ASPECTS OF CYCLOPHOSPHAMIDE TOXICITY IN PERINATAL MICE

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
ROBERT DOWNS SHORT JR.
1971

ABSTRACT

ASPECTS OF CYCLOPHOSPHAMIDE
TOXICITY IN PERINATAL MICE

By

Robert Downs Short Jr.

Cyclophosphamide is a potent teratogen in mice. Metabolites of the parent compound are active alkylating agents. There is evidence, however, that cyclophosphamide may produce toxic effects on developing tissues by a mechanism other than alkylation. These investigations were undertaken to further characterize the form of cyclophosphamide responsible for developmental toxicity in mice and to define biochemical lesions responsible for its teratogenic action.

Cyclophosphamide administration to day-old mice produced a reduced growth rate, increased lethality, and morphologic abnormalities. The administration of an equimolar dose of nor-nitrogen mustard to day-old mice did not affect development. The ability of perinatal mice to form alkylating metabolites of cyclophosphamide was examined in an *in vitro* system. These studies indicated that neither the fetus, placenta, neonatal liver, nor neonatal body was able to produce alkylating metabolites to the same degree as the adult liver. Cyclophosphamide developmental toxicity, therefore, occurred at a time when the affected organism had not fully developed an ability to activate the parent compound. These

observations suggest that a non-alkylating form of cyclophosphamide was responsible for perinatal toxicity in mice.

The *in vitro* study, in addition, showed that cyclophosphamide activation proceeded at a greater rate in nursing females than in virgin females. Furthermore, there were no differences between males and females in their ability to activate cyclophosphamide. Phenobarbital pretreatment was shown to increase activation while SKF 525-A pretreatment inhibited the formation of alkylating metabolites.

The incorporation of ^{14}C-leucine into embryonic protein was inhibited by 72 and 96 hours after a teratogenic dose of cyclophosphamide. There was, in addition, a decreased incorporation of the precursor into the maternal liver by 96 hours and the kidney by 72 hours after cyclophosphamide. The placenta, on the other hand, did not show an inhibition of incorporation at any of the times studied. The incorporation of this precursor into embryonic protein, when pregnant mice were treated 72 hours earlier with the drug, was inhibited at 30 and 60 but not at 15 and 120 minutes after precursor. Since the cyclophosphamide induced inhibition of protein synthesis occurred at a time when the embryo was visibly abnormal it was not possible to conclude that this was the mechanism of teratogenic action.

The synthesis of nucleic acids in embryos was studied by measuring the incorporation of ^{14}C-orotic acid, ^{14}C-uridine, and ^{14}C-thymidine into nucleic acids. There was no significant effect of cyclophosphamide on the rate of synthesis of nucleic acids in embryos at 3, 24, or 72 hours after drug treatment, although DNA content of the embryos was significantly reduced. The data suggested that cyclophosphamide induced effects in the embryo, in contrast to the mother, were temporarily dissociated or occurred by different mechanisms.

ASPECTS OF CYCLOPHOSPHAMIDE TOXICITY IN PERINATAL MICE

By

Robert Downs Short Jr.

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pharmacology

1971

ACKNOWLEDGMENTS

The author wishes to thank the graduate committee members: Drs. Theodore M. Brody, Hyram Kitchen, David A. Reinke and especially James E. Gibson for their assistance and guidance throughout the various phases of this project. The author, in addition, would like to thank Dr. K. S. Rao for valuable comments and suggestions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Cyclophosphamide was synthesized by Arnold and Bourseaux (1958) in an effort to provide an alkylating agent that required specific bioactivation by neoplastic tissues for the production of its cytotoxic action. The molecule consists of nitrogen mustard to which a cyclic phosphoramide ring structure has been attached. Higher concentrations of phosphatases and phosphamidases in tumor tissues (Gomori, 1948) were postulated as sites of activation of the inactive transport form. The activation of cyclophosphamide was proposed to occur by the hydrolytic cleavage of the P-N bond between nitrogen mustard and the phosphamide ring to yield nitrogen mustard, an active alkylating agent.

Cyclophosphamide has been successfully employed in the treatment of a broad spectrum of malignant and non-malignant diseases, including the lymphoproliferative diseases, such as Hodgkin's disease, the malignant lymphomas, chronic lymphatic leukemia and mycosis fungoides (Abele and Dodson, 1960). Cyclophosphamide has been shown to be unusually effective in treating leukemia in children, some of whom had developed a resistance to antimetabolite therapy (Brubaker *et al.*, 1962). Alkylating agents have been employed as potential inhibitors of the immune response and cyclophosphamide has been successfully used in the treatment of the nephrotic syndrome in children (Drummond *et al.*, 1968). Cyclophosphamide therapy may produce the following signs of toxicity: alopecia, leucopenia, hemorrhagic cystitis, and minor degrees of anorexia and nausea. The usual clinic dose of cyclophosphamide in man is

approximately 3 mg/kg/day p.o. or 15 mg/kg/week i.v. after a priming dose of 40-50 mg/kg i.v.

