AN ABSTRACT OF THE THESIS OF

IRENE LU (Name of student) for the Ph. D. (Degree)

in Pharmacology and Toxicology (Major) presented on 5/10/72 (Date)

Title: THE EFFECTS OF THE CARCINOSTATIC COMPOUND 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) ON THE HEPATIC MICROSOMAL ENZYME SYSTEMS IN RATS

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Abstract approved: _____
Dr. Robert E. Larson

Preliminary toxicity studies of BCNU in rats revealed that significant prolongations of pentobarbital hypnosis were associated with all doses employed. Investigations were undertaken to determine the underlying mechanism responsible for this observed effect. Studies on the in vitro metabolism of pentobarbital by hepatic microsomes of BCNU-treated animals demonstrated that significant impairment of enzyme activities had occurred as early as 7 days following a single dose. The effect was dose-related, and progressed through 21 days post treatment. Kinetic changes in the microsomal enzymes were characterized by a greatly diminished maximum velocity and an unaltered Michaelis constant. Repeated administrations of phenobarbital for 5 days following a single dose of BCNU greatly increased the pentobarbital metabolizing activity given on the seventh post-BCNU

day. These results suggested that the observed prolongation of pentobarbital sleeptime in rats following BCNU treatment could be largely explained by impaired metabolism of the pentobarbital in these animals.

The effect of BCNU on microsomal enzyme systems was not limited to pentobarbital oxidation. Inhibition of ethylmorphine N-demethylation, hexobarbital oxidation, aniline hydroxylation and p-nitrobenzoic acid reduction were evident at 13 days after a single dose of 30 mg/kg of BCNU. The extent of inhibition was equivalent in all systems considered. Therefore, it was apparent that BCNU affected both oxidation and reduction reactions. Moreover, metabolism of both type I and type II compounds as determined by their characteristic spectral properties upon binding to microsomes was impaired.

The general depression of microsomal enzyme activities resulting from BCNU treatment could not be accounted for by deficiencies of cofactors nor actual loss of the enzymes; however, the effect was found to be closely related to significant reductions of the microsomal hemoprotein, cytochrome P-450, which is known to play an important role in the final phase of the electron transport chain. Decreases of cytochrome P-450 levels in BCNU-treated rats were again dose-related and prolonged, as observed through 21 days post treatment. Interestingly, cytochrome b_5, the second of the two

hemoproteins in liver microsomes, and total microsomal protein were not significantly affected by BCNU. Parallel temporal effects on pentobarbital metabolizing activity and levels of cytochrome P-450 gave further evidence that the major effect of BCNU on the hepatic drug metabolizing systems was specifically upon cytochrome P-450.

A seven-day starvation in rats resulted in severe impairment of oxidation of pentobarbital by liver microsomes. Alterations of enzyme kinetics, levels of cytochrome P-450, cytochrome b_5 and total protein were equivalent to rats at 7 days following a single dose of BCNU (30 mg/kg). Despite the fact that BCNU-treated animals exhibited tremendous decreases in food consumption and severe weight losses, the effect of BCNU on drug metabolism was independent of the nutritional status of the animals. BCNU-treated rats which were force-fed with a nutritional liquid diet were capable of maintaining their body weights at the same level. Furthermore, no significant difference was noted with respect to kinetics of pentobarbital oxidation and concentrations of cytochrome P-450 between the force-fed BCNU-treated rats and the BCNU-treated rats without the nutritional supplement. Therefore, although the same apparent effects on drug metabolizing systems were observed with either starvation or BCNU treatment, they were thought to act by different mechanisms.

The Effects of the Carcinostatic Compound 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) on the Hepatic Microsomal Enzyme Systems in Rats

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1972

Approved:

Redacted for Privacy

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Date thesis is presented 5/10/72

Typed by Opal Grossnicklaus for Irene Lu

ACKNOWLEDGEMENTS

Sincere thanks are extended to my major professor, Dr. Robert E. Larson, for his guidance in my graduate training and invaluable advice throughout this investigation. Gratitude is also expressed to Drs. Gregory B. Fink, Lavern J. Weber, Ronald H. Winters, Donald R. Buhler and Donald J. Reed for their constructive criticisms and suggestions in expanding the depth of my research. Appreciation is also extended to my fellow graduate students for their interesting and stimulating discussions. I would like to thank Hudson D. White for his assistance, in many ways, with laboratory animals.

Special thanks are expressed to my husband, David, whose encouragement and understanding have made this work less difficult.

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I. GENERAL INTRODUCTION

Considerable interest has been stimulated by the compound 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) as a result of its unique effectiveness against a wide spectrum of tumors including intracerebrally implanted L1210 leukemia (Johnston et al., 1963; Southern Research Institute, 1964; Goldin et al., 1964; Gale, 1965a; Iriarte et al., 1966; Johnston et al., 1966; Reitemeier et al., 1966; Sugiura, 1967; Horwitz and Groth, 1967; Ausmen et al., 1968). The drug could be administered with equal effectiveness intraperitoneally, intravenously, subcutaneously or intragastrically. Being highly lipid-soluble and essentially non-ionized at physiologic pH, BCNU is able to penetrate the "blood-brain barrier" and rapidly equilibrate with the cerebrospinal fluid (Loo et al., 1966; Iriarte et al., 1966; De Vita et al., 1967). Neither the mechanism of its carcinostatic action nor the active moiety of this compound is understood.

The discovery of nitrosourea derivatives as potential anticancer agents originated from the finding that 1-methyl-1-nitroso-3-nitroguanidine (NSC-9369) possessed weak antineoplastic activity. Screening of a series of nitrosourea congeners showed that BCNU and 1-(2-chloroethyl)-3- cyclohexyl-1-nitrosourea (CCNU) were

among the most active. The similarity of the biological effects of BCNU and other nitrosoureas to those of the accepted biological alkylating agents had led to the suggestion that these compounds may act by an alkylating mechanism (Pittilo et al., 1964; Gale, 1965b; Wheeler and Bowdon, 1965; Green, 1966; Loo et al., 1966; Montgomery et al., 1967). The suggestion was further supported by cross-resistance studies (Wheeler and Chumley, 1967). Gale (1965a) showed that BCNU produced a marked reduction in the rate of DNA synthesis, increased the rate of RNA synthesis, while protein synthesis was only slightly affected at low drug concentrations. Nicotinamide adenine dinucleotide (NAD) levels were elevated in Ehrlich ascites tumor cells. Decreased activity of nicotinamide adenine dinucleotide glycohydrolase (NADase) in mice brains was also reported (Tsukagoski, 1968). In addition, the modes of decomposition of BCNU in aqueous solution were characterized as "anomalous" (Montgomery et al., 1967), meaning no diazoalkane was formed under any conditions studied. This represented a rather unique property to the compound among known carcinostatic alkylating agents. Conceivably one of the degradation products (such as 2-chloroethyl-isocyanate) could be the active alklyating agent.

Initial screening and clinical trials in man and animals consistently showed a pattern of delayed toxicity involving the liver, kidney and hematopoietic tissue following its administration (Southern

Research Institute, 1964; Rall et al., 1963; De Vita et al., 1964; De Vita et al., 1965). De Vita et al. (1967) studied the biological disposition of BCNU in man and animals using the ^{14}C-labelled drug. Radioactivity was excreted slowly in man and monkeys and rapidly in mice. However, these workers consistently failed to account for 30% of the administered dose over an eight-day period in both man and monkeys. Although BCNU had a half-life of 20 minutes in vitro and less than 15 minutes in vivo (Loo et al., 1966), prolonged plasma levels of the isotope were observed. The contrast of the short biologic half-life to the observed delayed toxicity certainly makes BCNU a unique and interesting compound.

Basic toxicological screening of BCNU was performed extensively in rats (Thompson and Larson, 1969). Marked loss of body weight associated with a much reduced food consumption was a prominent feature of the toxicity in animals treated with BCNU (Larson and Rall, 1965; Thompson and Larson, 1969). Delayed deaths were known to occur as long as 100 days following a single oral administration. Alterations of liver function were reported as demonstrated by elevations of serum bilirubin and enhanced bromsulfalein (BSP) retention. Furthermore, by one week post treatment, all doses of BCNU studied resulted in a significant prolongation of pentobarbital hypnosis. Enzyme induction with phenobarbital after BCNU treatment greatly diminished the latter effect.

Severe liver toxicity was noted in rats receiving single doses of BCNU. The prolongation of pentobarbital sleeptime followed a characteristic latency, which became more protracted as the dose of BCNU was reduced. The effect could not be correlated well with changes in the volume of dilution of the pentobarbital suggesting a defective metabolism of the drug as a major explanation for the observed effect. The present study was undertaken to determine if this was indeed true. By studying the hepatotoxicity at a subcellular level, an attempt was made to delineate the basic mechanisms of the toxicity associated with BCNU, the knowledge of which may be of significant importance to the avoidance of potential dangers of concurrent medications. Furthermore, through better understanding of the molecular disturbance, protective means may be found to modify the severe toxicity.

II. STUDIES ON THE EFFECT OF BCNU ON THE IN VITRO OXIDATIVE METABOLISM OF PENTOBARBITAL AND HEXOBARBITAL

Introduction

A prominent feature of the effect of BCNU in rats following a single oral administration was a severe hepatotoxicity, as expressed by distinct hyperbilirubinemia, increased BSP retention, and significant prolongation of pentobarbital sleeptime (Thompson and Larson, 1969). The effect was dose-dependent, and became more protracted as the dose was lowered. The increase in pentobarbital hypnosis could not be totally explained by alterations in body fluid compartment, which suggested strongly that an impairment of the metabolism of the compound occurred. The present study was conducted to find out if this was indeed so, and furthermore, to characterize the kinetics and time course of the effect. In addition to pentobarbital, hexobarbital was employed to double check on the metabolizing system.

Methods

In vivo Studies

Pentobarbital and Hexobarbital Sleeptime. Male rats, of the Sprague-Dawley strain and initially weighing 150 to 200 grams were

used throughout the investigation. The animals were routinely housed in groups of 3 in metal cages measuring 16" x 10" x 7", and were given free access to food and water. BCNU was dissolved in corn oil and administered to the animals intraperitoneally. The volume of injection was 0.1 ml/100 gm body weight. Controls received an equivalent volume of the vehicle only. The dose of BCNU chosen for the study was 30 mg/kg, representing an LD_{50}, within an observation period of 30 days (Thompson and Larson, 1969). On the thirteenth day post treatment, pentobarbital or hexobarbital were administered i.p. at doses of 45 mg/kg and 150 mg/kg respectively. The duration of hypnosis, hereafter sleeptime, was defined as the period of time between which the animals lost and then regained their righting reflex.

Enzyme Induction. For induction of hepatic microsomal enzymes, phenobarbital, at a dose of 100 mg/kg, was administered intraperitoneally to the animals daily for 5 days beginning on the seventh day after BCNU (Orrenius, 1965). Pentobarbital or hexobarbital sleeptimes were determined 24 hours following the last injection of phenobarbital. Statistical analysis of the data was performed utilizing the Student's t-test. A probability level of $P < 0.05$ was chosen as the critical level of significance.

In vitro Metabolism Studies

Preparation of Tissue. Rats were sacrificed at specific times

following administration of BCNU. They were killed by a blow on the head, after which their livers were immediately removed and placed on ice. Five grams of each liver were homogenized in 2 volumes of ice cold 1.15% KCl with a Potter homogenizer and a Teflon pestle. The 9000 xG fractions were obtained by centrifuging the homogenate at 8500 rpm in a refrigerated Lourdes Betafuge for 60 minutes in a 9RA-24 rotor. The supernatant was used for all metabolism studies.

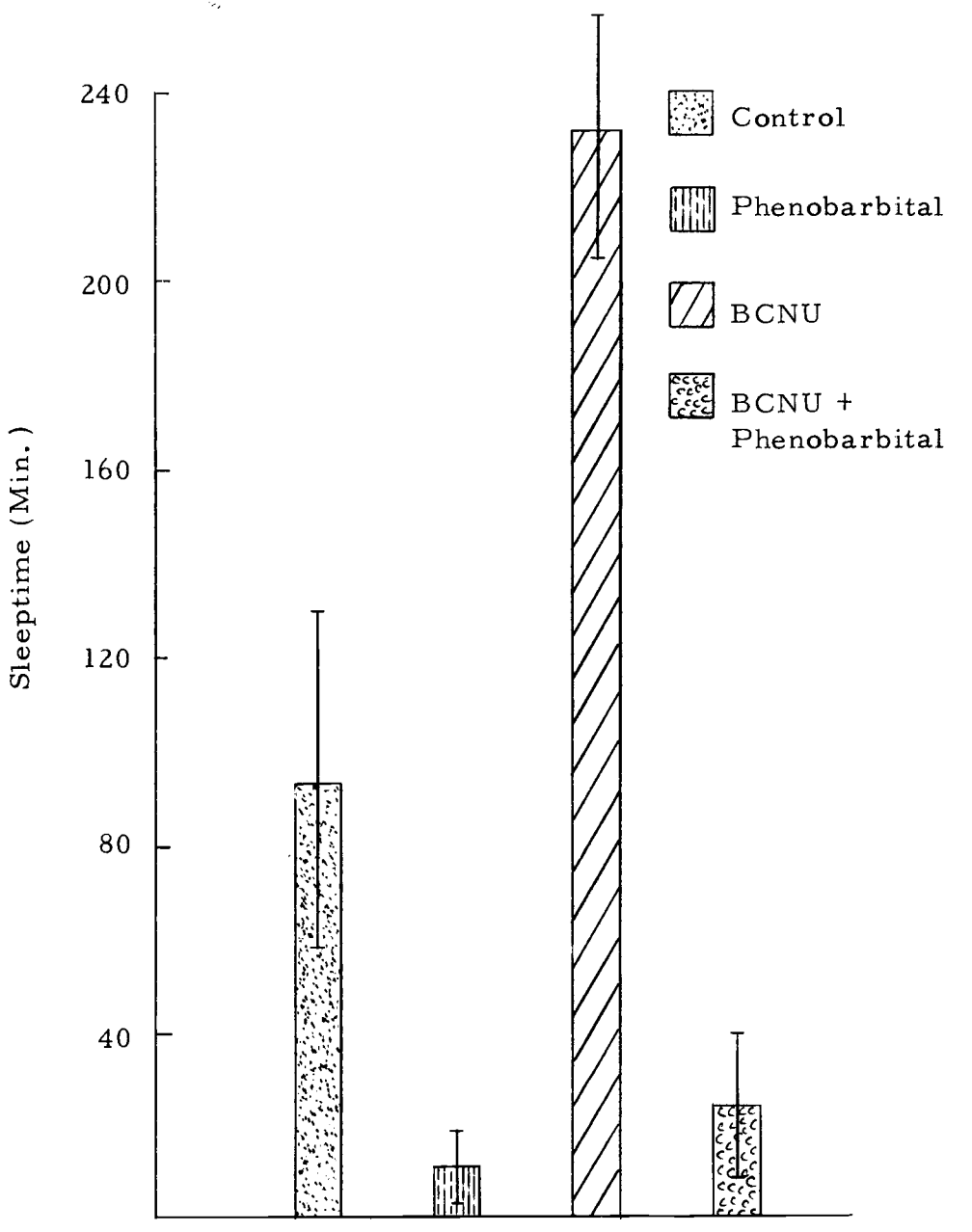
Incubation Mixture. To each 25-ml Erlenmeyer flask was added the following: $NADP^+$ (2 μmoles), glucose-6-phosphate (20 μmoles), nicotinamide (40 μmoles), magnesium chloride (20 μmoles), varying concentrations of the substrate (pentobarbital or hexobarbital), 2 ml of microsomal preparation (equivalent to 1 gm of liver), 2 enzyme units of glucose-6-phosphate dehydrogenase. The contents of each flask were adjusted to pH 7.4 with 0.1M Tris buffer to produce a final volume of 5 ml (Rubin, Tephly and Mannering, 1964). The complete reaction mixture was incubated at 37° C in a Dubnoff metabolic incubator with constant shaking for 45 minutes under an atmosphere of oxygen. At the end of the incubation period, the amount of unchanged substrate was determined according to the method of Brodie et al. (1953), and from it, the amount of substrate metabolized was calculated.

Extraction of Pentobarbital and Hexobarbital (Brodie et al., 1953). At the end of incubation, the reaction mixture was transferred to a 50-ml centrifuge tube, to which were added 1 ml of pH 5.0 phosphate buffer, 1 gm of sodium chloride, and 30 ml of hexane containing 1.5% isoamyl alcohol. The tube was shaken for 45 minutes and centrifuged. Twenty milliliters of the organic phase was then transferred to another 50-ml centrifuge tube containing 4 ml of pH 11.0 phosphate buffer. The tube was again shaken for 10 minutes and centrifuged. Finally, 3 ml of the aqueous phase was placed in a quartz cuvette and the optical density at 240 nm (for pentobarbital) or 245 nm (for hexobarbital) was determined.

Results

A three-fold increase in the duration of pentobarbital hypnosis was observed in rats 12 days after a single intraperitoneal injection of BCNU, 30 mg/kg (Figure 2-1). The effect was dose-related, and the onset, degree and persistence of the effect had been characteristically defined by Thompson and Larson (1969). Pretreatment with optimal enzyme inducing doses of phenobarbital shortened the sleeptime of control animals significantly. Phenobarbital pretreatment to BCNU-treated rats also reduced the sleeptime to a level comparable to that of the induced controls. The mean sleeptimes of both groups of induced animals were not significantly different statistically

Figure 2-1. The effect of a single i.p. dose of BCNU (30 mg/kg in corn oil) on the duration of pentobarbital hypnosis in rats at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 following BCNU treatment. At least 16 animals were assigned to each group. The bars were mean responses and the brackets were standard deviations. A three-fold increase in sleeptime was observed in BCNU-treated rats. Repeated doses of phenobarbital to controls and BCNU-treated rats reduced the sleeptime to comparable levels.


240
200
160
120
80
40
Sleeptime (Min.)
Control
Phenobarbital
BCNU
BCNU +
Phenobarbital

at P = 0.05. These results suggested that an impairment in the metabolism of pentobarbital would be a possible explanation for the observed effects in the BCNU-treated animals.

The kinetics of pentobarbital metabolism by the liver 9000 xG fractions was represented by a Lineweaver-Burk plot (Figure 2-2). It was apparent that in BCNU-treated rats the enzyme kinetics were quite different from controls. The results resembled closely an enzyme kinetic pattern of non-competitive inhibition. The Michaelis constants (K_m) were almost identical, but the maximum velocities (V_{max}) varied greatly, the BCNU-treated livers having a much smaller value. Therefore, these data explained at least in part the prolongation of pentobarbital sleeptime previously observed in these animals.

On the other hand, phenobarbital induction had remarkable effects on the microsomal enzymes of rats receiving a single injection of BCNU previously (Figure 2-3). The Lineweaver-Burk plot was essentially superimposable on that of the induced controls. This was reflected in the sleeptime determination in which the induced BCNU-treated rats and induced controls "slept" the same length of time. Phenobarbital induction in control animals brought about a ten-fold increase in both the K_m and V_{max}. It was interesting to note that phenobarbital induction in animals given BCNU resulted in a seventy-fold increase in the maximum velocity.

Figure 2-2. The effect of a single i.p. dose of BCNU (30 mg/kg) on the kinetics of in vitro pentobarbital metabolism by liver 9000 xG fractions in rats. Enzyme activities were determined at 13 days post treatment. Changes in enzyme kinetics were characterized by a greatly reduced V_{max} (0.17 μmole/gm/45 min. compared to 1.0 μmole/gm/45 min. in controls) and unaltered K_m (4.1×10^{-3} M compared to 3.8×10^{-3} M in controls).

