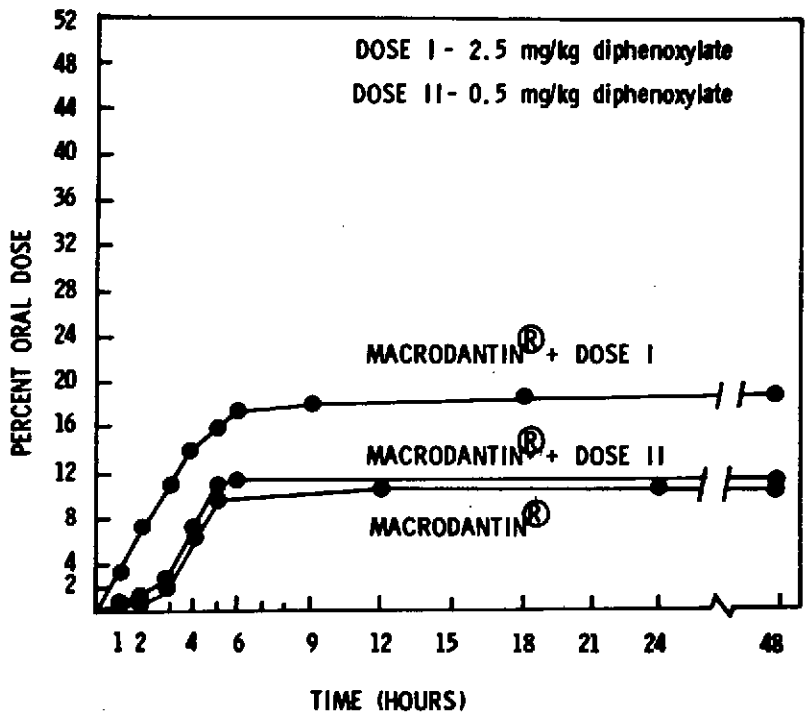

FIGURE 22

Cumulative excretion of Macrodantin® in urine of Dog 100 with/without diphenoxylate. Chemical method used to analyze urine.

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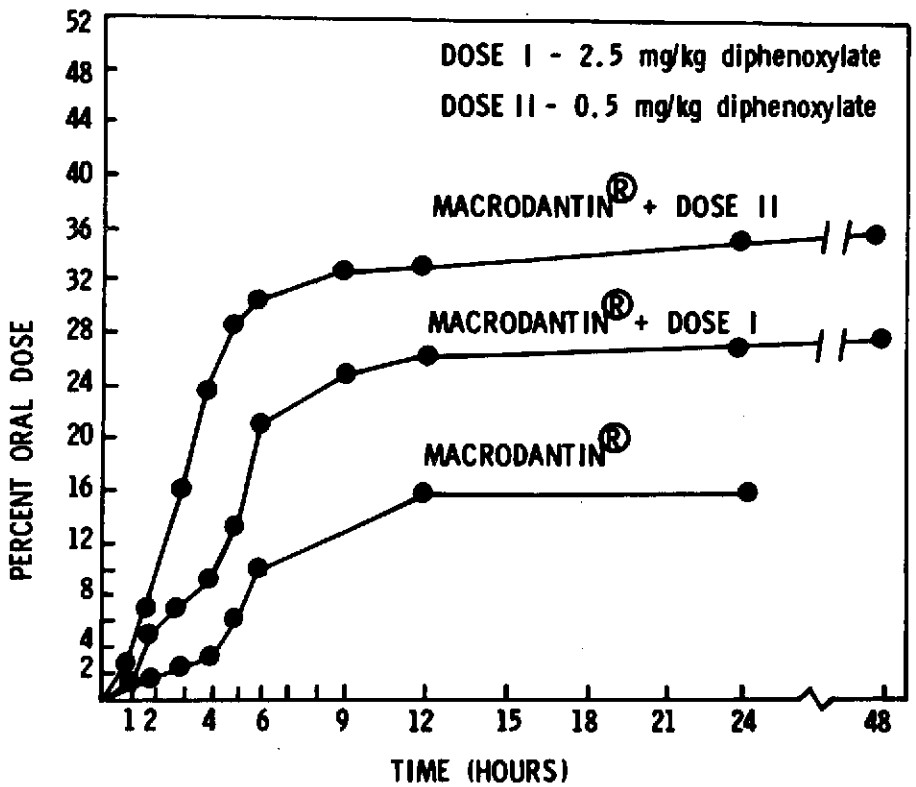

FIGURE 23

Cumulative excretion of Macrodantin® in urine of Dog 101 with/without diphenoxylate. Chemical method used to analyze urine.