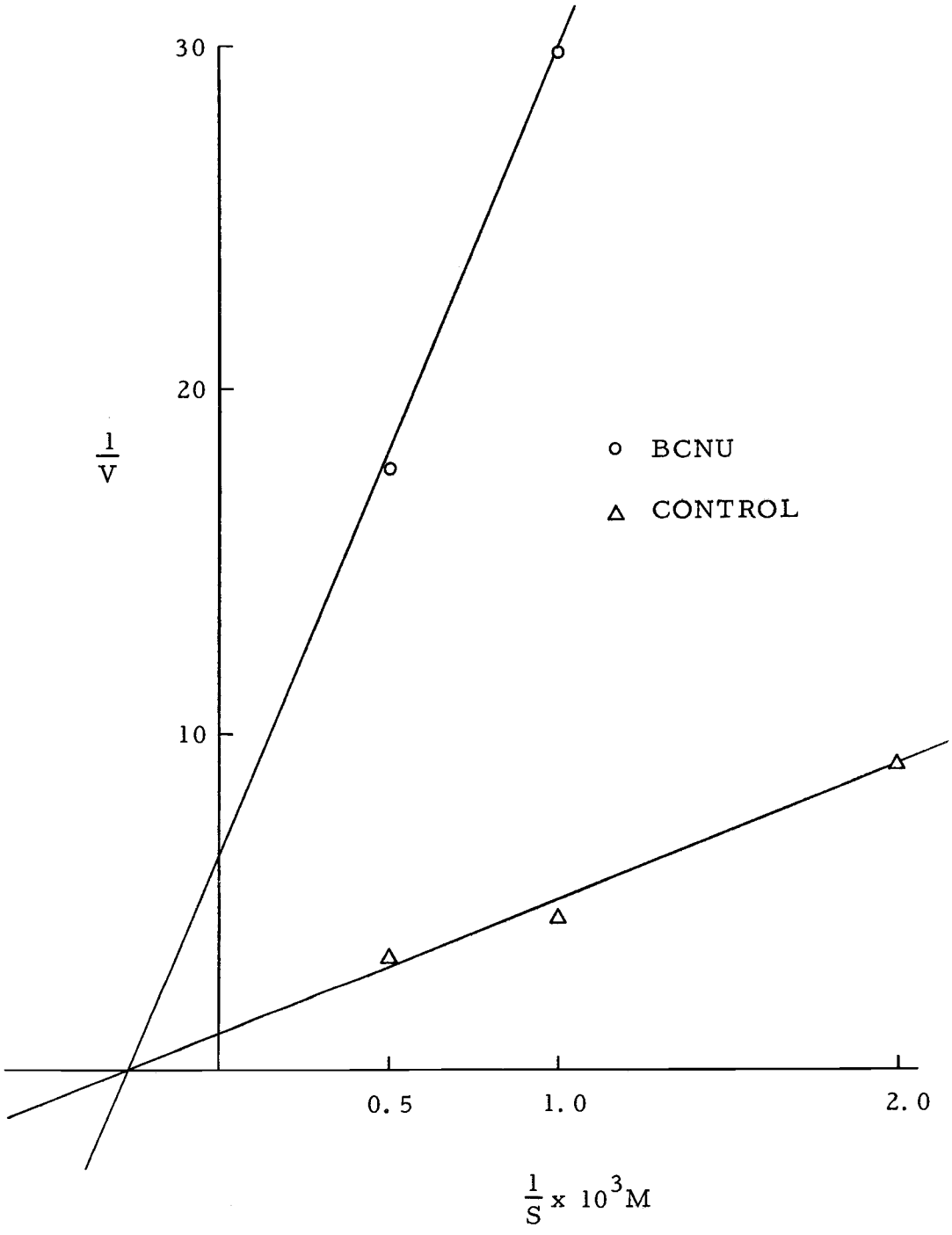
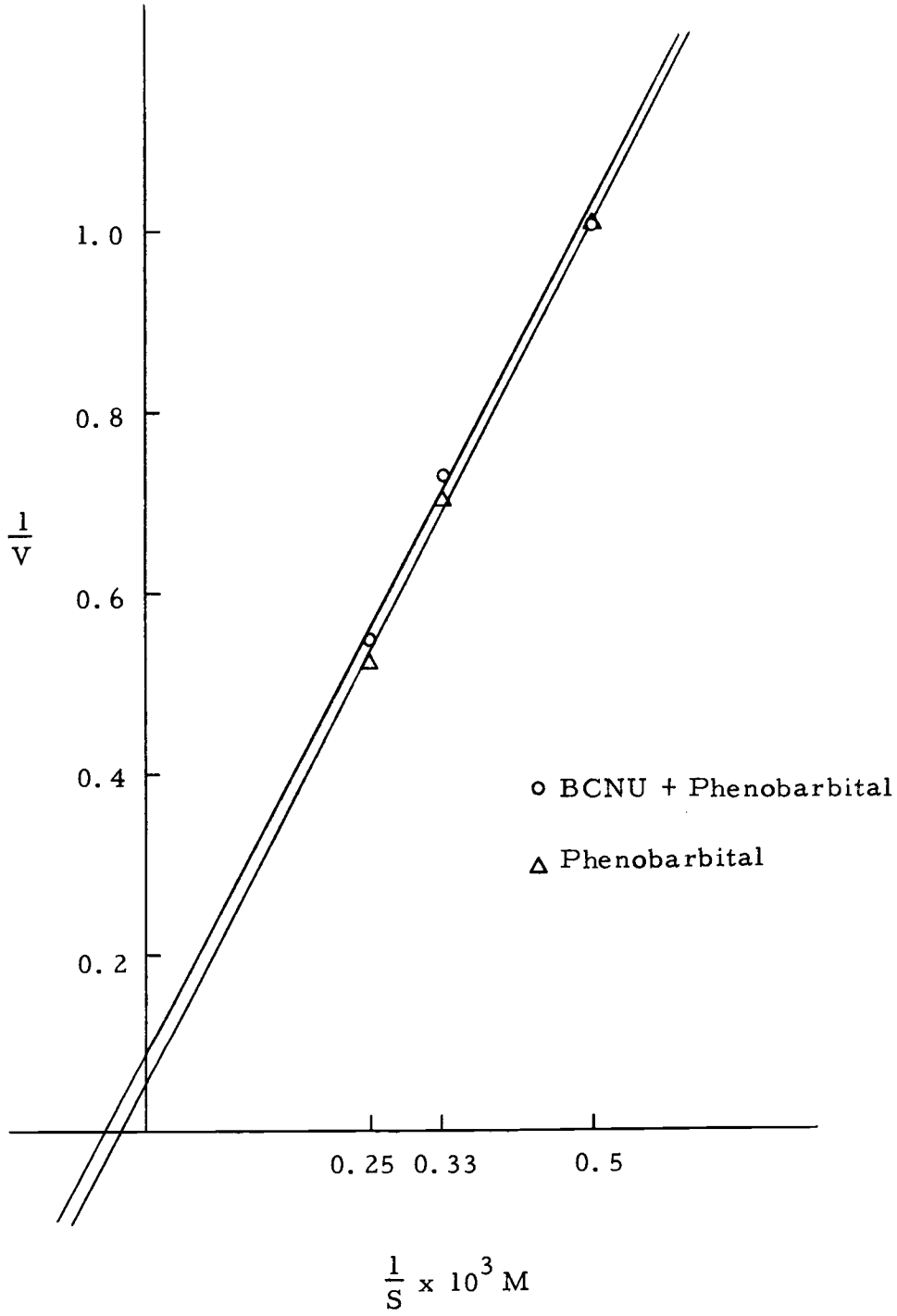

30
20
10
1/V
BCNU
CONTROL
0.5
1.0
2.0
1/S x 10^3M

Figure 2-3. The effect of phenobarbital induction on the kinetics of pentobarbital oxidation by liver 9000 xG fractions in rats receiving a single dose of BCNU (30 mg/kg) at 13 days post dosing. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 after BCNU treatment. Rats were sacrificed 24 hours after the last injection of phenobarbital. No significant difference was noted between controls and BCNU-treated animals following the induction.


1.0
0.8
0.6
0.4
0.2
1/V
0.25
0.33
0.5
1/S x 10^3 M
o BCNU + Phenobarbital
Δ Phenobarbital

The results of a time course study of the effect of BCNU on the in vitro oxidation of pentobarbital by liver postmitochondrial fractions up to 21 days are represented by Figure 2-4. Two doses of BCNU (20 and 30 mg/kg) were employed. Control livers consistently metabolized about 58 micrograms of pentobarbital per gram of liver per 45 minutes. Livers from BCNU-treated rats metabolized significantly less. The effect was dose-dependent and became more magnified as time progressed. By 21 days, the livers were affected to the same degree with either dose.

Since the termination of effects of hexobarbital depended entirely upon its metabolism whereas for pentobarbital a combination of metabolism and redistribution was involved, a major portion of the experiment was repeated using hexobarbital as the substrate instead of pentobarbital. Effects on hexobarbital sleeptime are shown in Figure 2-5. As expected, the duration of hypnosis was very much extended in BCNU-treated animals. Phenobarbital induction in these animals again reduced the sleeptime to the level of induced controls. Kinetics of the side chain oxidation of hexobarbital in vitro by the liver 9000 xG fractions was also determined for BCNU-treated rats with and without enzyme induction by phenobarbital (Figures 2-6 and 2-7). The results were very similar to that noted for pentobarbital. A non-competitive type of inhibition was demonstrated for BCNU-intoxicated rats, but phenobarbital

Figure 2-4. The temporal effects of single doses of BCNU on the oxidative metabolism of pentobarbital by liver postmitochondrial fractions in rats. Five animals were sacrificed at each sampling time for each dose of BCNU. The brackets were standard deviations. Significant and dose-related depressions of enzyme activity were observed at 7 days post treatment. Furthermore, the effect was found to persist through 21 days after the administration of BCNU.

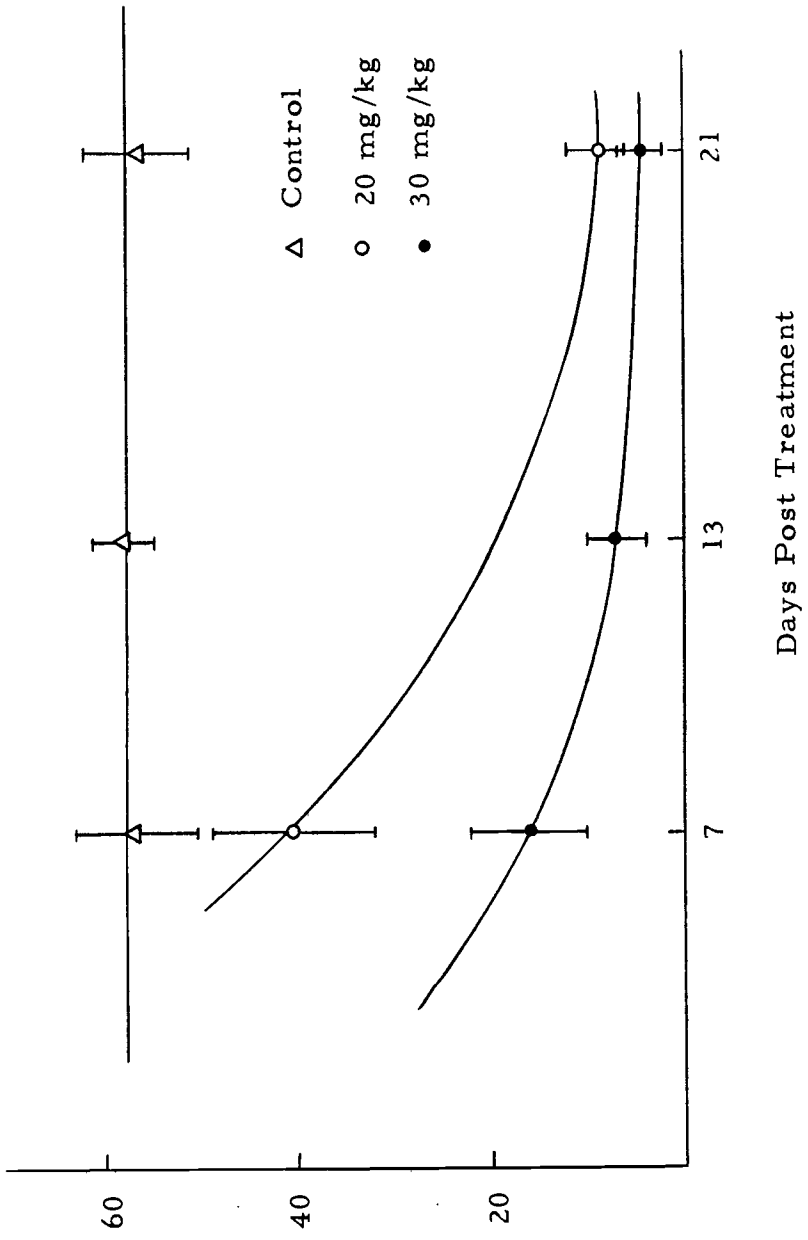

Mcgm Pentobarbital Metabolized/gm Liver/45 Min.
60
40
20
Control
20 mg/kg
30 mg/kg
7
13
21
Days Post Treatment

Figure 2-5. The effect of a single i.p. dose of BCNU (30 mg/kg in corn oil) on the duration of hexobarbital hypnosis in rats at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 following BCNU treatment. At least 12 animals were assigned to each group. The bars were mean responses and the brackets were standard deviations. More than a three-fold increase in sleeptime was observed in BCNU-treated rats. Phenobarbital induction in controls and treated rats reduced the sleeptime to comparable levels.

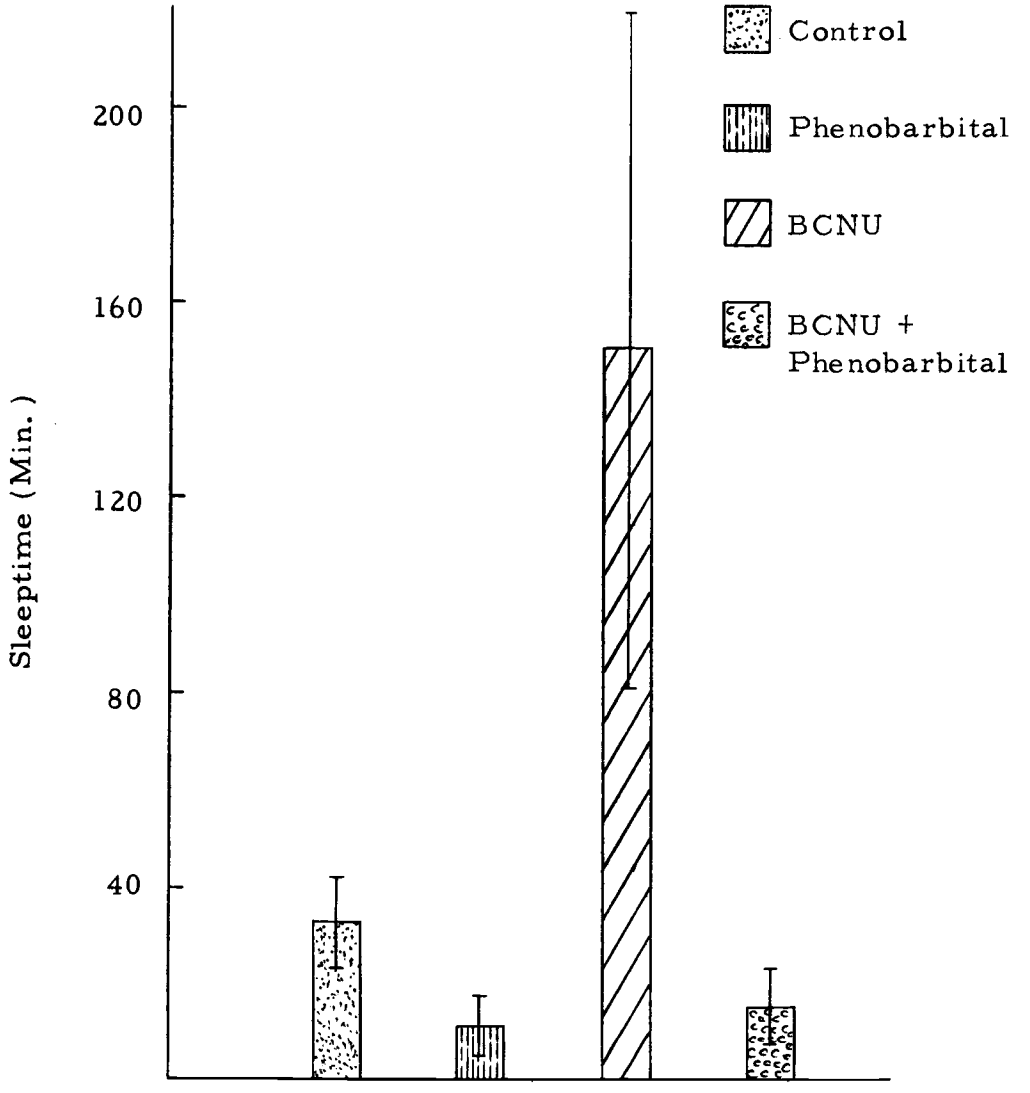

Control
Phenobarbital
BCNU
BCNU + Phenobarbital
Sleeptime (Min.)
200
160
120
80
40

Figure 2-6. The effect of a single i.p. dose of BCNU (30 mg/kg) on the kinetics of hexobarbital metabolism by liver 9000 xG fractions in rats. Enzyme activities were determined at 13 days post treatment. Changes in enzyme kinetics were characterized by a reduced V_{max} (10 μmoles/gm/45 min. compared to 5 μmoles/gm/45 min. in controls) and unaffected K_m (5.3×10^{-2}M compared to 5.1×10^{-2}M in controls).

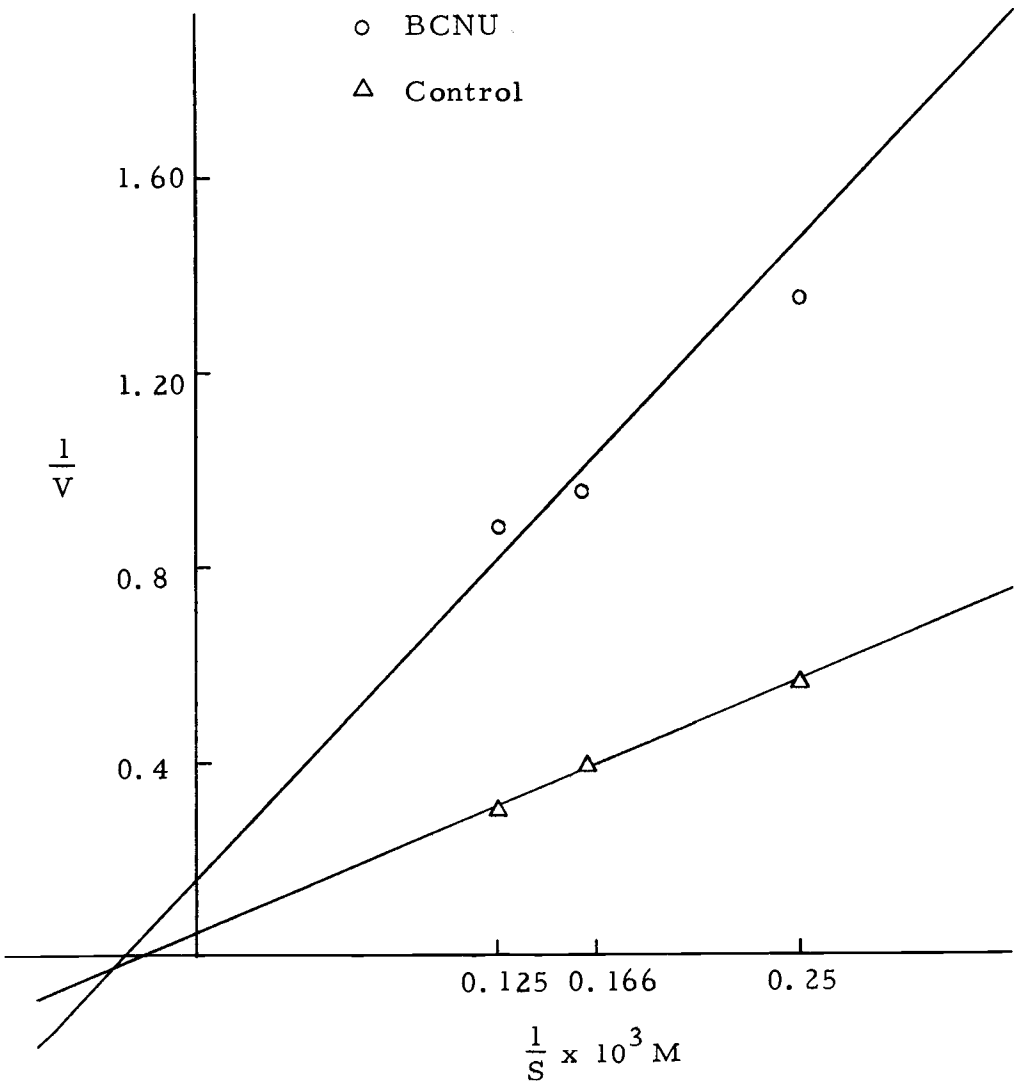
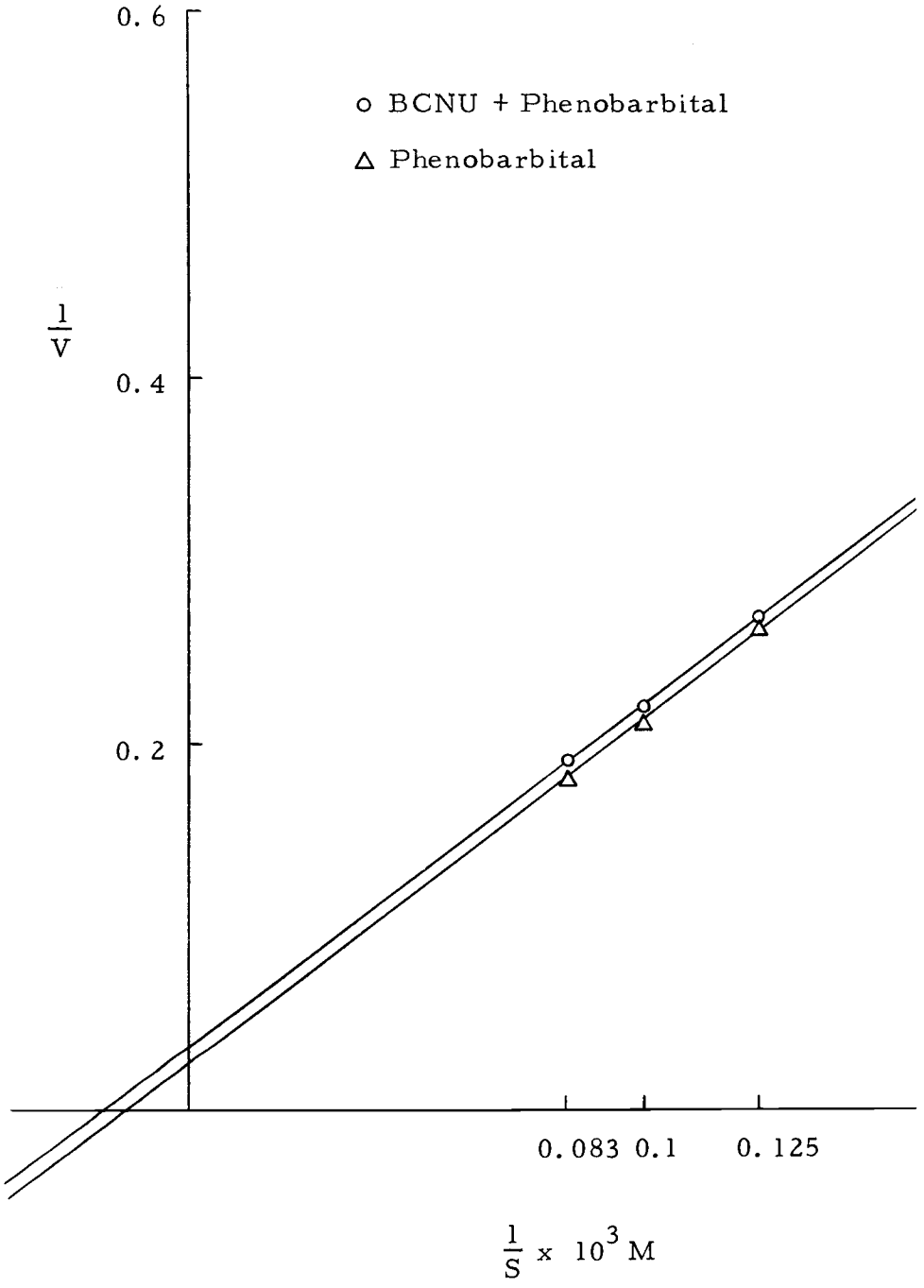

o BCNU
Δ Control
1.60
1.20
0.8
0.4
1/V
0.125
0.166
0.25
1/S x 10^3 M

Figure 2-7. The effect of phenobarbital induction on the kinetics of hexobarbital oxidation by liver post-mitochondrial fractions in rats receiving a single dose of BCNU (30 mg/kg) at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days. Rats were sacrificed 24 hours after the last phenobarbital injection. No significant difference was noted between controls and BCNU-treated animals following the induction.


0.6
o BCNU + Phenobarbital
Δ Phenobarbital
1/V
0.4
0.2
0.083
0.1
0.125
1/S x 10^3 M

induction in these animals was able to bring the enzyme kinetics back to control characteristics.

Discussion

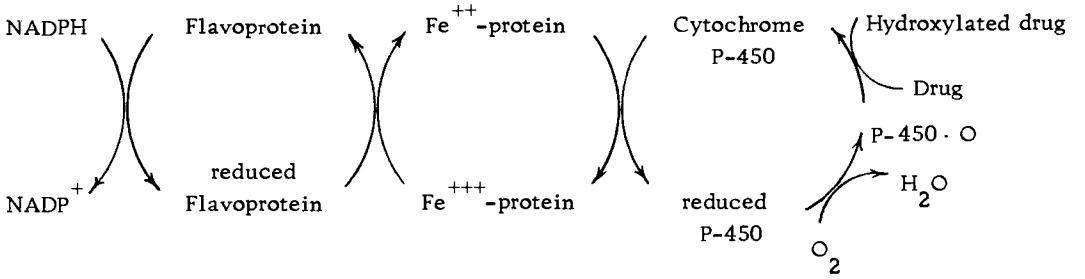
The findings on sleeptime agreed well with that of Thompson and Larson (1969). It had been established that the termination of effects of pentobarbital or hexobarbital depended largely on their metabolism by liver microsomal enzymes. This system is well known for its ability to metabolize a wide variety of foreign compounds (Gillette, 1963). It was then reasonable to assume that the longer the drug effect persisted, the less efficient the liver was in metabolizing the compound. Therefore, a measure of the duration of hypnosis induced by either pentobarbital or hexobarbital would give some indication of the efficiency of the drug-metabolizing system. In BCNU-treated rats, both pentobarbital and hexobarbital sleeptimes were increased approximately three-fold over the controls. Although alterations of body fluid compartments in BCNU-treated animals could perhaps partly explain the observed effects, the prolongation of sleeptimes suggested strongly that impaired metabolism was also involved. Consequently, the side chain oxidations of pentobarbital and hexobarbital by liver microsomes of BCNU-intoxicated rats were determined under *in vitro* conditions. The results were found to parallel the sleeptime observations. The

enzymes were greatly depressed in terms of the amount of substrate metabolized per unit time. At 13 days after a single dose of 30 mg/kg of BCNU, the 9000 xG fractions equivalent to one gram of liver metabolized only 7 micrograms of pentobarbital in 45 minutes, compared to 58 micrograms for control livers. Thus, there appeared to be an 85% inhibition in the treated animals. The effect was also observed with 20 mg/kg of BCNU but was of a smaller magnitude at this dose.

This immediately led to a few speculations. First, the impaired metabolism could result from a deficiency of the metabolizing enzymes, due to either excessive protein breakdown or decreased rate of synthesis. The enzyme induction studies with phenobarbital was carried out to test if rats given BCNU were capable of synthesizing more enzymes under the challenge. Phenobarbital, a classical hepatic enzyme inducing agent, is known to exert its enzyme inducing effects by a combination of decreased enzyme destruction and increased enzyme synthesis (Omura et al., 1969). With electron microscopy, a massive proliferation of the smooth endoplasmic reticulum in liver cells, the site where most of the drug-metabolizing enzymes are located, was seen with phenobarbital pretreatment (Remmer and Merker, 1963). Phenobarbital also markedly elevated the level of one of the two hemoproteins, cytochrome P-450 (Omura and Kuriyama, 1969). Indeed, it was shown that in BCNU-treated

rats, phenobarbital induction significantly increased the oxidative enzyme activities as compared with their non-induced counterparts. This was reflected in a marked reduction of the pentobarbital and hexobarbital sleeptimes to levels comparable to phenobarbital-induced controls. In addition, the maximum velocities (V_{max}) of the enzyme were greatly increased. In other words, the protein synthesizing mechanism in BCNU-treated rats was probably not affected. This assumption is in agreement with the findings of Gale (1965a) that BCNU only slightly affected protein synthesis.

Secondly, the observed effects of BCNU on pentobarbital and hexobarbital metabolism could be caused by a deficiency of one or more cofactors for the system rather than the enzyme itself. The microsomal mixed function oxidase is an NADPH-dependent system. The electron flow pathway was outlined as follows by Omura et al. (1965):



It was apparent that a deficiency of pyridine coenzymes, magnesium ions or any factors that mediated the conversion of $NADP^+$ to NADPH in the animals would be expected to affect the rate of metabolism of

the barbiturates. Since, however, the reaction mixtures were provided with excess amounts of $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and magnesium ions, the concentration of cofactors was not considered as the limiting factor in the altered rate of barbiturate metabolism.

Toward the final phase of the proposed electron transport chain, an unusual hemoprotein, cytochrome P-450 plays a key role in the system. It seems to be the final oxygen-activating enzyme, reducing one oxygen atom to water and at the same time, incorporating an oxygen atom into a drug substrate (Cooper et al., 1965). This opens up an attractive possibility that BCNU might have altered the level of cytochrome P-450 leading to depressed enzyme system.

Thirdly, enzymes can only function if they retained their tertiary structure. Any change in their conformation could result in loss of activity. It is possible that BCNU, being classified as an alkylating agent, interacted with the liver mixed function oxidase, producing a less active enzyme. If this was true, even though the enzymes may be present, they would not be functioning at their normal capacity. The kinetics studies of pentobarbital and hexobarbital metabolism showed a pattern resembling non-competitive inhibition. The maximum velocity was drastically reduced in BCNU-treated rats while the Michaelis constant remained unchanged. These kinetic data suggested that the enzymes of animals that had been

exposed to BCNU were changed in some manner. This would be consistent with the above-mentioned hypothesis that BCNU altered the conformation of the enzymes by alkylating them.

It has been estimated that the mean half-life of proteins of the endoplasmic reticulum is about 2 to 2.5 days (Arias et al., 1969). Consequently, by 21 days, the liver should be completely replenished with newly synthesized enzymes. If BCNU was interacting with the enzymes directly, one would expect its effect to disappear by 21 days following its administration, with the complete turnover of liver proteins. In contrast, the study of the time course of BCNU action demonstrated that at 21 days after a single injection of BCNU (20 or 30 mg/kg), the microsomal enzymes had progressively less activity. The effect was dose-related initially, but by 21 days, the same magnitude of effect was achieved by either dose. This persistence of effects was strongly indicative of a permanent damage. Since BCNU was known to act at the DNA level (Gale, 1965a), it was not hard to visualize a change in the nucleotides of the DNA molecules, thereby altering the genetic code. In this way, the newly produced enzyme would not be the same. Unfortunately, as attractive as this theory was, it was shown that following phenobarbital induction, BCNU-treated rats had enzyme activities identical to those of the induced controls. An alternative explanation would be that BCNU or its metabolite was recycled in the system and alkylated each molecule

of the enzyme or cytochrome P-450 as it was synthesized. The failure of De Vita and his coworkers (1967) to account for 30% of the administered dose of labelled BCNU in monkeys yields evidence supportive of this hypothesis. Further experiments are necessary to confirm this possibility.

III. EFFECT OF BCNU ON THE MIXED FUNCTION OXIDASE

Introduction

The mixed function oxidase system of the liver is known to catalyze a wide variety of reactions (Gillette, 1963; Gillette and Gram, 1969; Gillette, 1971). The range of compounds metabolized included steroids (Conney et al., 1968), fatty acids (Lu and Coon, 1968; Wada et al., 1968), many drugs of varied structures, polycyclic carcinogens (Silverman and Talalay, 1967), heme and insecticides (Estabrook et al., 1971). One must therefore conclude that the mixed function oxidase enzyme system has an extremely broad specificity. At present, workers in this area are debating whether the mixed function oxidase complex of the endoplasmic reticulum represents a single type (Hildebrandt, Remmer and Estabrook, 1968) of enzyme system or a large variety of different types of enzyme complexes, each with different specificity. Pathways that are frequently studied are: 1) side chain oxidations of hexobarbital and pentobarbital, 2) N-demethylation of ethylmorphine, 3) p-hydroxylation of aniline, 4) nitroreduction of p-nitrobenzoic acid. The effect of BCNU on the oxidations of hexobarbital and pentobarbital has been studied rather thoroughly. It would be interesting to see if BCNU also affected the other reactions mentioned above. The objective of this study was to show the degree of specificity of BCNU for the mixed function oxidases.

Methods

Incubation mixtures were prepared as described previously in the text. N-demethylating activity of ethylmorphine was determined by measuring the amount of formaldehyde produced. Aromatic hydroxylation of aniline was determined by measuring p-aminophenol; and nitro-reductase activity was obtained by estimating p-aminobenzoic acid that was produced following incubation of substrates with liver 9000 xG fractions or microsomal preparations.

The Colorimetric Estimation of Formaldehyde

At the end of the incubation period, 1 ml of 5% $ZnSO_4$, 1.5 ml of saturated solution of barium hydroxide, and 0.5 ml of saturated sodium borate solution were added to the reaction mixtures (Cochin and Axelrod, 1959). The tubes were centrifuged at 4000 rpm for 10 minutes. A 2-ml aliquot of the protein-free supernatant was treated with 2 ml of double-strength Nash reagent (Nash, 1953). The cuvettes were then heated in a water bath at 60°C for 30 minutes, after which the optical density at 415 nm was determined.

Estimation of p-aminophenol

The reaction mixtures were incubated with aniline as the substrate at 37°C for 30 minutes under air (Guarino et al., 1969). Para-aminophenol that was formed was extracted into 15 ml of peroxide-free

ether saturated with sodium chloride. Ten milliliters of the ether extract was then returned to an aqueous phase by shaking with 4 ml of a 0.1M NaOH solution containing 1% phenol. The aqueous phase was allowed to stand for 30 minutes and the optical density measured at 620 nm.

Nitro-reduction of p-nitrobenzoic Acid

One milliliter of 9000 xG supernatant, equivalent to 0.5 gram of liver, was incubated with p-nitrobenzoic acid (3 μmoles), $NADP^+$ (0.3 μmole), nicotinamide (100 μmoles), Tris buffer, pH 7.2 adjusted to a final volume of 5 ml, at 37° C for 3 hours under nitrogen (Fouts and Brodie, 1957). Following incubation, 15 ml of 6.67% trichloroacetic acid was added. After centrifugation, a 5-ml aliquot of the protein-free supernatant was reacted according to the Bratton and Marshal method (1939) by adding 1 ml of 0.1% sodium nitrite; when 3 minutes had elapsed, 1 ml of 0.5% ammonium sulfamate was added. After another 2 minutes, 1 ml of 100 mg% N-(1-naphthyl)-ethylenediamine dihydrochloride was also added. The color products were then extracted into 15 ml of isoamyl alcohol and the optical density at 540 nm was recorded.

Results

BCNU was found to produce a general depression on the microsomal mixed function oxidase system. N-demethylation activity was determined by measuring the amount of formaldehyde formed from the substrate ethylmorphine. It was demonstrated that control rats were able to produce 0.37 micromole of formaldehyde per half gram of liver per 15 minutes at a substrate concentration of 2.5×10^{-3}M whereas rats receiving BCNU could only produce 0.03 micromole under equivalent conditions (Figure 3-1). It was clear that the enzyme activities in BCNU-treated animals were very much reduced. When enzyme inducing doses of phenobarbital were given to control animals, a three-fold increase in formaldehyde production was noted. In contrast to what was seen in the oxidation of pentobarbital and hexobarbital, phenobarbital administration to BCNU-treated rats produced little change in the enzyme activities as compared with the non-induced BCNU-treated animals. The enzymes were still inhibited.

From the data illustrated in Figure 3-2, it was apparent that the hydroxylation of aniline was also impaired in rats 13 days after a single administration of BCNU. Their livers were metabolizing at 1/3 the rate of control livers. Moreover, when controls were pretreated with phenobarbital, a doubling in the rate of the reaction was observed. Similarly, when BCNU-treated rats were exposed

Figure 3-1. Effect of a single i.p. dose of BCNU (30 mg/kg) on microsomal ethylmorphine N-demethylase activity at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 after BCNU administration. Each bar was the mean ± standard deviation of at least 5 animals. The enzyme was significantly depressed in BCNU-treated rats. Phenobarbital induction greatly increased enzyme activity in controls, but only slightly increased enzyme activity in BCNU-treated animals.