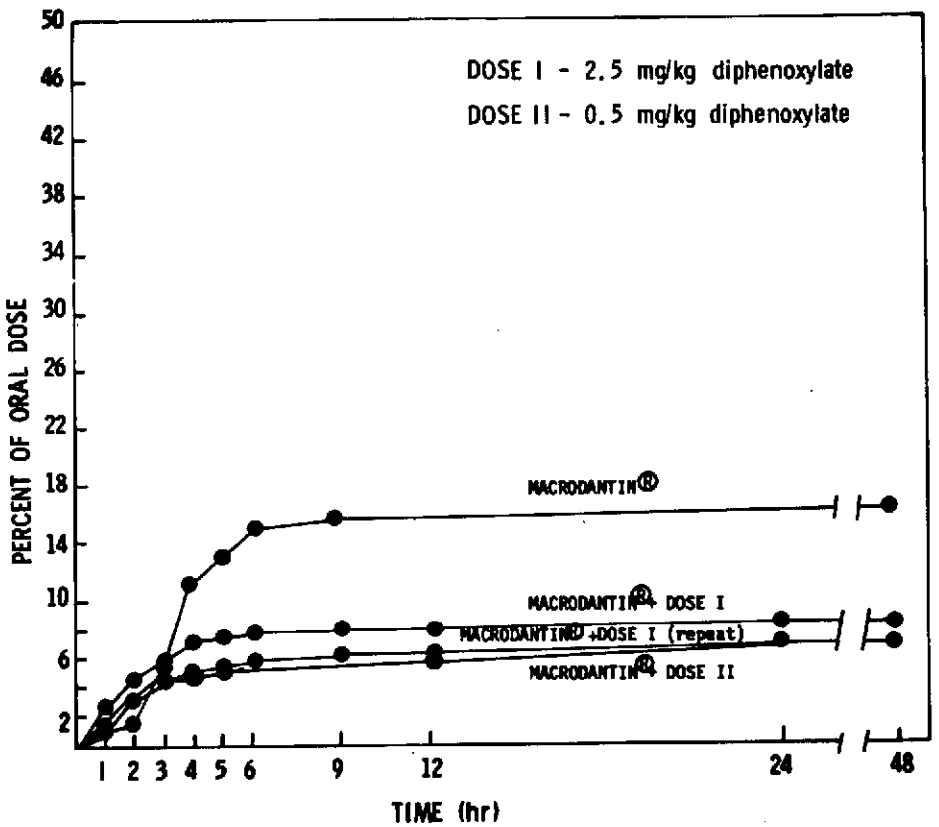

FIGURE 24

Cumulative excretion of Macrodantin® in urine of Dog 102 with/without diphenoxylate. Chemical method used to analyze urine.

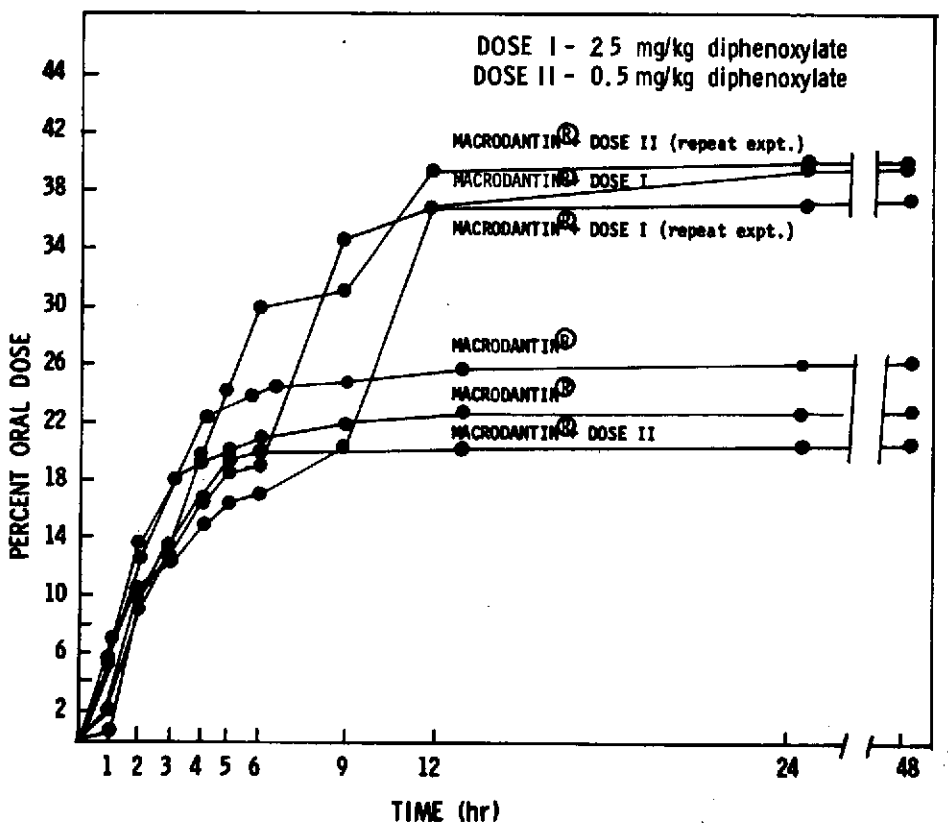

FIGURE 25

Cumulative excretion of Macrodantin® in urine of Dog 103 with/without diphenoxylate. Chemical method used to analyze urine.

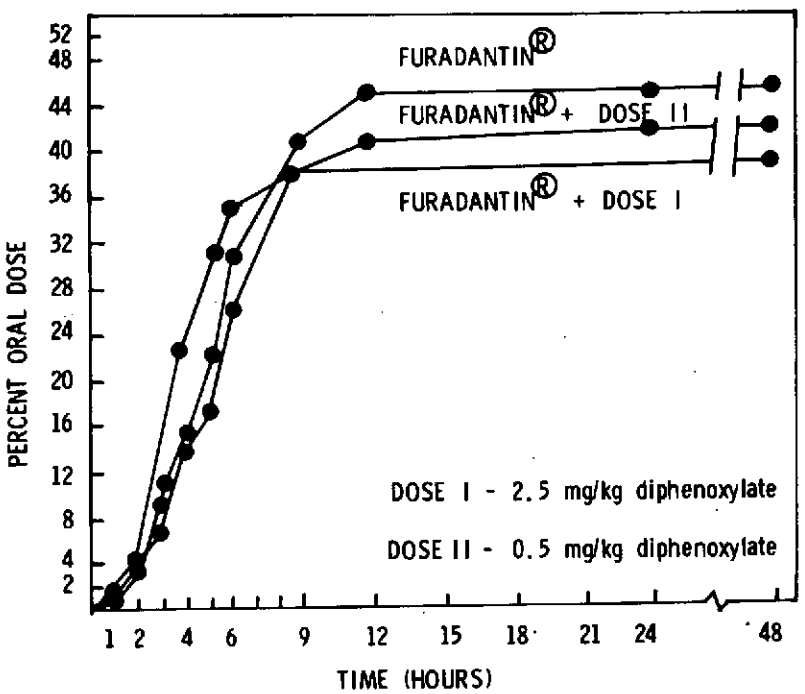

FIGURE 26

Cumulative excretion of Furadantin® in urine of Dog 100 with/without diphenoxylate. Chemical method used to analyze urine.