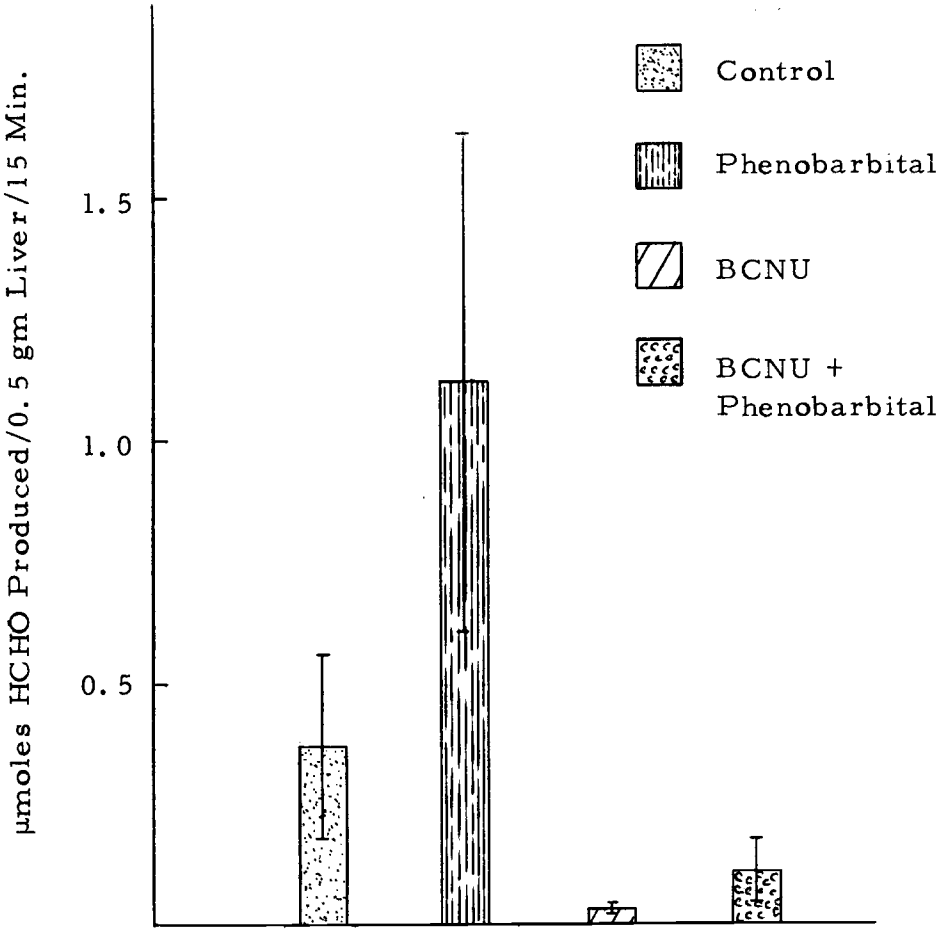
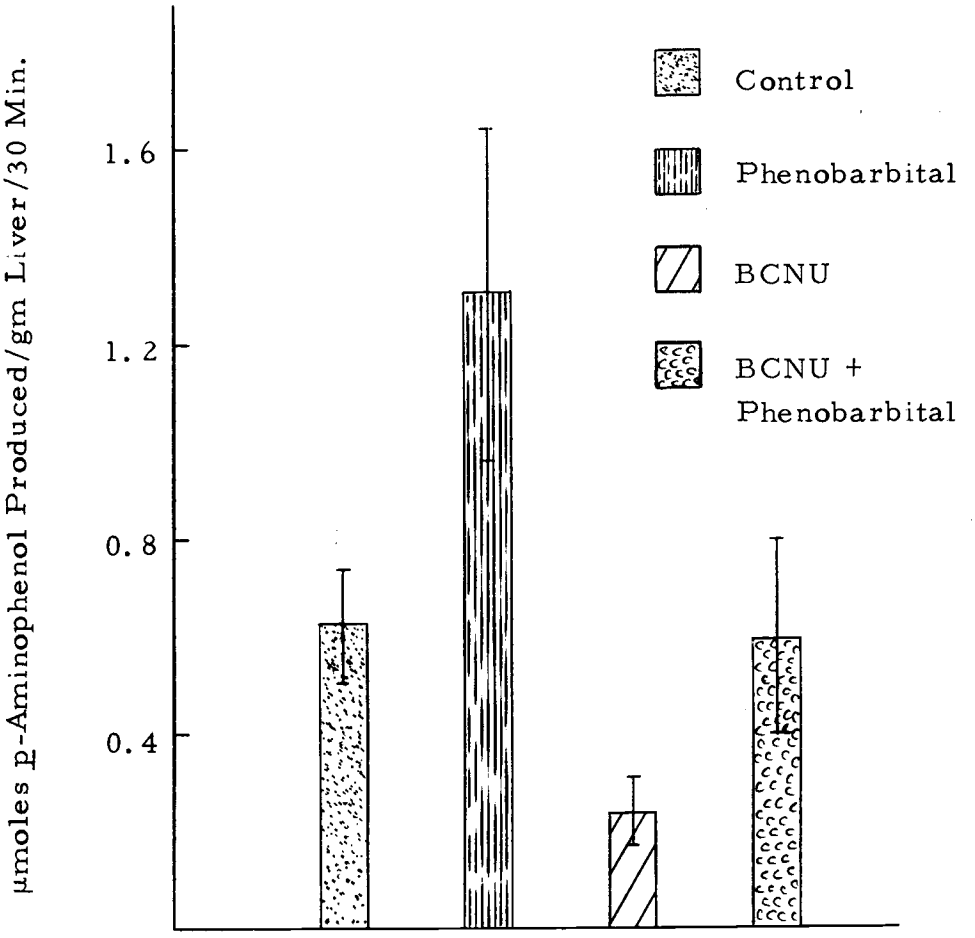

µmoles HCHO Produced/0.5 gm Liver/15 Min.
1.5
1.0
0.5
Control
Phenobarbital
BCNU
BCNU +
Phenobarbital

Figure 3-2. Effect of a single i.p. dose of BCNU (30 mg/kg) on the hydroxylation of aniline by liver 9000 xG fractions in rats at 13 days post treatment. At least 5 animals were used in each group. The bars were mean responses and the brackets were standard deviations. Enzyme activity was greatly depressed in BCNU-treated animals. Repeated administrations of phenobarbital resulted in significant increases of enzyme activity in both controls and BCNU-treated rats.


µmoles p-Aminophenol Produced/gm Liver/30 Min.
1.6
1.2
0.8
0.4
Control
Phenobarbital
BCNU
BCNU +
Phenobarbital

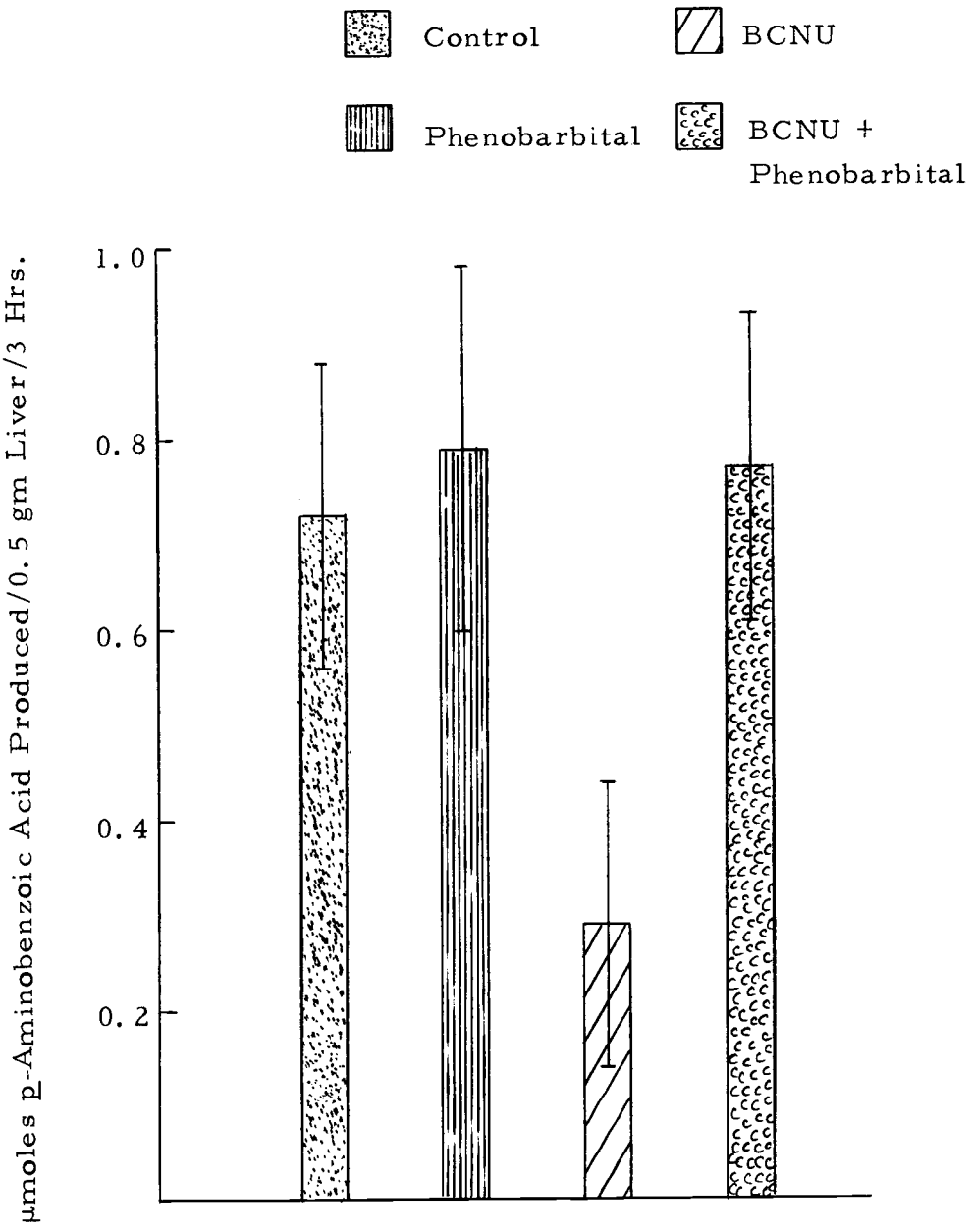
to enzyme inducing doses of phenobarbital, a two-fold increase in the rate of product formation over that of non-induced BCNU-treated rats was obtained; however, comparing both groups of induced animals, a significant difference in the rate of aniline hydroxylation was noted.

In addition to oxidative pathways, the effect of BCNU on nitroreduction was considered; the substrate used was p-nitrobenzoic acid. As expected, BCNU produced an inhibition of the reductase activity (Figure 3-3). The magnitude of inhibition was about 60%, which was approximately the same as in all other pathways studied. Repeated administrations of phenobarbital had no significant inducing effects on control rats. Nevertheless, phenobarbital pretreatment in rats given BCNU brought the reductase activities in these animals to control values. It appeared that phenobarbital did induce the reductase in BCNU-treated rats although it did not induce this enzyme in controls.

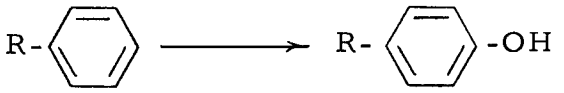
Discussion

BCNU appeared to affect both oxidative and reductive pathways catalyzed by the liver microsomal system. According to Brodie and La Du (1958), all of the oxidative reactions considered could be written as hydroxylation reactions in the following manner:

Figure 3.3. Effect of a single i.p. dose of BCNU (30 mg/kg) on the nitro-reduction of p-nitrobenzoic acid by liver 9000 xG fractions in rats at 13 days post treatment. At least 5 animals were assigned to each group. The bars were mean responses and the brackets were standard deviations. Enzyme activity was greatly depressed in BCNU-treated animals. Optimal enzyme inducing doses of phenobarbital did not increase enzyme activity in controls, but significantly increased activity in BCNU-treated rats.


Control
BCNU
Phenobarbital
BCNU + Phenobarbital
µmoles p-Aminobenzoic Acid Produced/0.5 gm Liver/3 Hrs.
1.0
0.8
0.6
0.4
0.2

$$R\text{-}CH_3 \xrightarrow{P\text{-}450\cdot O} R\text{-}CH_2OH$$ Aliphatic oxidation



Aromatic hydroxylation

$$R\text{-}NH\text{-}CH_3 \longrightarrow [R\text{-}NH\text{-}CH_2OH] \longrightarrow R\text{-}NH_2 + HCHO$$

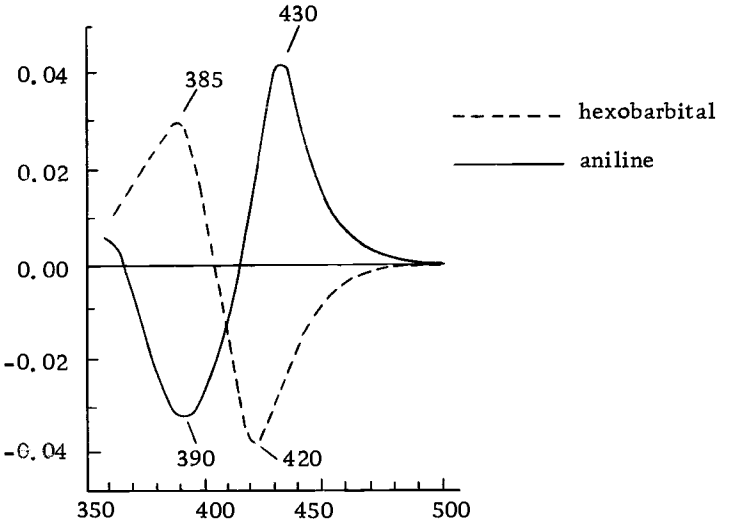
N-dealkylation

Studies with ^{18}O have shown that the oxygen introduced into the substrate molecule is derived from air, and not from the hydroxyl group of water (Ullrich and Staudinger, 1969). The protein P-450 is acting as an oxygen transferase, while reducing one oxygen atom to water (Omura et al., 1965). The observation that BCNU produced a general depression of the different oxidative reactions led to the impression that either the various pathways were mediated by limited non-specific enzyme systems, and BCNU had affected all of them, or, BCNU was affecting one factor that was common to all pathways, which was very likely to be cytochrome P-450.

Reduction of nitro compounds is also catalyzed by liver microsomes. The common requirement of NADPH for the oxidation of drugs and the reduction of nitro compounds suggested that certain parts of the electron transport system might also participate in the reduction reactions (Gillette and Gram, 1969). It was further demonstrated that inhibition of p-nitrobenzoate closely paralleled the formation of the carbon monoxide complexes of the cytochromes in mouse liver microsomes (Gillette and Gram, 1969). Moreover, the

decrease in activity caused by pretreatment of rats with CCl_4 paralleled the decrease in cytochrome P-450. Therefore, since decreased activities in both drug oxidation and nitro-reduction were obtained with BCNU treatment, it became strongly suggestive that inhibition of cytochrome P-450 was a possible underlying mechanism.

It is known that various substances added to liver microsomes cause two types of spectral changes (Remmer et al., 1966; Imai and Sato, 1966). The spectral change seen with type I compounds is characterized by a trough at 420 nm and an absorption peak at 385-390 nm and that of type II compounds by an absorption peak at 430 nm and a trough at 390 nm.



In general, primary aromatic and aliphatic amines cause type II changes, whereas secondary amines cause both type I and type II changes and most other substances including tertiary amines cause

type I changes (Gillette and Gram, 1969). Type I and type II binding could be explained on the basis of two binding sites on a single hemoprotein, with different compounds combining preferentially with one or the other site, or it could be explained on the basis of the existence of two hemoproteins which combined selectively with different drugs. In our investigation with BCNU, both type I (hexobarbital, ethylmorphine) and II (aniline) substrates were used. BCNU was shown to depress both. In accordance with the above hypotheses, one could propose that BCNU either inhibited both hemoproteins, or it had affected both binding sites on a single hemoprotein. The nature of the inhibition awaits further experimentation.

The enzyme inducing effects of phenobarbital were again demonstrated in animals with and without BCNU pretreatment. In both controls and BCNU-treated rats, ethylmorphine N-demethylase and aniline hydroxylase activities were increased by more than 100% following phenobarbital as compared with their non-induced counterparts. However, because of impaired metabolism of these compounds as a result of BCNU treatment, there were parallel lower enzyme activities in BCNU-treated animals after induction when compared to induced controls. The enhanced activities could be due to an increase in the concentration of protein or cytochrome P-450, or both. It is necessary at this point to investigate this aspect closely.

IV. EFFECTS OF BCNU ON TWO HEMOPROTEINS OF LIVER MICROSOMES

Introduction

The electron-transfer system in liver microsomes is characterized by two hemoproteins, cytochrome b_5 and P-450. It is well documented that cytochrome P-450 acts as the oxygen-activating enzyme (Cooper et al., 1965; Omura et al., 1965) as well as the site of substrate interaction (Imai and Sato, 1966; Remmer et al., 1967) for oxidations of various foreign compounds. The role of cytochrome b_5 is thought to be in oxidative desaturation of fatty acids (Sato, Nishibayashi and Ito, 1969). Estabrook et al. (1971) recently included cytochrome b_5 in the reduction-oxidation cycle, suggesting b_5 as the electron donor to the oxygenated intermediate of the reduced cytochrome P-450 substrate complex.

Previous experimentation had led to the feeling that cytochrome P-450 level might be significantly affected by administration of BCNU to rats, therefore, it became imperative that the content of cytochrome P-450 in BCNU-treated animals be determined. An attempt was also made to characterize the dose relationship of BCNU on P-450 levels observed through a period of 3 weeks. Furthermore, the content of cytochrome b_5 was measured, since it is the only other hemoprotein present in liver microsomes and it may also be

involved in drug oxidations. To complete the picture, total microsomal protein was also evaluated.

In addition, to investigate the possibility that BCNU had produced some changes in the spectral properties of the hemoprotein such that the spectral peak was shifted to some wavelength other than 450 nm, complete difference spectra of the cytochrome P-450-CO complex were determined. This study may be necessary to allow better interpretations of the effect of BCNU on cytochrome P-450.

Methods

Preparation of Tissue

Five grams of liver were obtained from each rat and homogenized in 10 ml of 1.15% KCl. The 9000 xG fraction was prepared as previously described in the text. The postmitochondrial fraction was then centrifuged at 39,000 rpm (100,000 x G) with a Beckman model L3-50 preparative ultracentrifuge and a type 40.2 rotor for 90 minutes to get the microsomal pellet. The pellet was rinsed once and then suspended in KCl and recentrifuged at 100,000 xG for 90 minutes. The samples were maintained at 0° C at all times. The microsomal pellet was finally resuspended a second time in KCl so that 1 ml was equivalent to the microsomes from 0.5 gm of liver.

Determination of Cytochrome P-450 (Omura et al., 1964)

The microsomal suspension was diluted four-fold in 0.1M phosphate buffer, pH 7.0. A few milligrams of sodium dithionite was added. The suspension was then divided and placed in 2 cuvettes. Carbon monoxide gas, generated by mixing concentrated formic acid with concentrated sulfuric acid, was bubbled for one minute through the suspension in one cuvette while the other cuvette served as reference. The absorbances at 450 nm and 500 nm were measured and the optical density difference calculated. The resulting difference was divided by the extinction coefficient, 91 $mM^{-1} cm^{-1}$ (Omura and Sato, 1963), and by the concentration of protein in the suspension. Consequently, the amount of cytochrome P-450 present was expressed as nanomoles of cytochrome per milligram of protein.

Difference Spectra of Carbon Monoxide Binding to Cytochrome P-450

Rats were treated with 30 mg/kg of BCNU intraperitoneally. At 7 days post treatment, the rats were sacrificed and the liver microsomes isolated as previously described. The final resuspension of microsomes was made in 1.15% KCl solution such that 1 ml of suspension was equivalent to 1 gm of liver. The preparation was further diluted fifteen-fold with 0.1M phosphate buffer, pH 7.4. One milliliter of diluted microsomes was placed in each

of 2 cuvettes. A few crystals of sodium dithionite were added to each cuvette. Carbon monoxide gas was bubbled into the sample cuvette for 15 seconds. Air was bubbled into the reference cuvette for the same amount of time. The difference spectrum was recorded with a Cary 15 spectrophotometer. Total microsomal protein was determined by the method of Lowry et al. (1951).

Determination of cytochrome b_5 (Alvares et al., 1969)

The microsomal preparation was diluted in 0.1M Tris buffer, pH 7.4. Three milliliters of suspension were placed in each of 2 cuvettes. Fifteen microliters of NADH (final concentration = 0.2 mM) was added to the sample cuvette and the difference spectra at 409 and 425 nm were recorded. The concentration of cytochrome b_5 was calculated in the same manner as cytochrome P-450, except the extinction coefficient used for cytochrome b_5 was 185 $mM^{-1} cm^{-1}$ (Eling et al., 1970).

Total Protein Determination

Total protein was estimated according to the method of Lowry et al. (1951). One-tenth milliliter of diluted microsomal suspension was delivered into a cuvette. One milliliter of alkaline copper solution, prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1N NaOH with 1 ml of 0.5% $CuSO_4$ in 1% sodium tartrate, was added. The contents of

the cuvette were mixed thoroughly and allowed to stand at room temperature for 10 minutes. Subsequently, 0.1 ml of diluted Folin reagent was added and the sample was mixed within 1 or 2 seconds. After 30 minutes, the optical density at 500 nm was determined.

Determination of Methemoglobin (Frankel and Reitman, 1963)

Blood samples were obtained by cardiac puncture and diluted ten times with distilled water. An initial optical density reading at 800 nm was noted. Then a few crystals of KCN was added to the cuvette and mixed. A second reading at 800 nm was obtained. The difference in optical density was multiplied by 36 to give the concentration (gm %) of methemoglobin in the blood.

Serum Iron Determination (Frankel and Reitman, 1963)

To 1 ml of plasma was added 2 ml of 2N HCl with vigorous stirring. After 10 minutes, 2 ml of 20% trichloroacetic acid was added, again stirred and centrifuged. Four milliliters of supernatant fluid was pipetted into a cuvette containing 2 drops of thioglycollic acid, 0.5 ml 0.4% 2, 2-dipyridyl and 2.5 ml saturated sodium acetate. The contents of the cuvette was mixed thoroughly and the optical density at 525 nm was determined.

Results

The level of cytochrome P-450 in microsomes was significantly lowered at 13 days after a single dose of BCNU (30 mg/kg) (Figure 4-1). Controls had an average of 1.46 nanomoles cytochrome P-450 per milligram of protein, while treated animals had only 0.56 nanomole per milligram protein. The hemoprotein was reduced to one-third its control level. Repeated administrations of phenobarbital produced a three-fold increase in control rats, as reported by a number of investigators (Gillette and Gram, 1969; Omura and Kuriy Kuriyama, 1969). Phenobarbital induction in BCNU-treated rats also resulted in a two-fold increase of the hemoprotein.

It was further demonstrated that the reduction of P-450 content was dose-related (Figure 4-2). By 7 days post treatment, the concentration of cytochrome P-450 was significantly lowered at a dose of 20 mg/kg. The effect was more magnified with the higher dose of BCNU (30 mg/kg). When the effect was followed on a time course basis, it was noted that it reached its maximum by 13 days and maintained at that level until 21 days. By 21 days, the same magnitude of effect was achieved by either dose.

Difference spectra of carbon monoxide binding to cytochrome P-450 of controls and BCNU-treated rats are shown in Figure 4-3. As both control microsomes and microsomes obtained from

Figure 4-1. The effect of a single dose of BCNU (30 mg/kg) on the level of microsomal cytochrome P-450 in rats at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 after BCNU treatment. The results were pooled from at least 3 experiments. Each bar was the mean ± standard deviation of 15-17 animals. A 60% reduction in P-450 concentration was noted in BCNU-intoxicated rats. Phenobarbital induction led to a three-fold and two-fold increase respectively of the hemoprotein in controls and BCNU-treated rats.

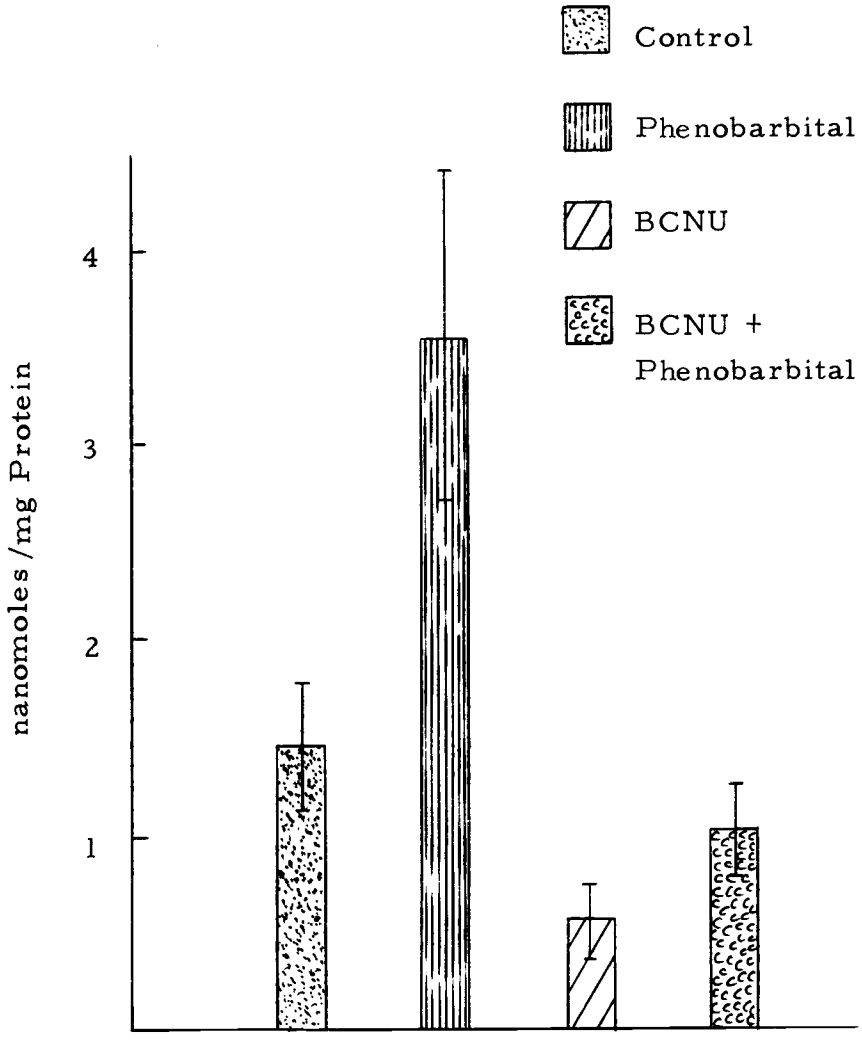

Control
Phenobarbital
BCNU
BCNU +
Phenobarbital
nanomoles /mg Protein
4
3
2
1

Figure 4-2. The temporal effects of single doses of BCNU (in corn oil) on levels of microsomal cytochrome P-450 in rats. Five animals were sacrificed at each sampling time for each dose. The brackets were standard deviations. Significant and dose-related reductions of cytochrome P-450 were evident at 7 days post treatment. The effect was found to persist through 21 days after BCNU administration.

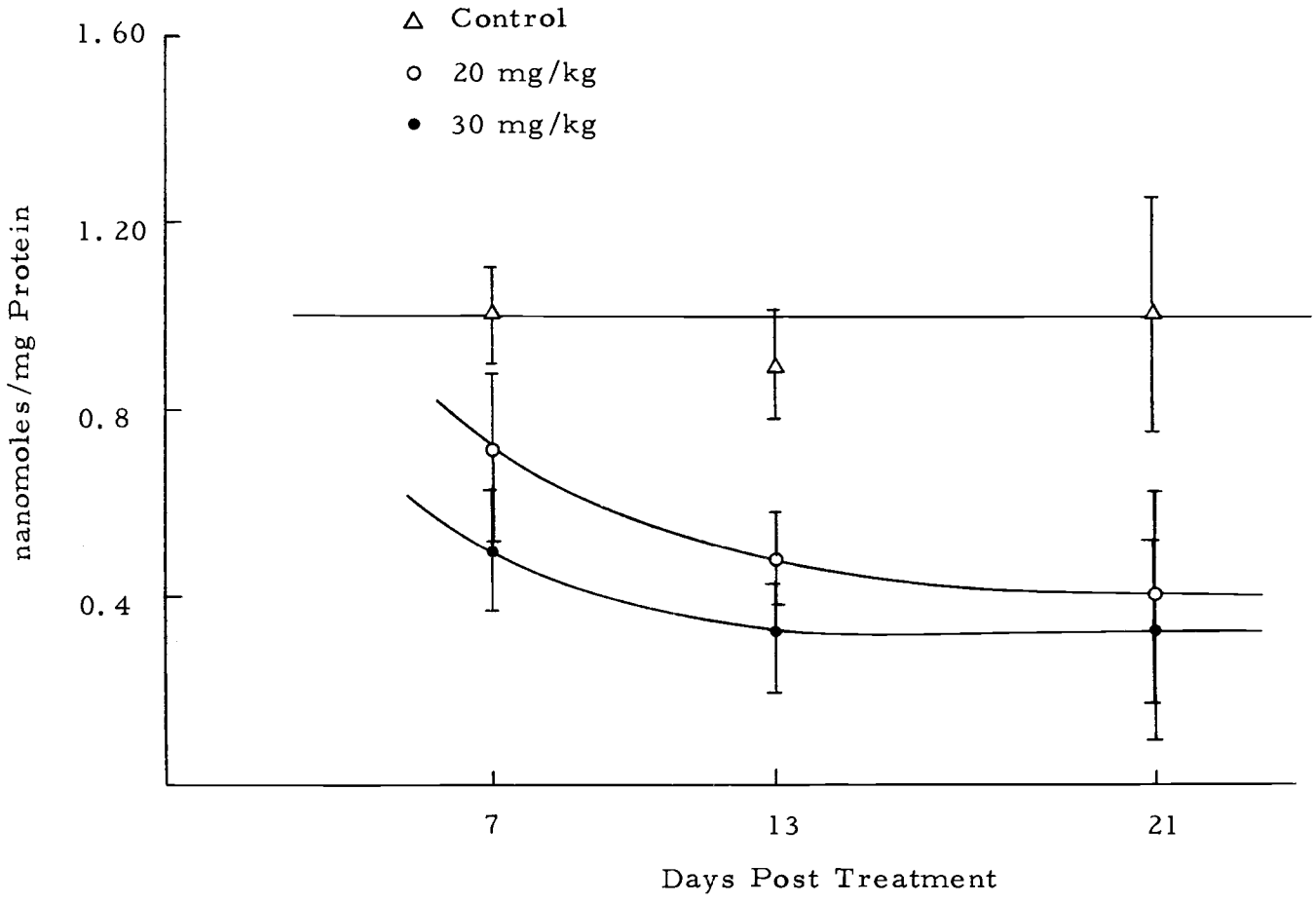
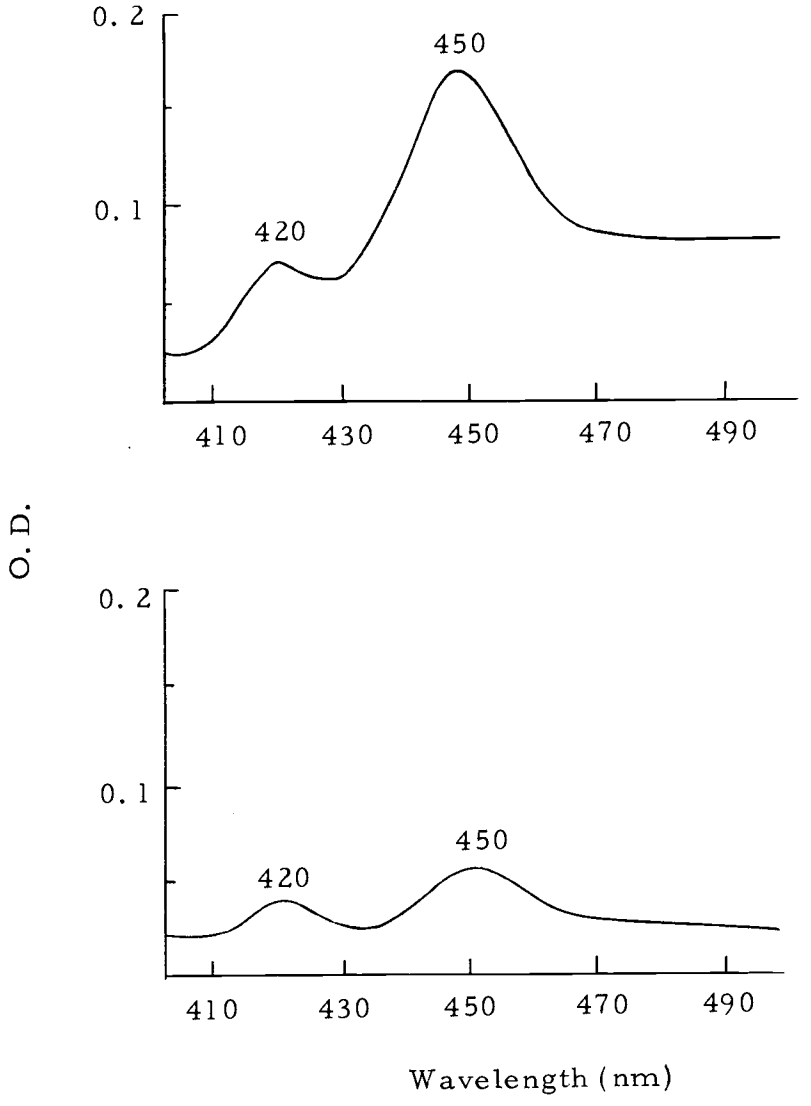

Control
20 mg/kg
30 mg/kg
1.60
1.20
0.8
0.4
nanomoles/mg Protein
7
13
21
Days Post Treatment

Figure 4-3. Effect of a single dose of BCNU (30 mg/kg in corn oil) on the difference spectra of carbon monoxide binding to microsomal cytochrome P-450 in rats. Microsomes were obtained at 7 days post treatment, and contained approximately 2 mg protein/ml. The spectrum of P-450-CO complex of BCNU-treated rats compared to control microsomes showed reductions in both the 450 and 420 peaks.


0.2
0.1
450
420
410
430
450
470
490
O.D.
0.2
0.1
450
420
410
430
450
470
490
Wavelength (nm)

BCNU-treated rats showed characteristic spectra, two spectra, one representative of each group, are illustrated. Each diluted sample contained approximately 2 milligrams of protein per milliliter. The spectrum of control microsomes revealed a typical peak at 450 nm, representing cytochrome P-450, and a much diminished peak at 420 nm, representing cytochrome P-420. The microsomes from BCNU-treated rats, on the other hand, showed considerable reductions in both the 450 and 420 peaks. These results led us to conclude that there was no significant conversion of cytochrome P-450 to cytochrome P-420 in the BCNU-treated animals, and the apparent reduction of this hemoprotein represented a true depression.

BCNU's effect on cytochrome b_5 presented a slightly different picture (Figure 4-4). Control livers contained 0.36 nanomole per milligram of microsomal protein whereas treated rats had 0.28 nanomole per milligram of protein. These values could not be proven different statistically, although there was some tendency for BCNU-treated animals to have lower levels of the cytochrome. Phenobarbital induction did not produce significant increases of this hemoprotein in either controls or treated rats. These results were not different from those reported by Omura and Kuriyama (1969).

To complete the picture, total microsomal protein was determined (Figure 4-5). BCNU administration did not reduce the concentration of protein significantly. When phenobarbital was given,

Figure 4-4. The effect of a single dose of BCNU (30 mg/kg) on microsomal cytochrome b_5 levels in rats at 13 days post treatment. Phenobarbital was administered at 100 mg/kg daily for 5 days beginning on day 7 after BCNU. Each bar was the mean ± standard deviation of at least 5 animals. Cytochrome b_5 concentration was slightly depressed by BCNU. Phenobarbital induction had no significant effects on cytochrome b_5 contents in either controls or BCNU-treated rats.

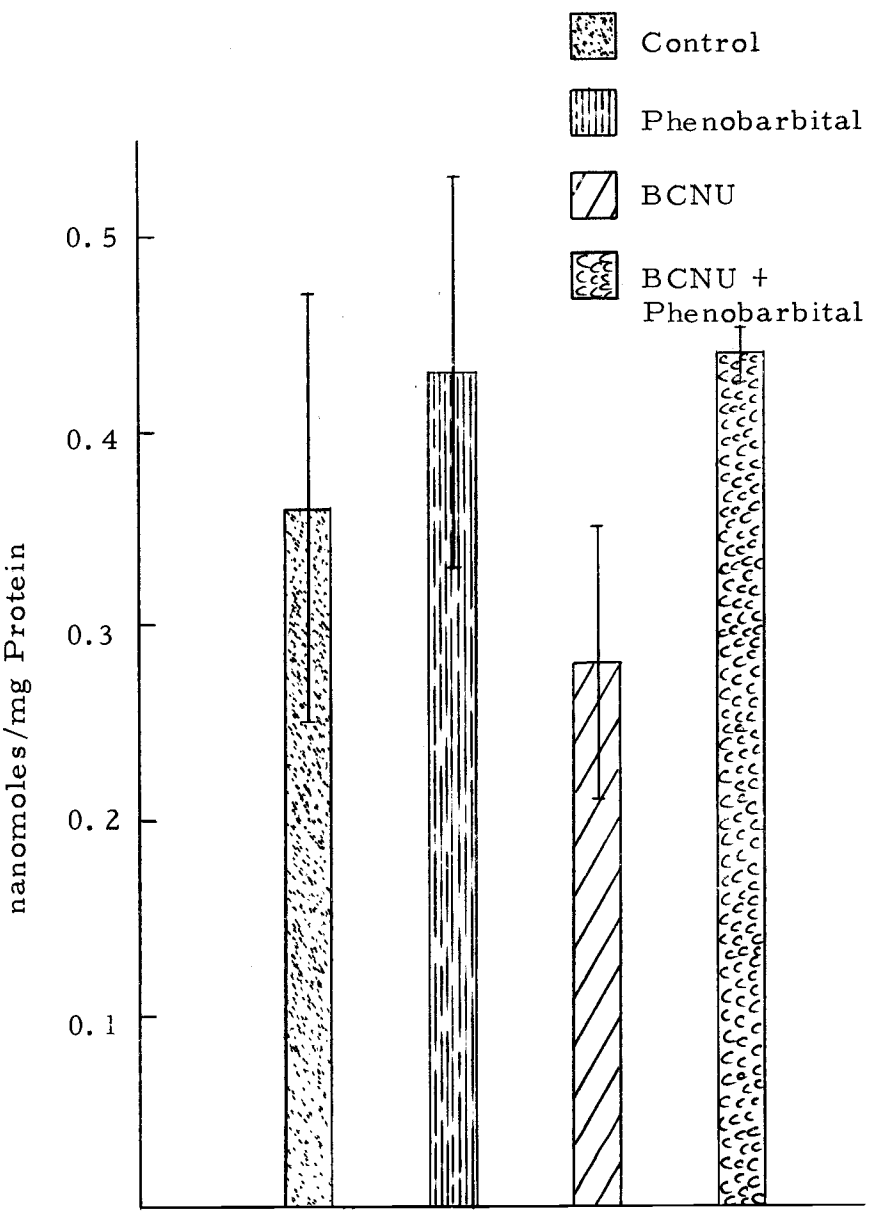
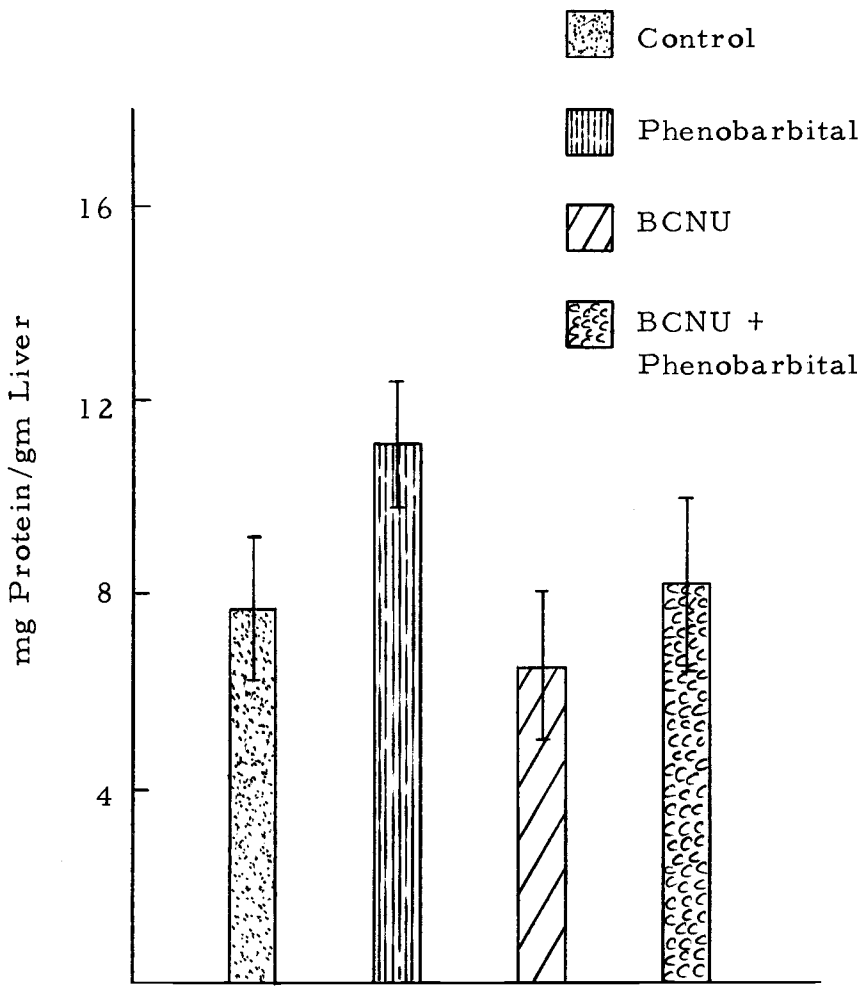

Control
Phenobarbital
BCNU
BCNU +
Phenobarbital
nanomoles/mg Protein
0.5
0.4
0.3
0.2
0.1

Figure 4-5. The effect of a single dose of BCNU (30 mg/kg in corn oil) on total microsomal protein in rats at 13 days post treatment. The results were pooled from at least 3 experiments. Each bar was the mean ± standard deviation of 16 animals. Phenobarbital was administered at 100 mg/kg daily for 5 days. Rats were sacrificed 24 hours after the last phenobarbital injection. Slight decreases of total protein, though insignificant, were demonstrated in BCNU-treated rats. Phenobarbital induction again brought significant increases in both groups.