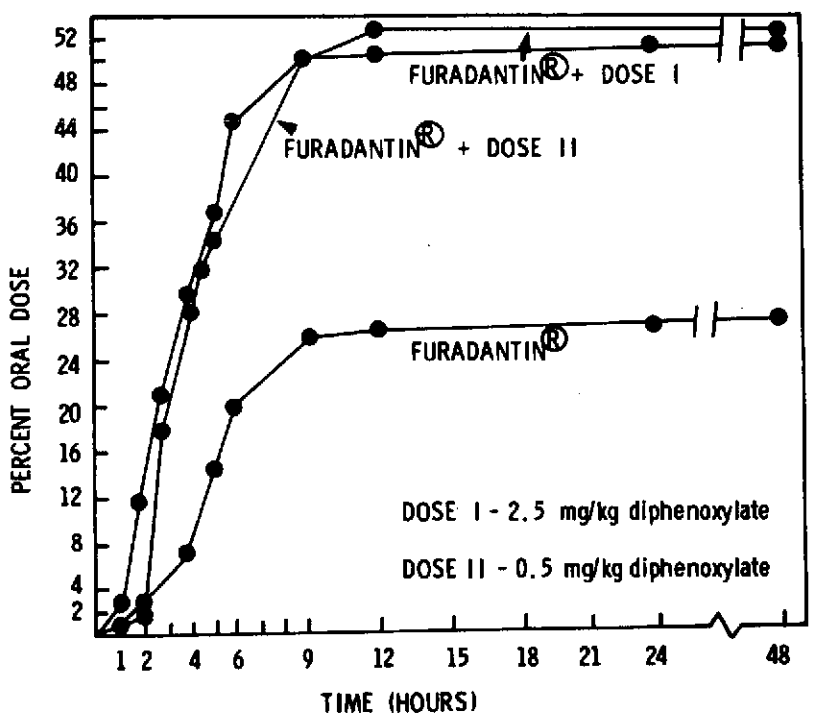

FIGURE 27

Cumulative excretion of Furadantin® in urine of Dog 101 with/without diphenoxylate. Chemical method used to analyze urine.

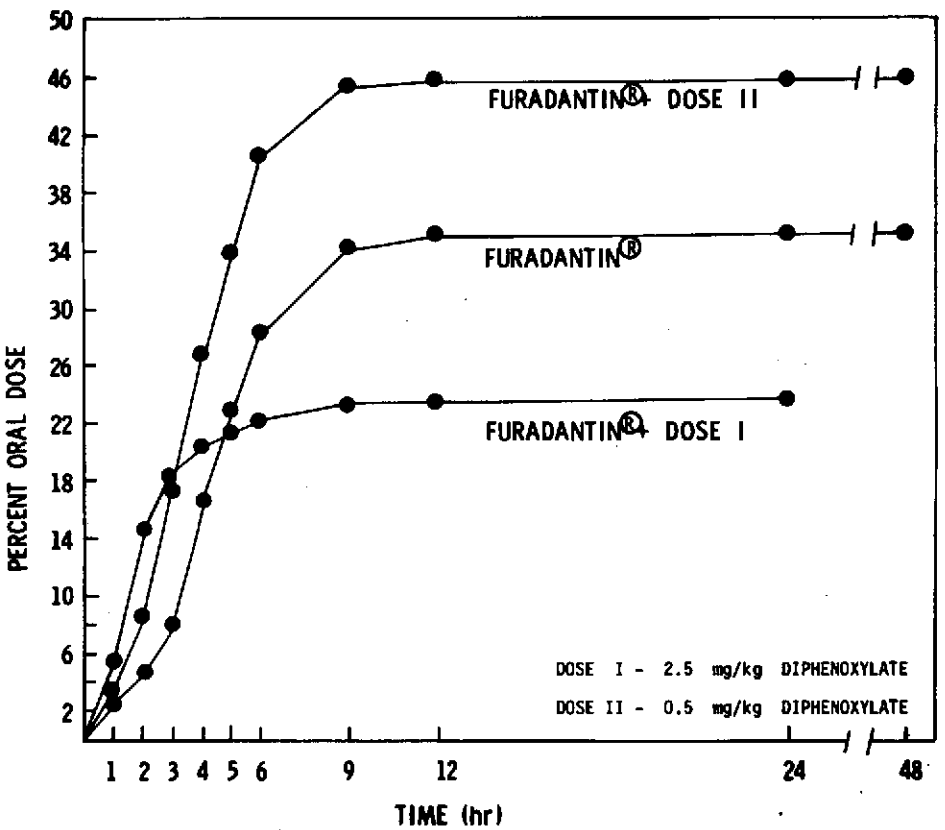

FIGURE 28

Cumulative excretion of Furadantin® in urine of Dog 102 with/without diphenoxylate. Chemical method used to analyze urine.