Control
Phenobarbital
BCNU
BCNU +
Phenobarbital
mg Protein/gm Liver
16
12
8
4

total protein increased from 7.68 to 11.1 mg/gm of liver in controls, and from 6.50 to 8.18 mg/gm of liver in BCNU-treated rats. The increase was significant in both cases.

It was now revealed that BCNU had pronounced effects on cytochrome P-450 and less severe effects on total microsomal protein. Since cytochrome P-450 is a hemoprotein, and since there has been no indication of any disturbance in the protein portion of the molecule, it appeared reasonable to consider carefully the heme portion of the cytochrome. Therefore, serum iron, together with total iron binding capacity was determined in animals receiving 30 mg/kg of BCNU (Table 4-1). Control animals exhibited 50 μg% of iron in their blood while treated animals had 65 μg%, showing that a deficiency of iron was not the limiting factor. Furthermore, total iron binding capacity had not changed.

Table 4-1. Effect of a single dose of BCNU (30 mg/kg) on serum iron and total iron binding capacity at 13 days post treatment.

	Serum iron	UIBC	TIBC	% Sat.
		(μg % ± S. E.)		
BCNU	65.2 ± 10.92 (7)[a]	408 ± 32.7	480 ± 31.2	15.2 ± 2.3
Control	50.1 ± 5.88 (9)	434 ± 28.6	484 ± 28.6	10.8 ± 1.6

UIBC = Unsaturated iron binding capacity
TIBC = Total iron binding capacity
% Sat. = % saturation
a = No. of animals per group

It was noted that blood samples withdrawn from BCNU-treated rabbits appeared dark brownish, suggesting that these animals might be experiencing methemoglobinemia. With reference to cytochrome P-450, iron in the oxidized state may act in a fashion entirely different than that in the reduced state with respect to substrate binding or the electron transport chain. Methemoglobin measurements were used as an index to determine the physical state of the iron in the heme moiety (Table 4-2). It was apparent that on both 7 and 13 days after BCNU, methemoglobinemia was not detected. In fact, significantly less methemoglobin was found in BCNU-treated rats.

Table 4-2. Temporal effects of a single dose of BCNU (30 mg/kg) on blood methemoglobin contents.

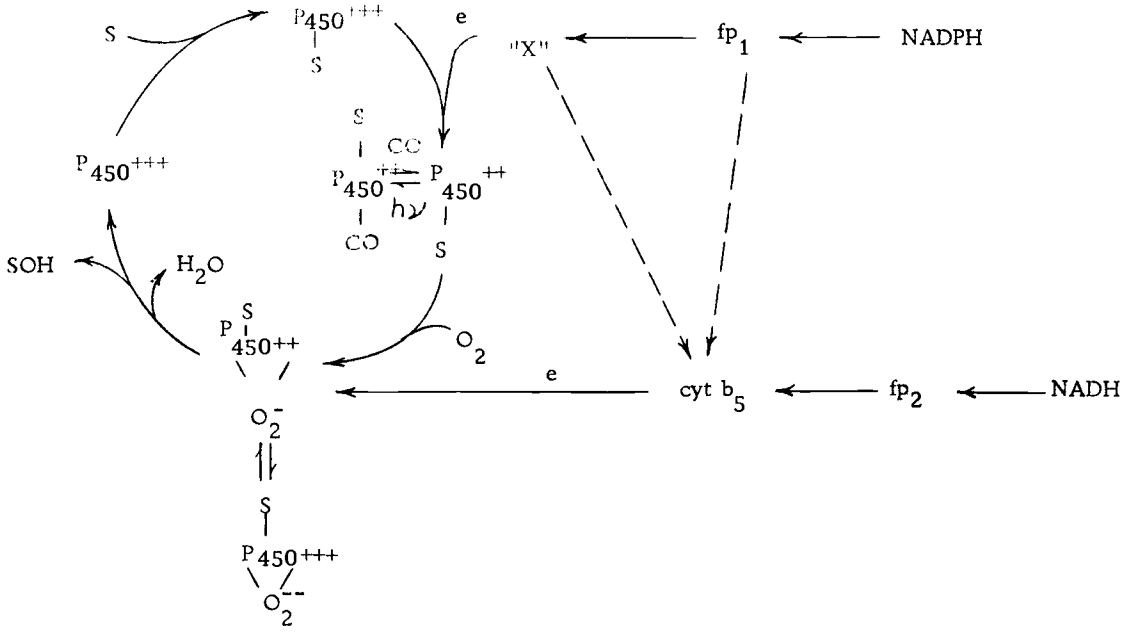
	(Methemoglobin in gm% ± S. E.)	
	Day 7	Day 13
BCNU	0.25 ± 0.045 (10)[a]	0.45 ± 0.119 (10)
Control	0.64 ± 0.066 (10)	0.74 ± 0.080 (10)

[a]Number of animals per group

Discussion

The depression of drug metabolizing enzymes in BCNU-treated rats could well be explained by significant reductions of cytochrome

P-450 in these animals. Estabrook et al. (1971) proposed a scheme of cytochrome P-450 reduction-oxidation cycle associated with hydroxylation reactions in the following manner:



It is generally recognized that cytochrome P-450 in its ferric state can react with substrates to give spectrally definable complexes (Schenkman et al., 1967b; Imai and Sato, 1966). This ferric-substrate complex then undergoes a reduction forming the ferrous-substrate complex. Furthermore, it was found by Gigon et al. (1969) that the oxidized cytochrome P-450-substrate complex was more rapidly reduced than the uncomplexed form of oxidized cytochrome P-450. The ferrous-substrate complex in turn reacts with oxygen to give a

hypothetical oxygenated intermediate after which it completes the cycle giving the hydroxylated product and water together with ferric cytochrome P-450. According to this scheme, it would not be difficult for one to visualize that if less cytochrome P-450 was available to the substrate, as demonstrated in BCNU-treated animals, less hydroxylated products would be formed. Therefore, inhibition of the mixed function oxidase would be, and in fact was observed.

This hypothesis is further supported by the parallel persistence of effects in pentobarbital metabolizing activity and the reduction of cytochrome P-450 for up to 21 days following BCNU.

When inducing doses of phenobarbital were administered to BCNU-treated rats, a two-fold increase in the amount of cytochrome P-450 was observed. This was not unusual since it had been well documented that phenobarbital increases the concentration of the hemoprotein by stimulating synthesis (Remmer and Merker, 1965; Ernster and Orrenius, 1965; Levin and Kuntzman, 1969c).

Recently, spectral studies led to the proposal of the existence of more than one form of cytochrome P-450. Levin and Kuntzman (1969) revealed a biphasic decrease of radioactive hemoprotein from liver microsomal CO-binding particles. Hildebrandt et al. (1968) demonstrated a marked spectral difference between the pigment induced by phenobarbital and that induced by the carcinogenic compound 3-methylcholanthrene. Further evidence was obtained by the

findings of Sladek and Mannering (1969a) that phenobarbital pre-treatment increased the N-demethylation of ethylmorphine and of the aminoazo dye 3-methyl-4-methylaminoazobenzene (3-CH_3-MAB), whereas 3-methylcholanthrene stimulated only the latter. It was subsequently suggested that 3-methylcholanthrene stimulated the formation of a new hemoprotein (Sladek and Mannering, 1966; Shoeman, Chaplin and Mannering, 1969), designated as cytochrome P_1-450. Cytochrome P_1-450 has been characterized by Alvares et al. (1967) to have a maximum absorption at 448 nm, slightly different than that of normally occurring cytochrome P-450.

Studies of the difference spectra of cytochrome P-450-CO complex clearly demonstrated that BCNU treatment resulted in a true depression of cytochrome P-450 in liver microsomes. Conversion of cytochrome P-450 to its inactive form cytochrome P-420 was not significant in BCNU-treated animals. These results suggested that more attention should be directed to the effect of BCNU on the rate of synthesis of cytochrome P-450, or more specifically on the biosynthetic pathway for heme. Conversely, the rate of degradation of the hemoprotein should be determined. A good way to pursue this investigation would be to follow the incorporation of δ-aminolevulinic acid-3-5-^{3}H and measure the radioactivity associated with cytochrome P-450 as described by Levin and Kuntzman (1969a). On the other hand, one can label the hemoprotein prior to

administration of BCNU, and then follow the rate of degradation of the pigment.

The effect of BCNU on cytochrome b_5 and total protein was much less severe than on cytochrome P-450. BCNU seemed to lower the concentration of b_5, but the values were not statistically different from controls. The effect on total microsomal protein was variable. In some animals, there were considerable reductions, while others were unaffected. Therefore, pooled data gave a mean value not significantly different from controls. In any case, it would be difficult to project measurements of total protein in the microsomes to what had actually occurred within a single enzyme system. Accurate analysis would require isolation of that particular enzyme.

This study did not provide us with an explanation of why cytochrome P-450 was affected by BCNU more significantly than cytochrome b_5. However, it was demonstrated by Estabrook and his coworkers (1971) that up to 25% of total protein per gram wet weight of liver was associated with the endoplasmic reticulum, and that the concentration of cytochrome P-450 was frequently 5 to 10 times greater per gram of liver than the content of any of the cytochromes associated with mitochondria. It was claimed, therefore, that cytochrome P-450 is the hemoprotein of highest concentration existing in the liver cell, making up as much as 20% of the protein of the endoplasmic reticulum. On this basis, since cytochrome P-450 was

present in the liver in greater abundance than cytochrome b_5, the effect of BCNU on cytochrome b_5 may be easily masked.

Furthermore, it is possible that the iron in the heme portion of cytochrome P-450 is held in the Fe^{++} state, and therefore will not react with the substrates as readily as the Fe^{+++} state. Electron paramagnetic resonance studies may help to reveal the state of oxidation of the iron in heme.

V. COMPARISON OF THE EFFECTS OF BCNU WITH THE EFFECTS OF STARVATION ON THE MICROSOMAL DRUG METABOLIZING SYSTEMS

Introduction

Many factors, such as drug pretreatment, hormones, and nutritional status are known to modify the activity of hepatic microsomal enzymes (Gram et al., 1970). Dixon et al (1960) demonstrated that starvation of male mice for 36 hours resulted in a marked depression in the oxidative metabolism of several drug substrates by liver microsomes. It has already been reported by Thompson and Larson (1969) that BCNU-treated animals significantly reduced their food consumption leading to severe losses of body weight. It was further observed that these treated animals had impaired drug metabolizing enzyme systems. Since the animals receiving higher doses of BCNU demonstrated very little food intake, one could not exclude the possibility that the observed impairment of metabolism was directly related to their dietary status. The object of this study was to determine the accuracy of this hypothesis. Furthermore, it may be important to see if the effect of BCNU could be alleviated by force-feeding the animals, since these findings may have possible therapeutic significance.

Methods

Male rats, initially weighing 300 to 350 grams, were deprived of food for 7 days but were given free access to water. On the eighth day, they were killed and their livers removed. The 9000 xG supernatant was obtained and used for kinetics studies of pentobarbital oxidation as described previously. Microsomes were isolated and the contents of cytochrome P-450, cytochrome b_5 and total protein determined.

A group of rats initially weighing 200 grams was treated with 30 mg/kg of BCNU intraperitoneally. Control received equivalent volumes of corn oil. All animals were deprived of food but were given free access to water. At 24 hours post treatment, the rats were provided with a complete nutritional liquid diet (Nutrament ®), approximately isocaloric with their standard Purina lab chow diet, given orally, three times daily amounting to a total of 21 ml per animal. The liquid diet contained the following:

387 calories per 375 milliliters which included:

25 gm	protein
11 gm	fat
47 gm	carbohydrates
2500 U. S. P. units	Vit. A
200 U. S. P units	Vit. D

37.5 mg	Vit. C
0.8 mg	thiamine
0.9 mg	riboflavin
10 mg	niacinamide
0.57 gm	calcium
0.51 gm	phosphorus
6 mg	iron
5 int. units	Vit. E
0.8 mg	pyridoxine
1 mcg	cyanocobalamine
4 mg	calcium pantothenate
0.45 gm	sodium
0.65 gm	potassium
0.5 mg	copper
1 mg	manganese

Daily changes in weights of these animals were recorded. On the thirteenth day after the administration of BCNU, (i.e. 12 days after the initiation of force-feeding), the animals were sacrificed and their enzyme activities determined. Cytochrome P-450 and total protein were also estimated.

Results

It is evident that as early as 7 days after a single dose of BCNU

(20 or 30 mg/kg), pentobarbital metabolism by hepatic microsomes was greatly depressed (Figure 5-1). The effect was dose-related. At 10 mg/kg, no significant changes were demonstrated. At 20 or 30 mg/kg, the maximum velocities were considerably reduced, as previously described. V_{max} and K_m values are summarized in Table 5-1.

Table 5-1. Effect of single doses of BCNU on the V_{max} and K_m values of pentobarbital metabolism by liver microsomes at 7 days post treatment. All animals were given free access to food and water.

	Dose of BCNU			
	Control	10 mg/kg	20 mg/kg	30 mg/kg
V_{max} (μmoles/gm/45 min.)	2.50	2.20	0.67	0.40
K_m (M)	7.75×10^{-3}	3.71×10^{-3}	3.67×10^{-3}	3.60×10^{-3}

Comparing the effects of BCNU with the effects of pure starvation on the kinetics of pentobarbital oxidation, it was noted that both the BCNU-treated and starved rats showed parallel kinetic alterations (Figure 5-2). The enzymes in both groups of animals were inhibited to approximately the same magnitude, as seen in the Lineweaver-Burk plot. With respect to cytochrome P-450 and b_5 levels, similar results were obtained (Figure 5-3). Controls were found to have 13.8 nanomoles of cytochrome P-450 per gram of liver. Seven days following a 30 mg/kg dose of BCNU, the cytochrome P-450 level was reduced to 7.0 nanomoles per gram of liver. A 50% reduction was

Figure 5-1. The effect of single graded i.p. doses of BCNU (in corn oil) on the kinetics of pentobarbital oxidation by rat liver 9000 xG fractions. Three rats were treated with each dose indicated. Enzyme activities were determined at 7 days post treatment. No significant changes were apparent at 10 mg/kg. With higher doses (20 and 30 mg/kg), the enzymes were inhibited; the inhibition was characterized by greatly reduced V_{max} and unaltered K_m.

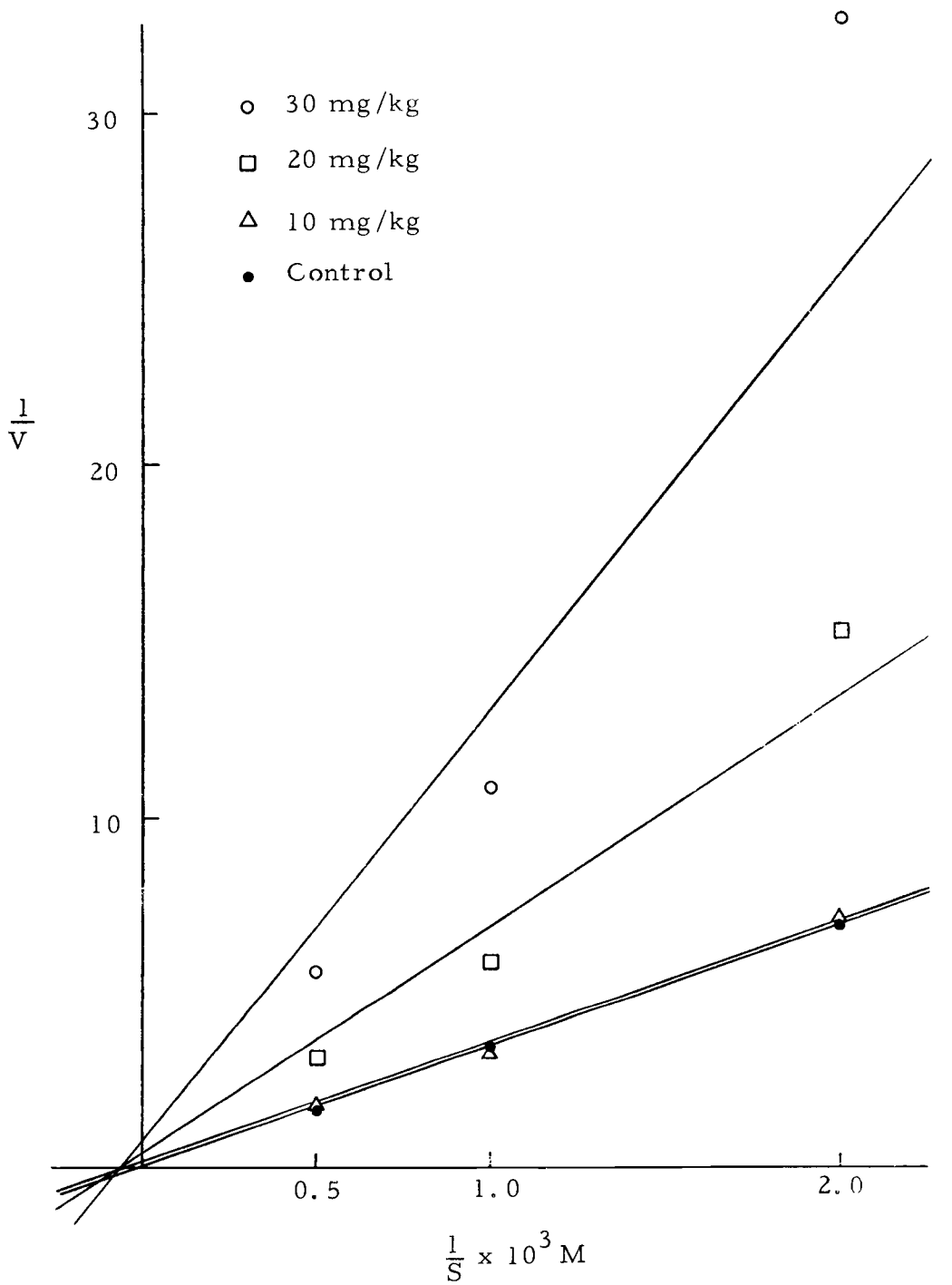

30 mg/kg
20 mg/kg
10 mg/kg
Control
30
20
10
1/V
0.5
1.0
2.0
1/S x 10^3 M

Figure 5-2. Comparison of the effect of a single dose of BCNU (30 mg/kg at 7 days post treatment) and the effect of a 7-day starvation on the kinetics of pentobarbital metabolism by rat liver postmitochondrial fractions. No significant difference was observed between BCNU-treated animals and starved animals. Both groups of rats had depressed enzyme systems.

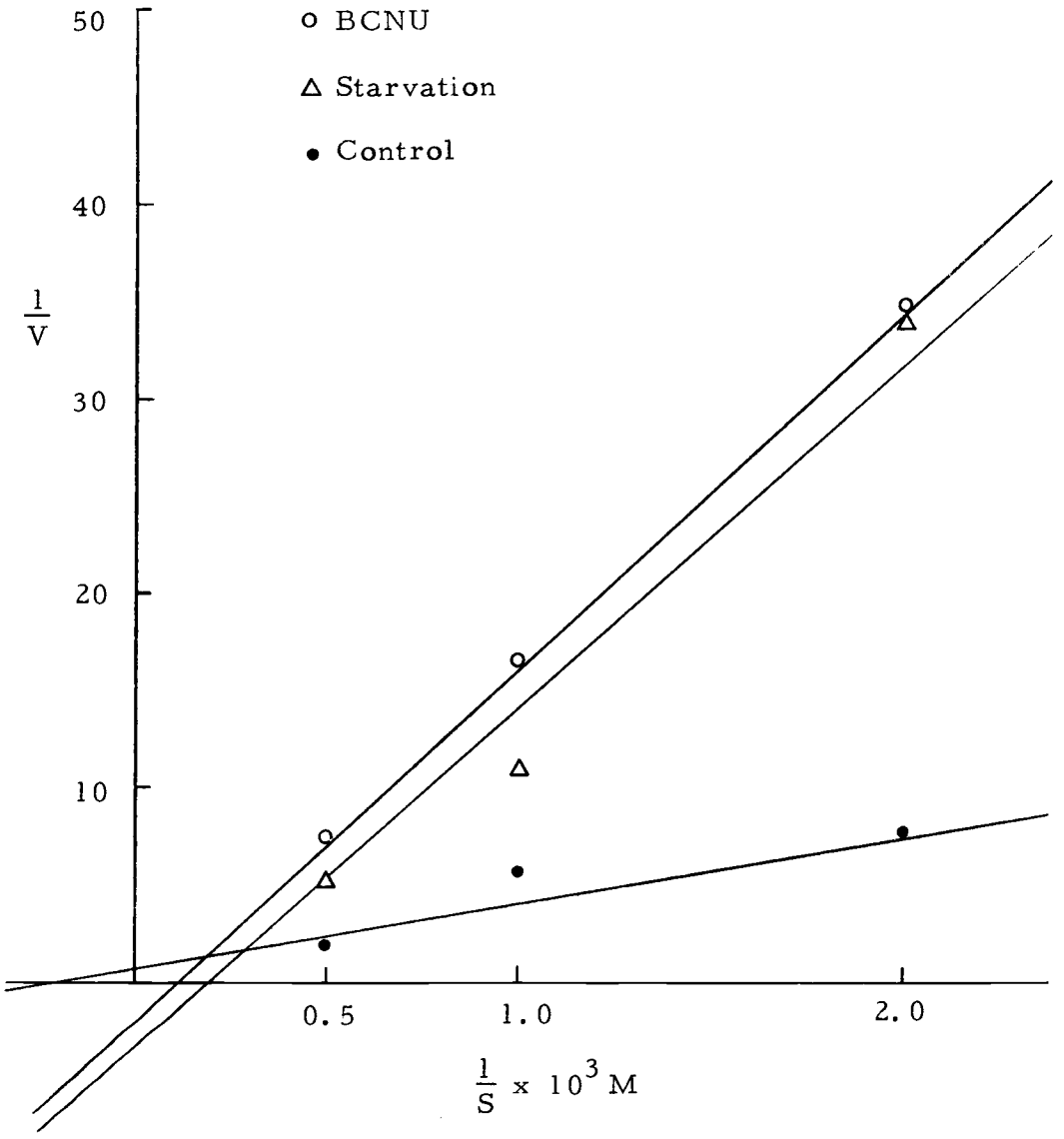
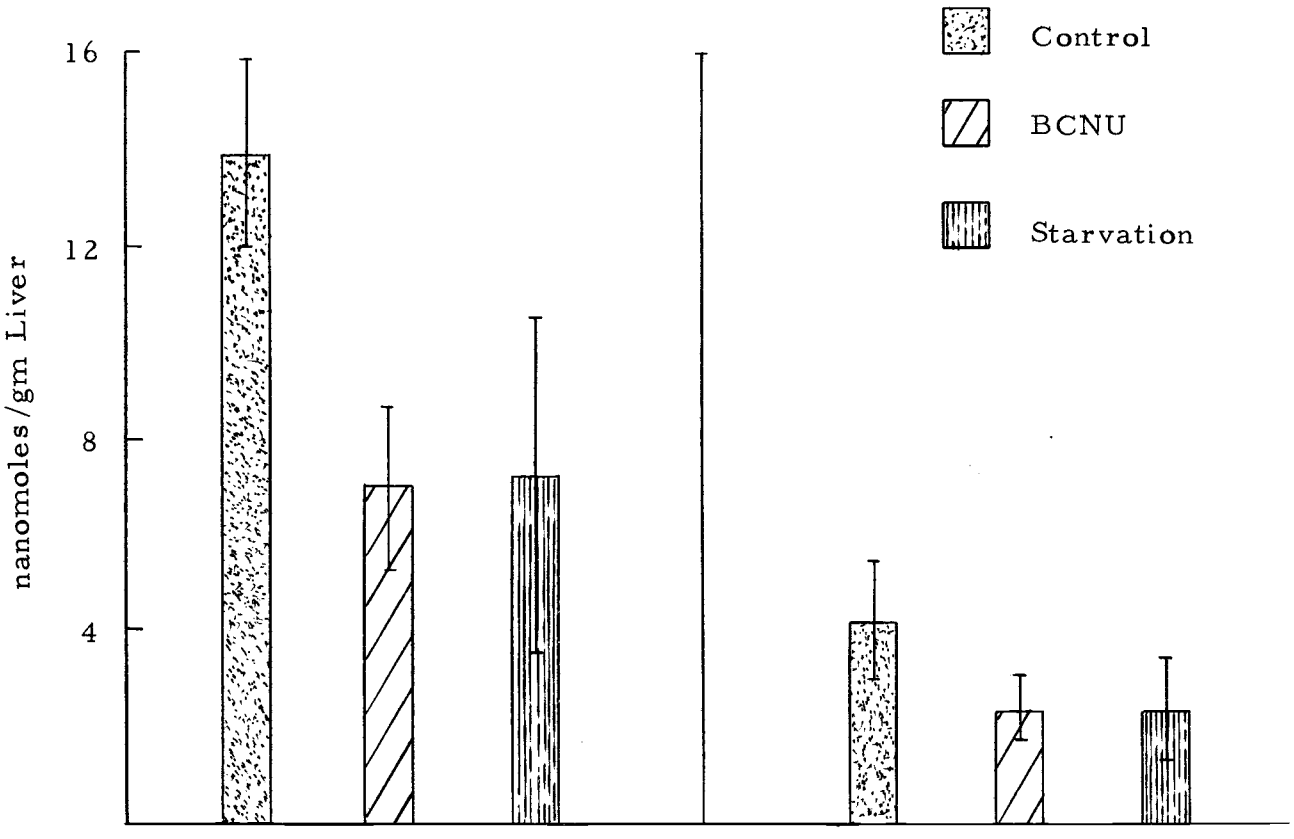

o BCNU
Δ Starvation
• Control
50
40
30
20
10
1/V
0.5
1.0
2.0
1/S x 10³ M

Figure 5-3. Comparison of the effect of a single dose of BCNU (30 mg/kg at 7 days post treatment) and the effect of a 7-day starvation on cytochrome P-450 (left) and b_5 (right) levels. Each bar was the mean ± standard deviation of at least 5 animals. Equivalent reductions of cytochrome P-450 and b_5 contents were noted for both BCNU-treated rats and starved rats.


16
12
8
4
nanomoles /gm Liver
Control
BCNU
Starvation

also demonstrated in rats which were starved for 7 days. Parallel decreases of cytochrome b_5 were apparent. Controls had 4.2 nanomoles cytochrome b_5 per gram of liver whereas both BCNU-treated or starved rats had only 2.4 nanomoles per gram of liver. The reduction was again about 50%. Therefore, it appeared that the ratio of cytochrome P-450 to cytochrome b_5 remained constant. However, it was also noted that when the cytochrome P-450 and b_5 levels were expressed in terms of nanomoles per milligram of protein, starved rats did not differ significantly from controls, but BCNU-treated rats had considerably lower values (Table 5-2).

Total protein was less severely affected (Figure 5-4). Protein concentrations in controls, BCNU-treated and starved rats were 12.0, 8.5 and 6.9 mg/gm liver respectively.

Table 5-2. Comparison of the effects of BCNU and the effects of starvation on cytochrome P-450 b_5 levels.

	Cytochrome P-450		Cytochrome b_5	
	nm/mg protein ±S.E.	nm/gm liver ± S.E.	nm/mg protein ± S.E.	nm/gm liver ± S.E.
Control	1.15 ±0.038	13.85 ± 0.779	0.35 ± 0.033	4.18 ± 0.512
BCNU (30 mg/kg, day 7)	0.87 ±0.075	7.03 ± 0.740	0.28 ± 0.020	2.39 ± 0.258
Starvation	1.08 ±0.168	7.22 ± 1.502	0.35 ± 0.052	2.36 ± 0.459

Weight changes of rats receiving a single dose of BCNU were followed up to 14 days post treatment (Figure 5-5). Controls continually showed weight increases. By 14 days, they had gained about

Figure 5-4. Comparison of the effect of a single dose of BCNU (30 mg/kg at 7 days post treatment) and the effect of a 7-day starvation on total microsomal protein in rats. Each bar was the mean ± standard deviation of at least 5 animals.

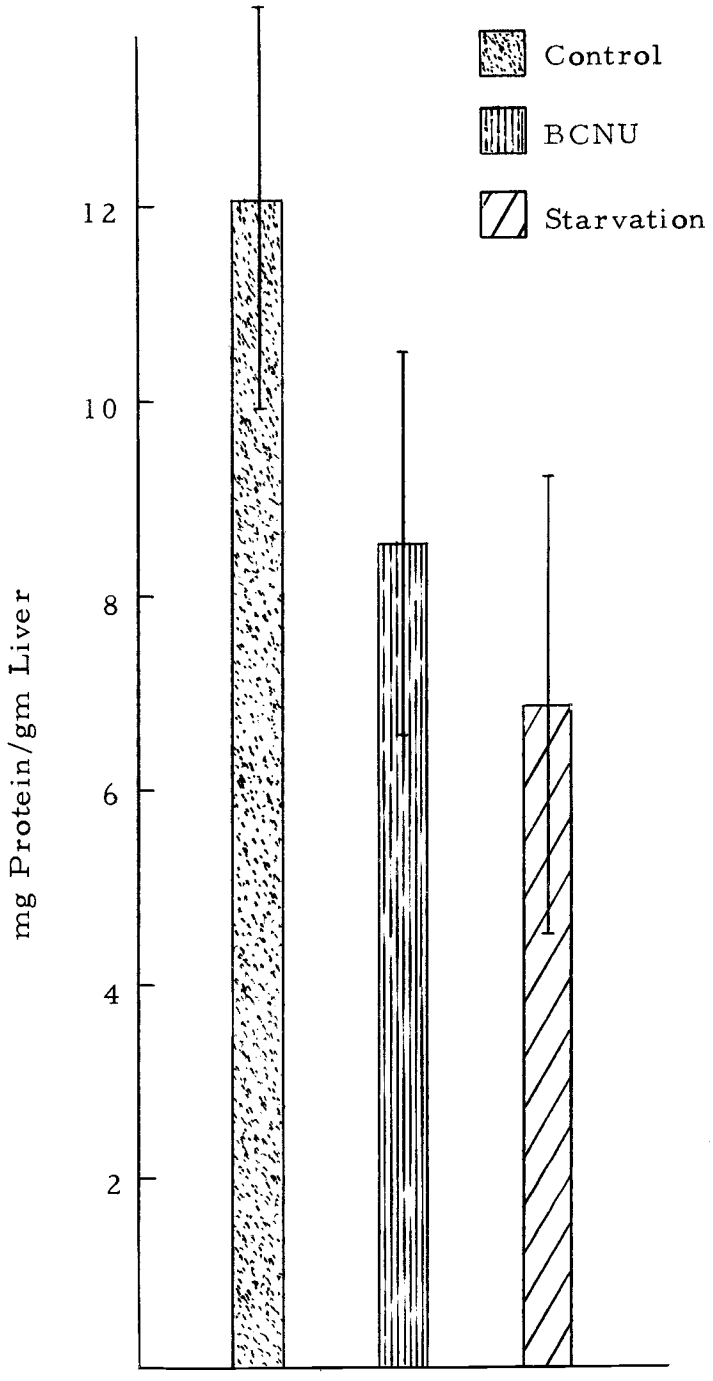
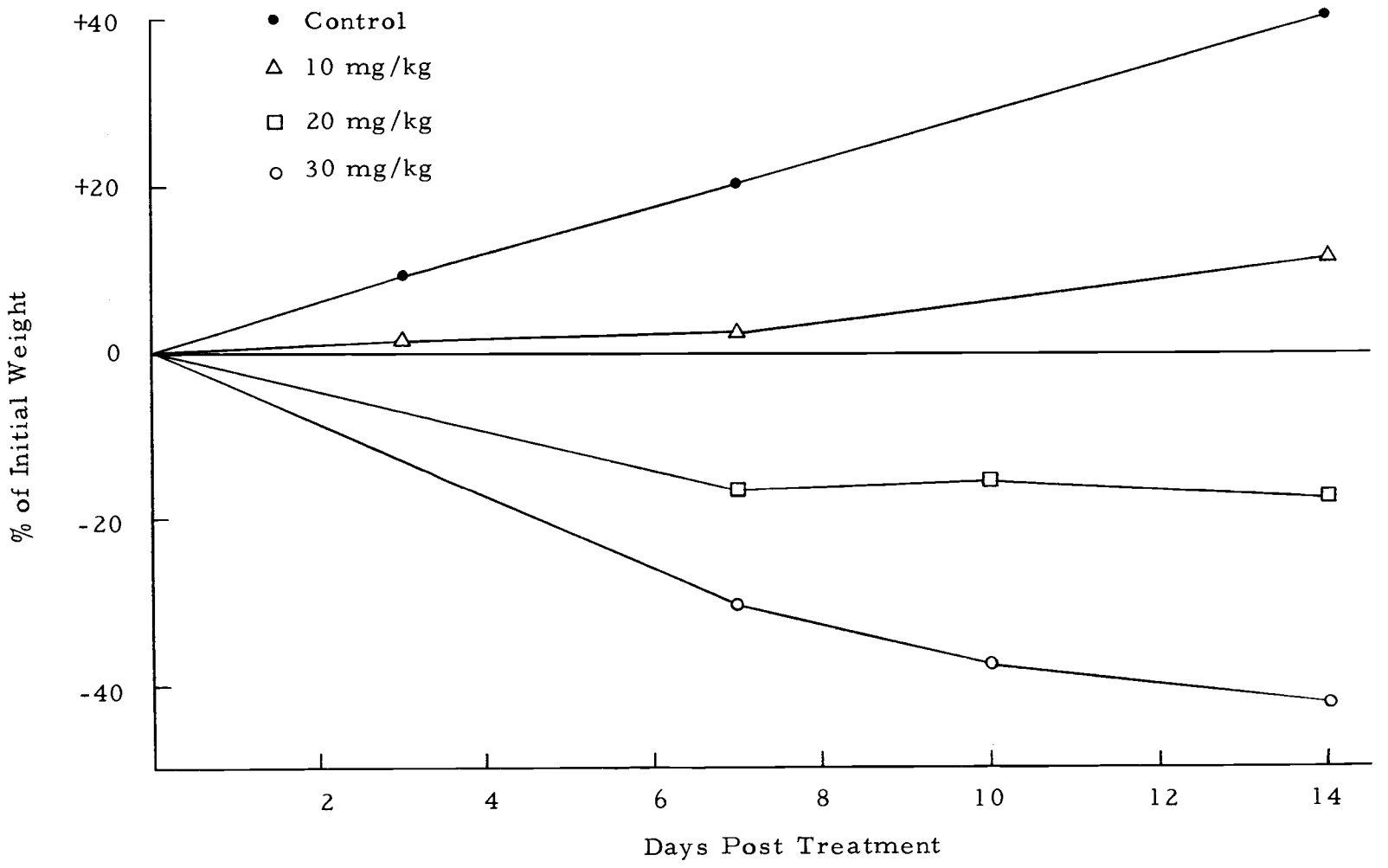

Control
BCNU
Starvation
mg Protein/gm Liver
12
10
8
6
4
2

Figure 5-5. The effect of single doses of BCNU (in corn oil) on changes in body weights in rats. At least 10 animals were treated with each dose. By 14 days, controls had gained 40% of their initial weights whereas rats treated with 10 mg/kg gained 10% during this time. Animals treated with 20 or 30 mg/kg consistently lost weight, the former losing 15% while the latter lost 40% of their initial body weights.


+40
+20
0
-20
-40
% of Initial Weight
Control
10 mg/kg
20 mg/kg
30 mg/kg
2
4
6
8
10
12
14
Days Post Treatment

40% of their initial weights. At 10 mg /kg, the animals maintained at the same weights for 7 days followed by a gradual increase. At the end of 14 days, these rats had gained about 10% of their initial body weights. In contrast to controls, rats given 20 or 30 mg/kg of BCNU continually lost weight. The effect was dose-related. By 14 days, animals receiving 20 and 30 mg/kg of BCNU had lost 15% and 40% respectively of their initial body weight.

The fact that rats treated with moderate doses of BCNU showed a semi-starvation phenomenon prompted the study of force-feeding these animals. Daily weight changes of each animal was recorded (Figure 5-6). Without any food or liquid diet, the animals were found to lose an average of 25 grams per day. With the nutritional supplement, however, the body weights of these animals were maintained at the same level from 5 to 14 days post treatment (or at the time of sacrifice).

To separate the effects of BCNU from the effects of starvation on the drug metabolizing system, kinetics studies of pentobarbital oxidation were performed in BCNU-treated rats which were force-fed. The results are represented in Figure 5-7. V_{max} and K_m values are shown in Table 5-3.

Figure 5-6. The effect of force-feeding on daily weight changes in rats receiving a single dose of BCNU (30 mg/kg). Controls (given corn oil) received equivalent volumes of the liquid diet only. Both groups were given free access to water. Force-feeding was initiated 24 hours following administration of BCNU. When completely deprived of food, controls and BCNU-treated rats were found to lose 25 grams/day. Subsequently no significant difference in weight changes was demonstrated between controls and BCNU-treated rats.

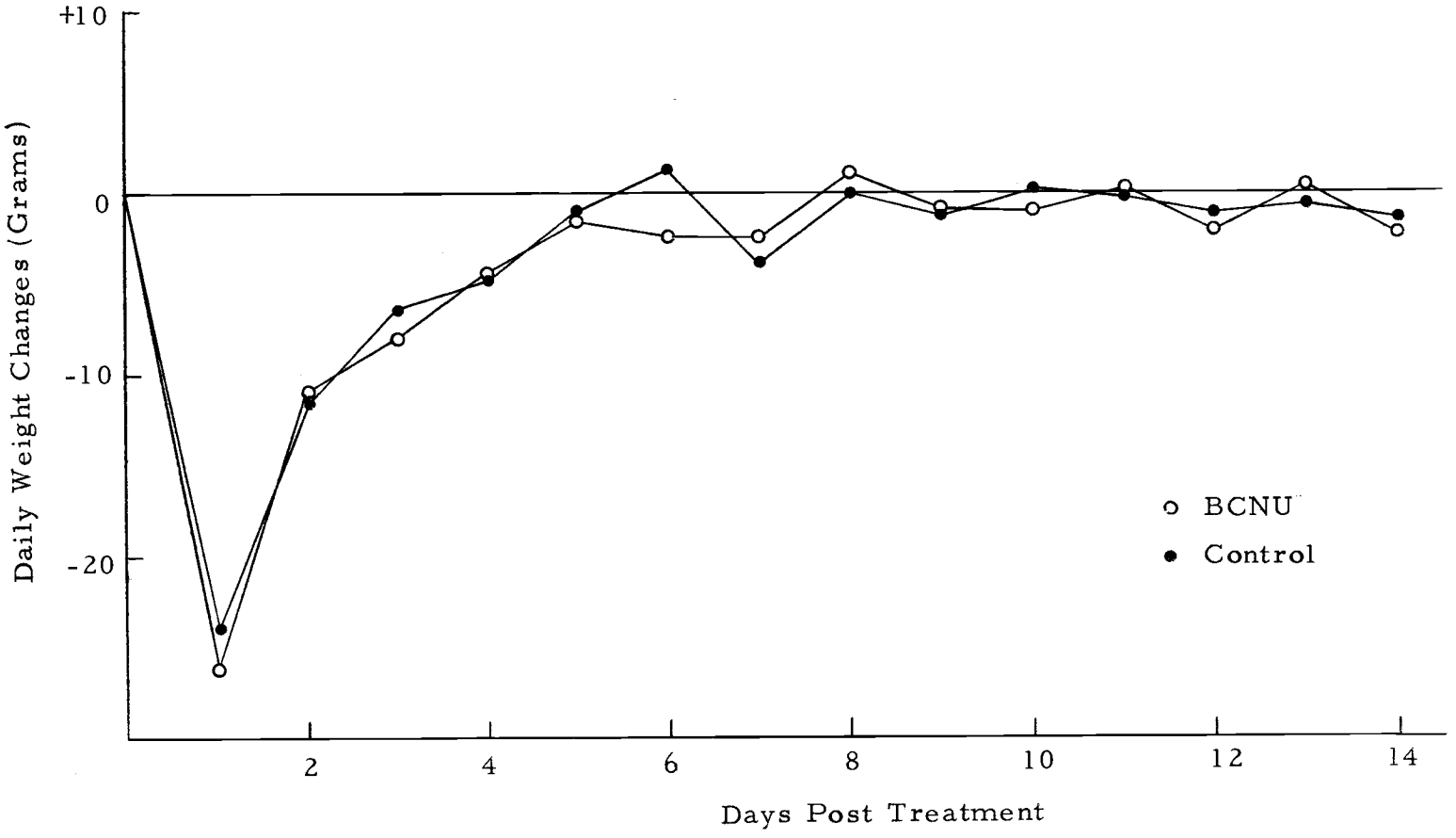

+10
0
-10
-20
Daily Weight Changes (Grams)
2
4
6
8
10
12
14
Days Post Treatment
BCNU
Control

Figure 5-7. Effect of force-feeding on the *in vitro* metabolism of pentobarbital by liver microsomes of rats treated with a single dose of BCNU (30 mg/kg). Controls received equivalent volumes of corn oil only. Enzyme activities were measured at 13 days post dosing. Three animals were assigned to each treatment. Parallel kinetic changes were noted for both force-fed BCNU-treated rats and rats receiving BCNU only (i.e. without nutritional supplement). Controls receiving the liquid diet alone showed no significant kinetic alterations. Therefore, the depression of microsomal enzymes associated with BCNU treatment was demonstrated to be independent of the nutritional status of the animals.