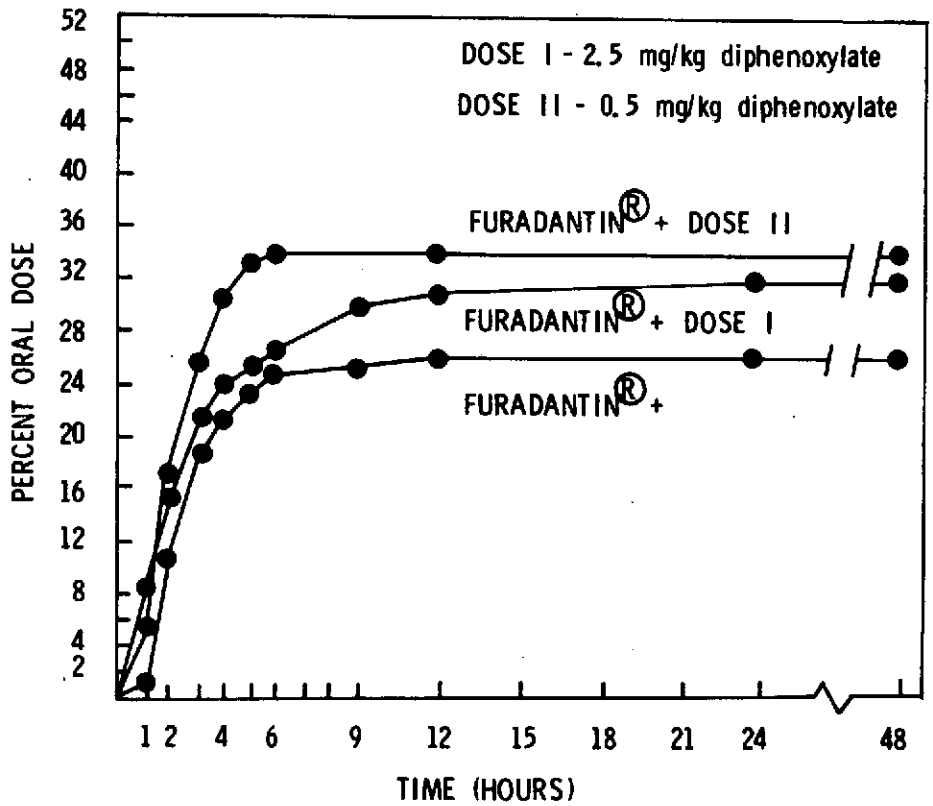

FIGURE 29

Cumulative excretion of Furadantin® in urine of Dog 103 with/without diphenoxylate. Chemical method used to analyse urine.

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experiments, measuring the dog's absorption of Macrodantin® only, significant drug levels did not appear until 2 hr after the animal received the Macrodantin®.

With Dog 103, 0.5 mg/kg diphenoxylate decreased absorption of Macrodantin®, while 2.5 mg/kg diphenoxylate increased total drug absorption (Figure 25). As mentioned above, Dog 103 appeared to handle both forms of nitrofurantoin similarly. Figure 25 shows that the pattern of excretion is similar for the control run and the run with the lower dose of diphenoxylate. The experiment involving the higher dose of diphenoxylate showed a disruption of that pattern, with large amounts of nitrofurantoin found in the 6-9 and 9-12 hr samples.

Figures 26-29 illustrate the experiments with diphenoxylate and the microcrystalline form of nitrofurantoin (Furadantin®). Dogs 101 and 103 (Figures 27 and 29) show a substantial increase in the amount of Furadantin® absorbed when the animals were predosed with diphenoxylate. The pattern of excretion is the same and both dose levels of diphenoxylate appear to have essentially the same effect. Dog 102 (Figure 28) showed an increase in the level of antibiotic in the urine following the low dose (0.5 mg/kg) of diphenoxylate but a decrease in drug level following the higher (2.5 mg/kg) dose. As seen in Figure 27, on the other hand, predosing with both levels of diphenoxylate in Dog 100 led to a decrease in the amount of drug absorbed but the pattern of excretion was the same.

Additional combination experiments were run to verify the results obtained in the noted exception cases. Results from these experiments confirmed the data for Dog 102 presented above but there was some discrepancy in the repeat experiments in Dog 103. This dog originally showed increased absorption of Furadantin® with both levels of diphenoxylate but in the Macrodantin® experiments showed increased absorption with the high dose and decreased absorption with the low dose of diphenoxylate. The results from the repeated experiment using the high dose of diphenoxylate were very similar to the original experiments

TABLE 19

CUMULATIVE PERCENT OF MACRODANTIN® AND FURADANTIN® EXCRETED BY DOGS WITH/WITHOUT DIPHENOXYLATE PRETREATMENT

Dog Number	Macrodantin® (100 mg total dose)	Macrodantin® (100 mg total dose) plus Diphenoxylate[a]	
		Low dose (0.5 mg/kg)	High dose (2.5 mg/kg)
100	11	12	18
101	15	35	28
102	16	8	8,7
103	26,22	20,40	38,40

	Furadantin® (100 mg total dose)	Furadantin® (100 mg total dose) plus Diphenoxylate	
		Low dose (0.5 mg/kg)	High dose (2.5 mg/kg)
100	46	42	38
101	27	56	58
102	36	46	22
103	27	34	32

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but the repeat experiment using the low dose showed an increase in absorption rather than the decrease that was first reported. Both dose levels of diphenoxylate increased absorption of Furadantin® and the repeated experiments showed the same results from Macrodantin®. The one experiment that indicated there was a decreased absorption of Macrodantin® was probably due to an uncontrollable variable: e.g., the dogs received the diphenoxylate orally at 4.00 p.m. the day before the clearance study was run and, although they were checked to see that the capsule was not regurgitated, this might occur.

The results of the dog study are summarized in Table 19. ^{14}C-Inulin clearance studies indicated that urinary functioning at the end of this study was similar to that measured at the start. The interaction between diphenoxylate and nitrofurantoin appears to be significant; diphenoxylate can effect an increased nitrofurantoin absorption by increasing the residence time of nitrofurantoin in the gut. However, two animals pretreated with diphenoxylate showed a decreased urinary output of nitrofurantoin. This may be due to several causes: longer residence in the gut may permit more metabolism of nitrofurantoin by the intestinal microorganisms; diphenoxylate may have affected biliary secretion of nitrofurantoin.

(b) Studies in Man. The results from a study in man, the design being similar to the study in dogs, are illustrated in Figures 30-35 and summarized in Table 20. As can be seen, the excretion of drug in controls varied greatly from individual to individual. Three out of the six subjects excreted more Furadantin® (48-78%) than Macrodantin® (38-62%), similar to our findings in dogs, while the reverse was true in the other three subjects: 26-42% for Furadantin® vs. 36-64% for Macrodantin®. No general statements can be made about the patterns of excretion for early or delayed absorption also seemed to vary for each dosage form. With the exception of two subjects, there did appear to be a difference in the way each individual absorbed the two dose forms and in the total amount of drug excreted. Subjects 1 and 3, however, appeared to handle both dosage forms similarly in these respects.