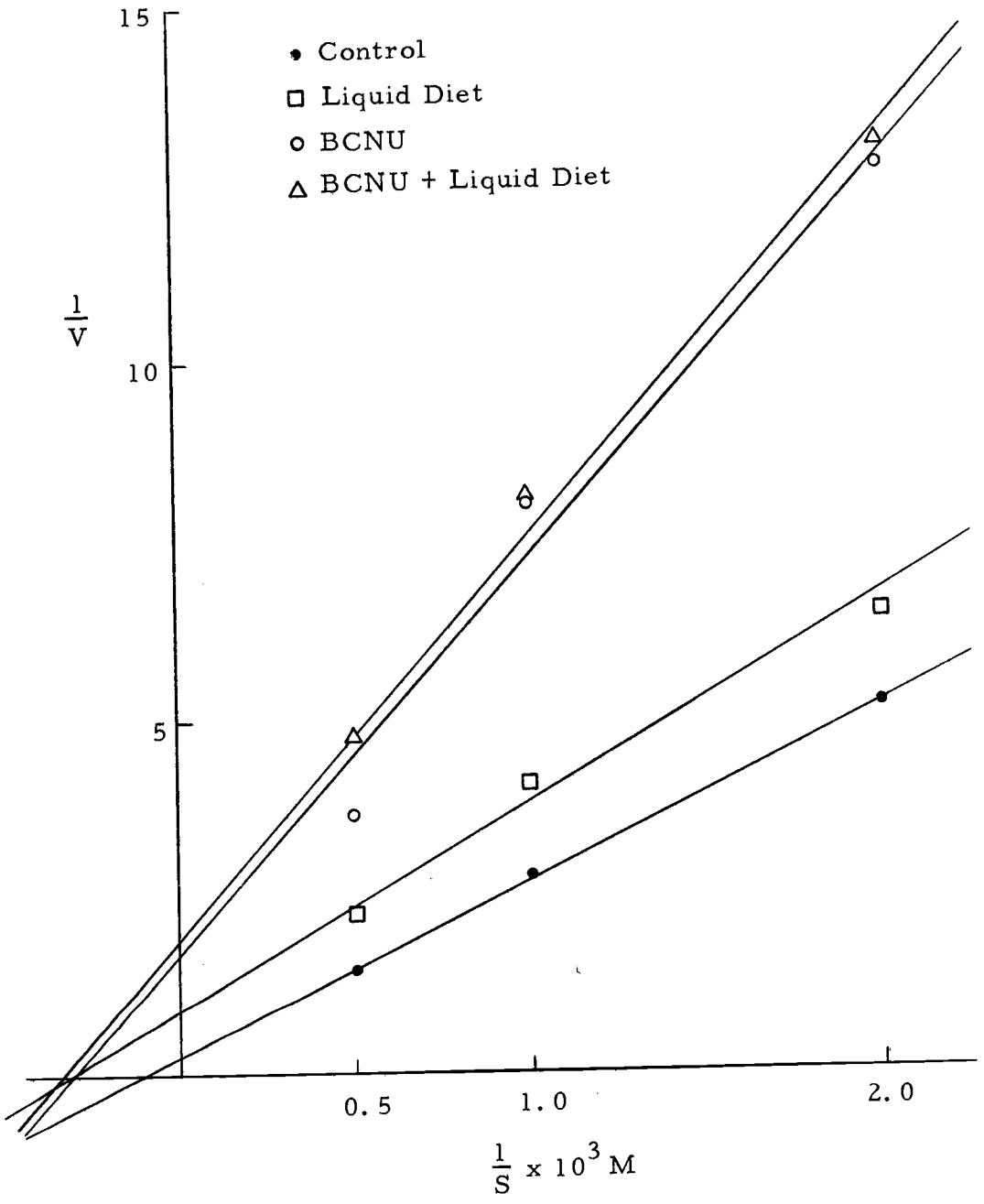

15
10
5
1/V
Control
Liquid Diet
BCNU
BCNU + Liquid Diet
0.5
1.0
2.0
1/S x 10^3 M

Table 5-3. Effect of force-feeding on the V_{max} and K_m of pentobarbital metabolism by liver microsomes in rats receiving a single dose of BCNU (30 mg/kg).

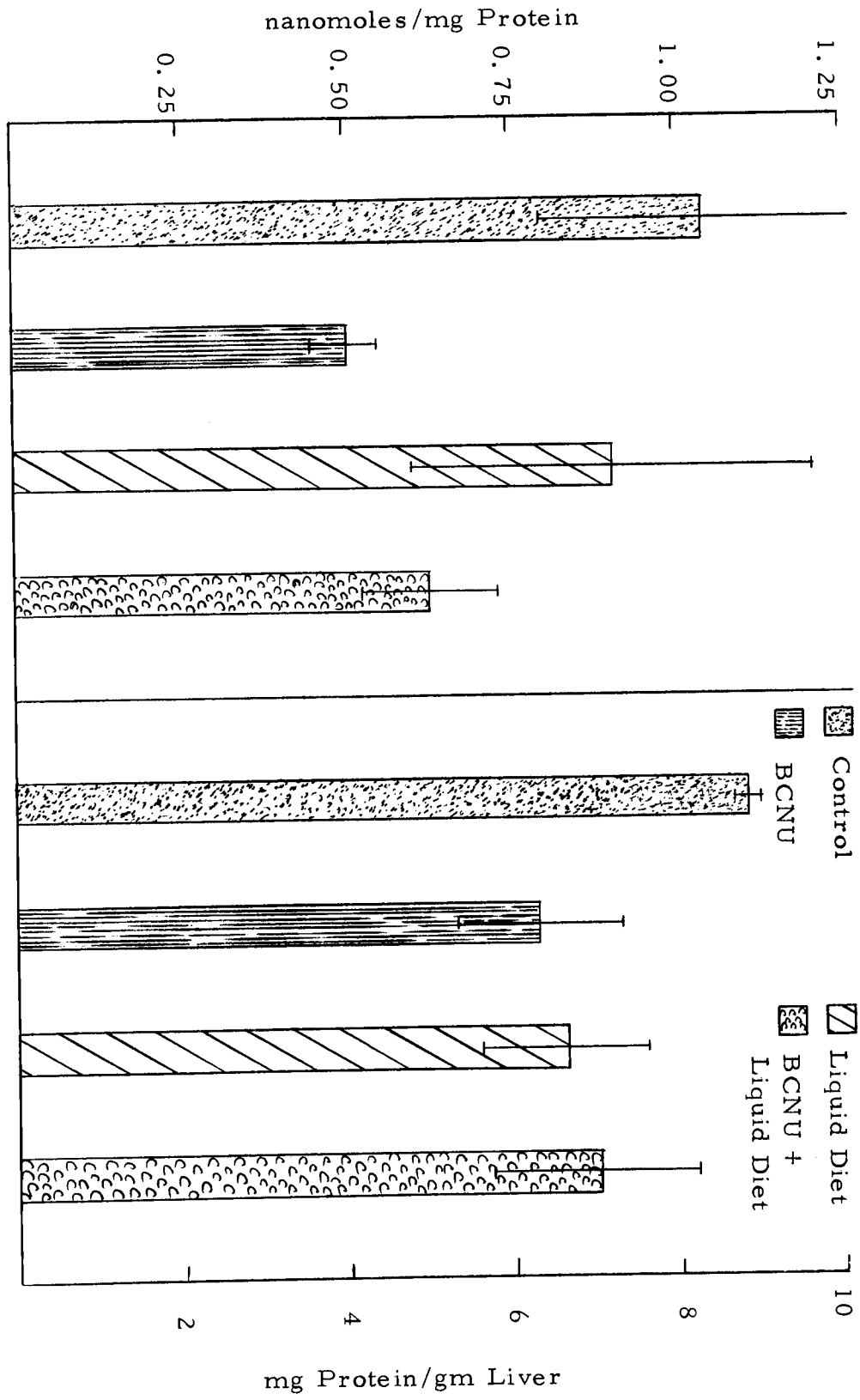
	Control	Liquid Diet	BCNU	BCNU + Liq. Diet
V_{max} (μmoles/gm /45 min.)	4.0	1.0	0.57	0.44
K_m (M)	10×10^{-3}	3.0×10^{-3}	3.2×10^{-3}	2.4×10^{-3}

It is evident that enzyme activities in both groups of BCNU-treated rats (i.e. with and without the liquid diet) were depressed to the same extent. Controls which received the liquid diet alone had only slightly reduced enzyme activities. This may be attributed to the significant initial loss of weight in these animals. Similar effects were noted for cytochrome P-450 levels (Figure 5-8). A 50% reduction of the hemoprotein was demonstrated in all BCNU-intoxicated rats, regardless of whether they were given the special liquid diet or the regular rat food. Controls which had only received the liquid diet did not show any changes in their P-450 levels. When total microsomal protein was considered, a slight depression was again observed in all groups of treated animals.

Discussion

It has long been recognized that starvation is one of the important factors that affects drug metabolism by liver microsomes

Figure 5-8. Effect of force-feeding on cytochrome P-450 levels (left) and total microsomal protein (right) in rats given a single dose of BCNU (30 mg/kg) at 13 days post treatment. Three animals were assigned to each group. The bars were mean responses and the brackets were standard deviations. Equivalent reductions of P-450 contents were noted in BCNU-treated rats receiving either regular food or the complete nutritional liquid diet. No significant changes in P-450 concentration were demonstrated in controls which were force-fed with the diet. With respect to total microsomal protein, slight but equivalent decreases were found in BCNU-treated rats with and without the nutritional supplement and force-fed controls.


nanomoles/mg Protein
0.25
0.50
0.75
1.00
1.25
Control
BCNU
Liquid Diet
BCNU + Liquid Diet
2
4
6
8
10
mg Protein/gm Liver

(Dixon et al., 1960; Kato and Gillette, 1965; Gram et al., 1970). Dixon et al. (1960) reported that starvation of male mice for 36 hours resulted in a marked depression in the metabolism of hexobarbital, chlorpromazine, pyramidon and acetanilid by hepatic microsomal enzymes. Moreover, p-nitrobenzoic acid and neoprontosil metabolisms were not affected by starvation. They further suggested that the decrease in enzyme activity was due to an actual loss in enzyme protein. It was also noted that within 24 hours after refeeding the animals the metabolism of various drugs returned to normal.

Depression of pentobarbital oxidation was demonstrated in rats which had been starved for 7 days as well as in those rats treated with a single dose of BCNU (30 mg/kg). Although both groups of animals had the same apparent enzyme kinetics and equivalent reductions in cytochrome P-450 and b_5 (as expressed in nanomoles per gram of liver), we are led to believe that they do not act by the same mechanism for the following three reasons.

First, starvation for 36 hours in mice (Dixon et al., 1960) or 72 hours in rats (Kato and Gillette, 1965) was shown not to affect reduction of p-nitrobenzoic acid. Moreover, Kato and Gillette (1965) reported that starvation of male rats impaired the metabolism of aminopyrine, hexobarbital, pentobarbital and morphine but enhanced the metabolism of methyl-aniline and aniline, while other NADPH-dependent enzymes remained unaffected. BCNU-treatment, however,

appeared to exhibit a general depression on all enzyme systems studied, including aniline hydroxylation and p-nitrobenzoic acid reduction.

Secondly, Gram and his coworkers (1970) demonstrated that a 72-hour starvation in male rats produced a slight increase of cytochrome P-450 content, expressed per milligram of microsomal protein. Our results showed that in the starved animals, cytochrome P-450 level, expressed in nanomoles per milligram of protein, was not significantly altered, whereas in BCNU-treated animals, P-450 concentration was considerably lowered. On the other hand, if cytochrome P-450 was expressed in terms of nanomoles per gram of liver, 50% reductions were seen in both groups. Similar effects were evident with respect to cytochrome b_5.

Thirdly, when comparing enzyme activities and cytochrome P-450 levels of BCNU-treated rats which were nutritionally supplemented to those which received the regular food, no difference was noted. In contrast to the findings of Dixon et al. (1960) that enzyme activities returned to normal within 24 hours after refeeding, the force-fed BCNU-treated rats had the same inhibited enzyme systems. This quite clearly demonstrated that the effect of BCNU on the drug metabolizing systems was independent of that of starvation, despite the fact that BCNU-treated rats were also in a semi-starved state. It is evident that there was no direct relationship between the two effects.

VI. SUMMARY AND CONCLUSIONS

The prolongation of pentobarbital and hexobarbital sleeptimes observed in rats following a single dose of BCNU was found to correlate well with impaired metabolism of these compounds by the liver microsomal enzyme systems. At a dose of 20 or 30 mg/kg of BCNU, significant depression of pentobarbital oxidation by liver 9000 xG fractions was noted as early as 7 days, and persisted through 21 days after a single administration. The effect was dose-related initially, but by 21 days, the same magnitude of response was produced by either dose. The contrast of its short biologic half-life to the prolonged effect of BCNU observed was consistent with the hypothesis that a long-lived active metabolite(s) was responsible for the toxic effects.

The impairment of oxidative metabolism of pentobarbital and hexobarbital was accompanied by kinetic changes of the enzyme, characterized by greatly reduced maximum velocity and unaffected Michaelis constant. The inhibition appeared to resemble closely a non-competitive type, suggesting strongly that a conformational disruption of the enzyme molecule had occurred, perhaps through interaction of BCNU with the enzyme. Supporting evidence for this possibility was the findings of Bowdon and Wheeler (1971) that BCNU binds extensively to lysine residues of protein. The implications of

the kinetic changes of the microsomal enzymes associated with BCNU treatment are not apparent at present. Nevertheless, these results yield suggestive evidence that BCNU may be acting by an alkylating mechanism, as many investigators had proposed.

In addition to oxidative metabolism of pentobarbital, BCNU was found to affect other metabolic pathways, such as N-demethylation of ethylmorphine, oxidation of hexobarbital, hydroxylation of aniline and nitro-reduction of p-nitrobenzoic acid. The various substrates investigated included type I and type II compounds, classified according to their characteristic spectral properties upon binding with microsomes. BCNU depressed all enzyme systems considered. Thus, both oxidation and reduction reactions, as well as metabolism of type I and type II substrates were affected. Furthermore, the depressed enzyme activities produced by BCNU could not be explained by deficiencies of cofactors (Mg^{++}, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) nor by deficiencies of the protein itself.

The effect of BCNU on the drug metabolizing systems in the rat appeared to be directed to a specific entity of the electron transport system, namely cytochrome P-450. A 50-60% reduction of the hemoprotein was noted by 7 days after a single dose of 30 mg/kg of BCNU. The depression was dose-related and prolonged, as followed through 21 days post treatment. Interestingly, cytochrome b_5, the

other of the two hemoproteins in liver microsomes, was only slightly affected by BCNU. The apparent reduction of cytochrome P-450 levels in BCNU-treated animals may reflect mechanisms interfering with synthesis of the hemoprotein, or conversely, enhancing its degradation. In view of this, following the rate of incorporation of labelled δ-aminolevulinic acid into microsomal fractions and decrease of radioactivity of labelled microsomes would be a reasonable approach to pursue. Along this line, the effect of BCNU on heme biosynthesis should also be determined.

BCNU is one of the antineoplastic agents which has received extensive clinical evaluation. Although hematologic toxicity was the major manifestation observed (Rall et al., 1963; De Vita et al., 1965; Reitemeier et al., 1966; Iriarte et al., 1966), severe liver toxicity has been reported to occur (De Vita et al., 1964; De Vita et al., 1965). Elevations of serum glutamic oxalacetic transaminase (SGOT), alkaline phosphatase and serum bilirubins were noted in a fair percentage of patients receiving BCNU treatment (De Vita et al., 1965). Alterations of these enzyme levels were also demonstrated in rats (Thompson and Larson, 1969). The effect of BCNU on the microsomal enzyme systems of these patients has not been assessed; however, it is not unreasonable to assume that humans treated with BCNU may experience hepatotoxic effects similar to those demonstrated in rats. Consequently, significant depression of cytochrome

P-450 may be expected to occur in these patients resulting in an impaired drug metabolizing system; these effects may have significant implications. Recent studies suggested that in addition to foreign compounds, steroid hormones (Mueller and Rumney, 1957; Conney and Klutch, 1963; Conney et al., 1968), fatty acids (Wakabayashi and Shimazono, 1963; Preiss and Bloch, 1964; Robbins, 1968; Lu et al., 1969; Ichihara et al., 1969) and heme (Tenhunen et al., 1968, 1969, 1970) are substrates for drug-metabolizing enzymes in liver microsomes, and require NADPH and oxygen. Furthermore, several of the enzymes necessary for the biosynthesis (Bloch, 1965; Gaylor and Mason, 1968) and metabolism (Danielsson and Einarsson, 1966; Danielsson and Tchen, 1968; Einarsson and Johansson, 1969) of cholesterol are located in liver microsomes, and also require NADPH and oxygen for activity. In many cases, cytochrome P-450 was demonstrated to be involved in these enzymatic reactions. In light of these observations, a compromised drug-metabolizing system, as resulted from BCNU treatment, may significantly affect regulation of the physiological actions of many normal body constituents which are substrates for these enzymes. Extrapolation of these effects may help to explain the many other perhaps interrelated toxic symptoms observed in rats as well as in humans following administration of BCNU.

In conclusion, the effect of BCNU on the microsomal enzyme

systems can be summarized as follows: 1) BCNU inhibited both oxidation and reduction reactions mediated by the hepatic drug-metabolizing system. 2) Enzyme kinetics were altered, resulting in greatly diminished maximum velocities but unchanged Michaelis constants. 3) The effect of BCNU on the enzymes was prolonged, exceeding 10 half-lives of endoplasmic protein turnover. 4) BCNU severely reduced the level of cytochrome P-450 in liver microsomes, possibly by interfering with the synthesis of the protein or by enhancing its degradation. 5) The effect of BCNU on hepatic drug metabolism was independent of the nutritional status of the animals.

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