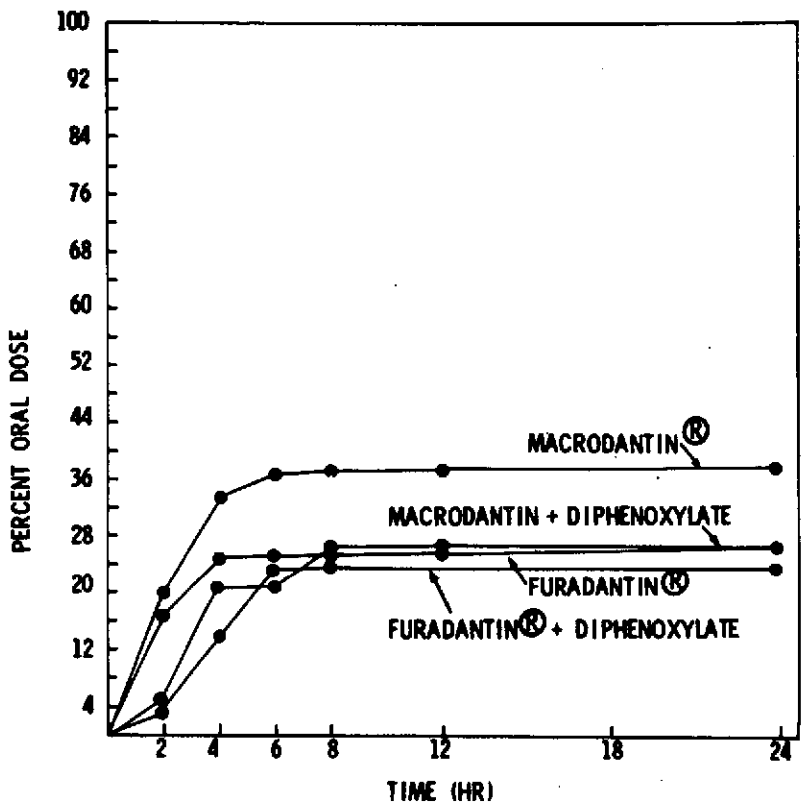

FIGURE 30

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 1. Chemical method used to analyze urine.

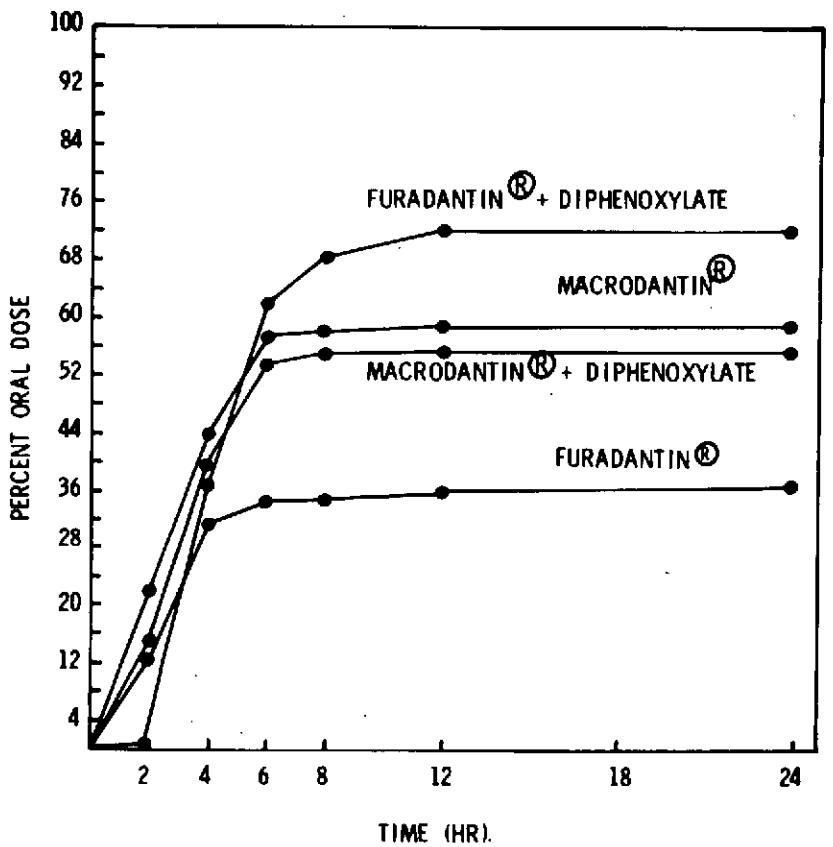

FIGURE 31

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 2. Chemical method used to analyze urine.

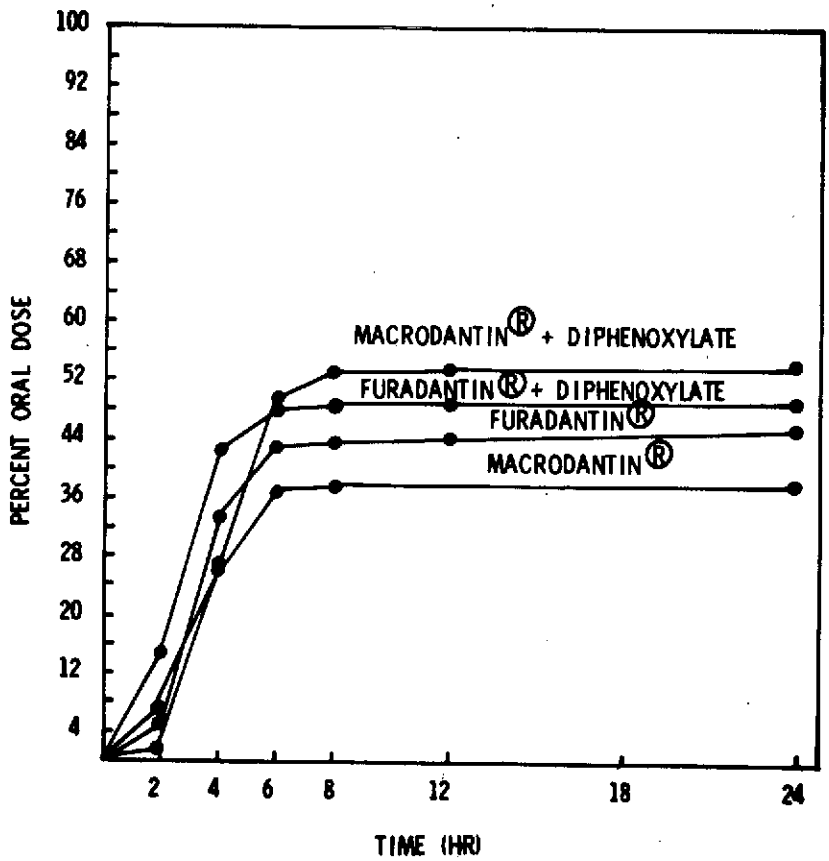

FIGURE 32

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 3. Chemical method used to analyze urine.

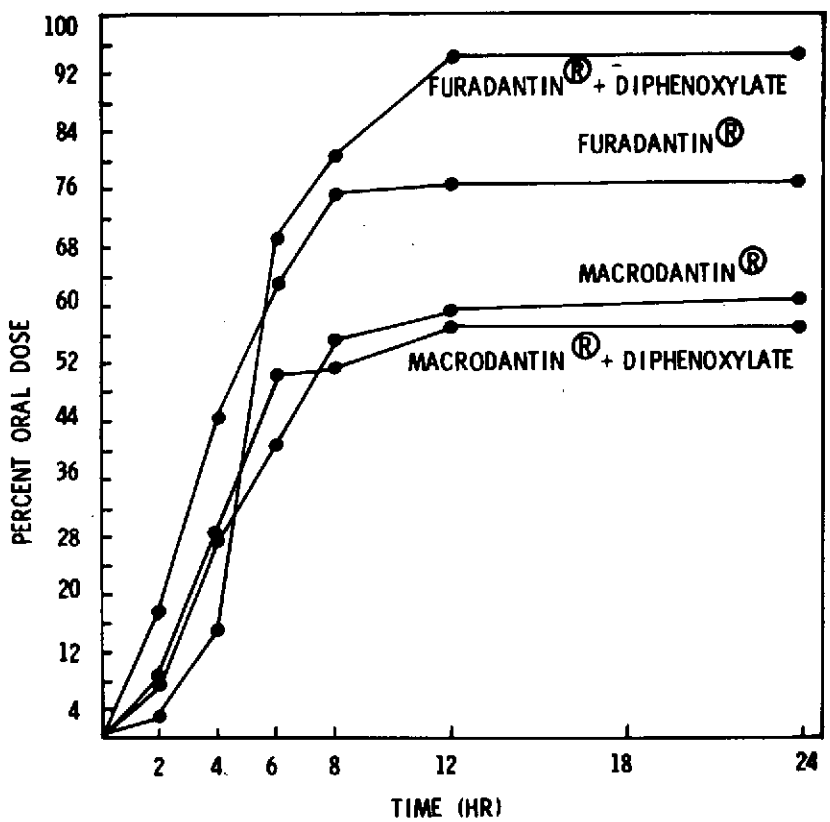

FIGURE 33

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 4. Chemical method used to analyze urine.

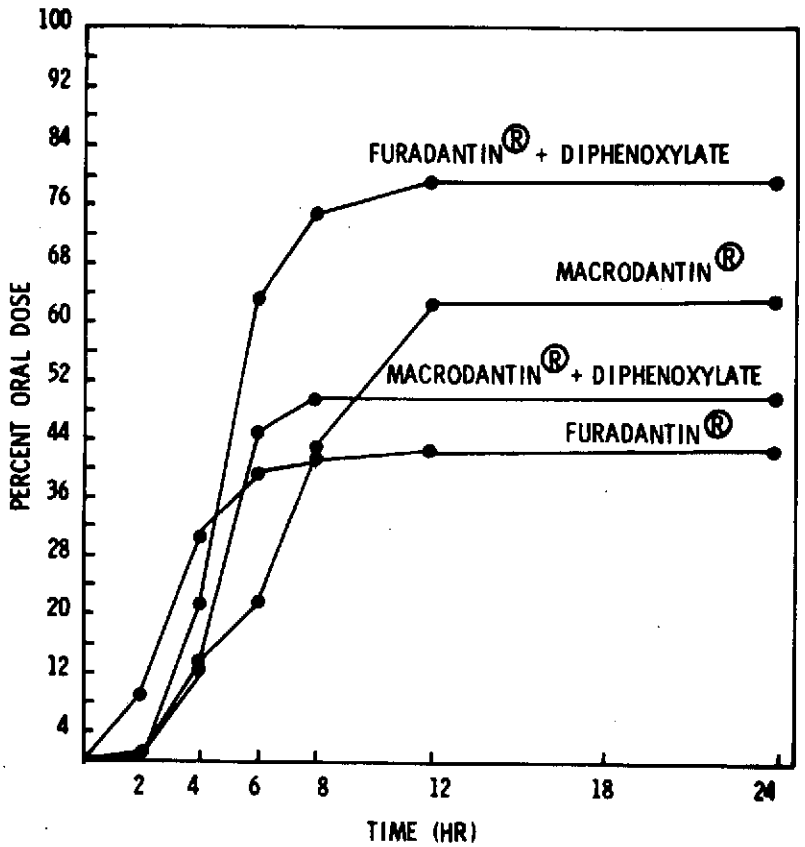

FIGURE 34

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 5. Chemical method used to analyze urine.

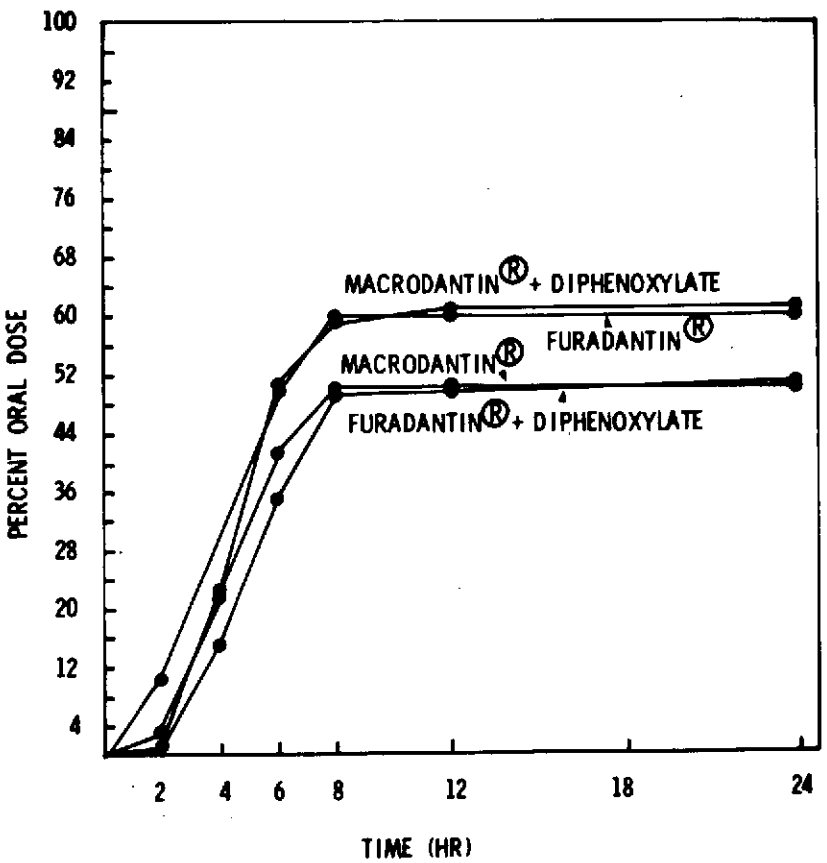

FIGURE 35

Cumulative excretion of Macrodantin® and Furadantin® with/without diphenoxylate in the urine of subject No. 6. Chemical method used to analyze urine.

TABLE 20

CUMULATIVE PERCENT OF MACRODANTIN® AND FURADANTIN® EXCRETED BY MAN WITH/WITHOUT DIPHENOXYLATE PRETREATMENT

Subject Number	Macrodantin® (200 mg total dose)	Macrodantin® plus Diphenoxylate[a]
1	36	27
2	60	56
3	38	54
4	60	56
5	64	50
6	52	62
	Furadantin® (200 mg total dose)	**Furadantin® plus Diphenoxylate[a]**
1	26	24
2	36	71
3	44	49
4	76	96
5	43	80
6	60	50

[a] 5 mg Diphenoxylate administered orally three times daily for three consecutive days prior to dosing orally with 200 mg of the nitrofurantoin dosage form.

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The effect of diphenoxylate on the bioavailability of both dosage forms of nitrofurantoin is also illustrated in Figures 30-35. The results for each subject are depicted separately as the data varied widely from individual to individual. In subjects Nos. 2 and 5 (Table 20) for example, predosing with diphenoxylate led to marked increase in the level of Furadantin® in the urine while results in the other subjects showed either no effect or a slight decrease in the level of antibiotic absorbed.

At this point, the mechanism of action and an explanation of the interaction between diphenoxylate and nitrofurantoin still remains unclear. Diphenoxylate apparently can increase the overall absorption of nitrofurantoin, given either in the macrocrystalline dosage form or the microcrystalline dosage form to dogs. The results of combination studies in man, however, are inconclusive. The interaction does not appear to be significant under the conditions described, affecting only two subjects out of six and with only one dose formulation (Furadantin®).

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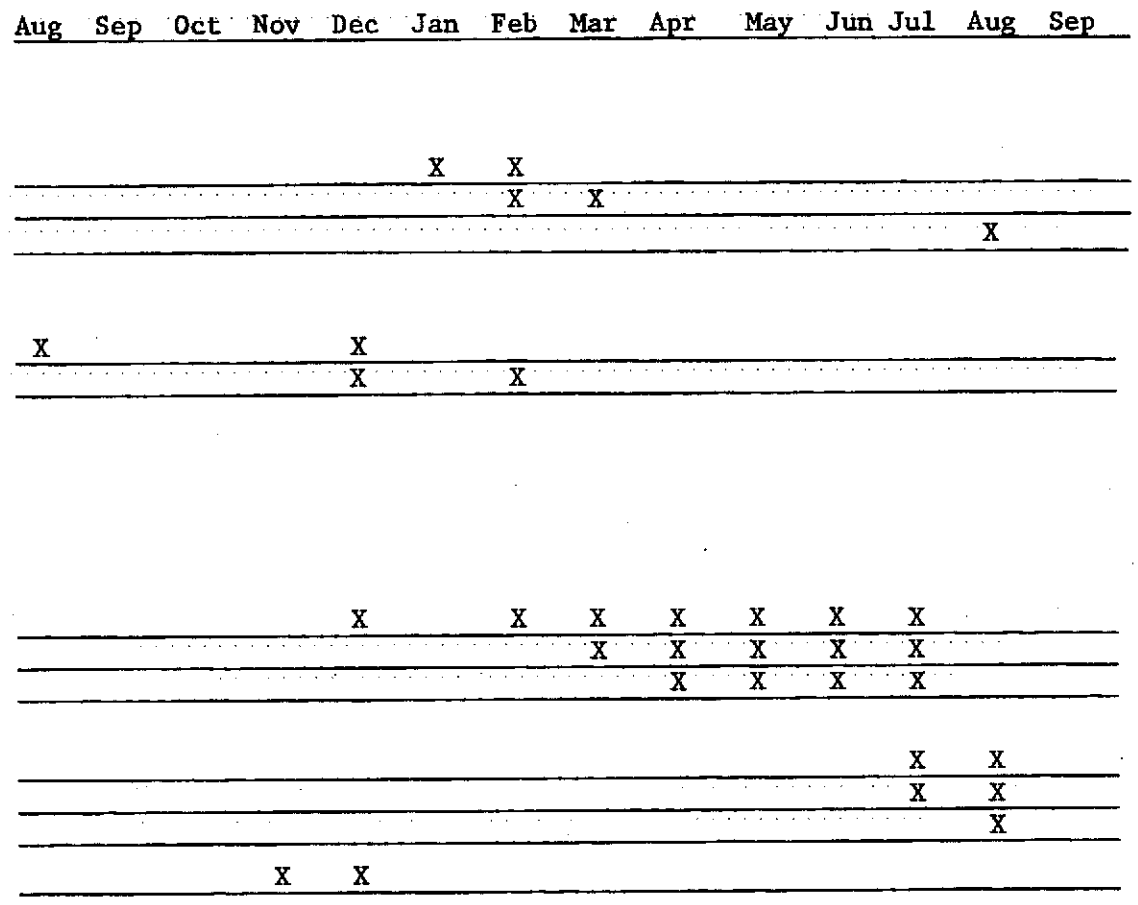

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V. APPENDIX

SCHEDULE OF WORK COMPLETED - CONTENTS OF MONTHLY REPORTS FROM AUGUST 1972 TO SEPTEMBER 1973

Description of Tasks	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
TASK I Potential interaction between sedative drug and nitrofurantoin														
A. Microsomal studies														
1. Hexobarbital oxidase	X	X												
2. Meperidine N-demethylase	X		X	X										
3. *p*-Nitrobenzoic acid reductase	X	X												
4. Cytochrome P-450 and b_5	X	X												
B. Pharmacological studies														
1. Chloral hydrate			X	X										
2. Hexobarbital			X	X										
3. Flurazepam					X	X								
TASK II Enzyme inducers and phenazopyridine														
A. Phenobarbital														
1. Rats-azoreductase and methemoglobin				X	X	X	X		X					
2. Rabbits-methemoglobin								X						
3. Dogs - methemoglobin									X					

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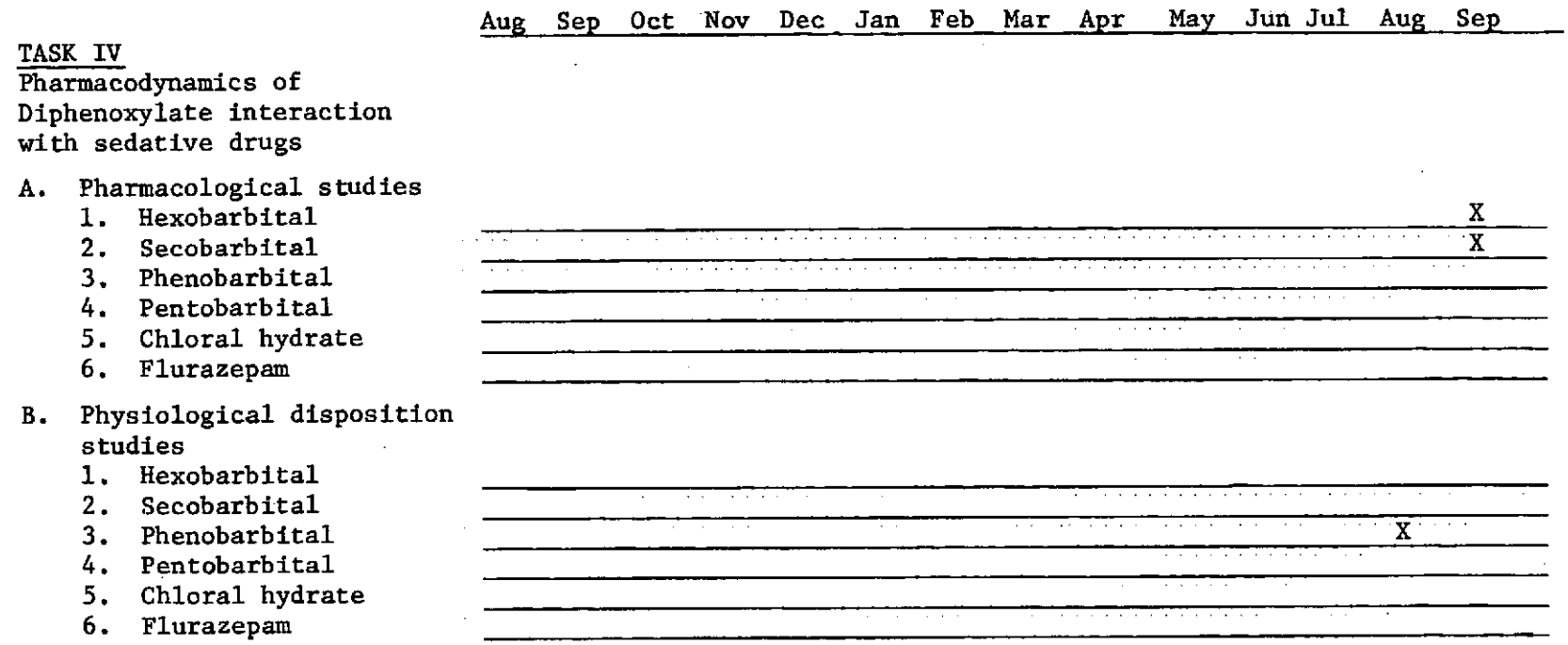

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