The requirement of bioactivation for the conversion of the inactive cyclophosphamide parent compound to active cytotoxic metabolites has been demonstrated by various techniques. Foley *et al.* (1961) demonstrated that cyclophosphamide lacked an *in vitro* inhibitory effect on mammalian cell cultures. The sera of rats treated with cyclophosphamide, however, had an inhibitory effect on the cultures. Homogenates of neoplastic tissues, on the other hand, were not able to conduct the activation reaction to a sufficient degree to produce an inhibitory effect on cell growth. Brock and Hohorst (1963) used the formation of nitrobenzyl pyridine reactive material as an estimation of cyclophosphamide activation. They demonstrated activation in mice, rats, guinea pigs, rabbits, cats, dogs, and humans. Activation was also demonstrated in liver slices and, to a lesser degree, in both adrenal cortical and lung tissue slices. Activation, however, was not demonstrated by acid prostrate phosphatase, alkaline renal phosphatase, or phosphamidases.

Brock and Hohorst (1967) suggested that activation occurred by an NADPH and oxygen dependent microsomal system as a result of experiments involving fractionation of rat liver homogenates. Cohen and Jao (1970) have subsequently shown, in addition, that *in vitro* activation by a liver microsomal system can be inhibited by the simultaneous addition of either parahydroxymercuribenzoate, cytochrome c, hexobarbital, or testosterone. These observations suggested that the activation process was inhibited by chemicals that interfered with activity of the mixed function oxidase system or were metabolized by this system. Cyclophosphamide activation was shown (Sladek, 1971) to be inhibited by carbon monoxide. The parent

compound, in addition, was shown to have a type 1 spectral interaction with cytochrome P450.

The previously described observations concerning cyclophosphamide activation led to the conclusion that the molecule existed as the inactive parent compound that required bioactivation for the production of cytotoxic effects. The bioactivation process, however, did not occur by the action of hydrolytic enzymes in tumor tissues but rather by the action of the mixed function oxidase system of the liver microsomal fraction.

It is generally agreed that alkylating agents react with nucleophilic centers in biological systems. Alkylating agents have the potential for a variety of interactions and effects since there are a variety of nucleophilic centers within cells, e.g., phosphate, amino, and sulfhydryl groups. The *in vitro* treatment of calf thymus DNA with nor-nitrogen mustard, for example, reduced its template activity in reactions catalyzed by *E. coli* DNA and RNA polymerase (Ruddon and Johnson, 1968). Nor-nitrogen mustard treatment, in addition, reduced the coding capacity of poly A, poly U, and poly C in an *E. coli* cell free protein synthesizing system (Johnson and Ruddon, 1967). The *in vivo* treatment of hamster plasmacytomas with cyclophosphamide produced a maximal decrease in DNA and RNA synthesis by 24-48 hours. The decrease in DNA synthesis was accompanied by a decrease in DNA nucleotidyl transferase activity in a crude cell free system (Wheeler and Alexander, 1969).

The first report of teratogenic effects of alkylating agents in mammals appeared in 1948 (Haskin). A single administration of nitrogen mustard (0.5 and 1.0 mg/kg) to pregnant rats produced developmental abnormalities. The abnormalities observed included reduced size and weight, cleft palate, fusion of the digits, abnormal posture of the hind

limbs and short tail. Cyclophosphamide has been shown to be teratogenic in chicks (Gerlinger *et al.*, 1963), rabbits (Gerlinger, 1964), rats (Kreybig, 1965), and mice (Gibson and Becker, 1968a).

The intraperitoneal administration of 20 mg/kg cyclophosphamide to pregnant Swiss-Webster mice on gestational days 10 through 14 produced an increased number of resorptions and a variety of teratogenic effects (Gibson and Becker, 1968a). The surviving fetuses demonstrated cleft palates, exencephaly, digital defects, fusion of the long bones, hydrocephalus and hydronephrosis. The widest spectrum of anomalies was produced by the administration of cyclophosphamide on day 11. Fetal mortality curves were biphasic with peaks occurring when the drug was administered on days 10 to 11 and days 14 to 15. Doses of 5 and 10 mg/kg resulted in increased resorption and decreased growth rates in a dose-related manner but failed to produce detectable anomalies.

The role of the parent compound and its metabolites in the production of cyclophosphamide teratogenicity was investigated by means of enzyme induction or inhibition (Gibson and Becker, 1968b). Pretreatment of pregnant mice with phenobarbital or SKF 525-A produced a corresponding increase or decrease in plasma alkylating products after cyclophosphamide administration. Since the teratogenic effects of cyclophosphamide were decreased by phenobarbital pretreatment and increased by SKF 525-A pretreatment it was proposed that teratogenicity was associated with the parent compound rather than metabolites. This suggestion was supported by the observation that the distribution of ^{14}C-cyclophosphamide, under conditions of altered maternal metabolism, indicated that the increased teratogenic effects were associated with levels of the parent compound rather than metabolites. The levels of metabolites in the embryo were observed to remain relatively constant regardless of the pretreatment.

This observation may indicate that there is a placental barrier to the transport of the more polar cyclophosphamide metabolites (Gibson and Becker, 1971a). In another study, Gibson and Becker (1971b) found that certain theoretical alkylating metabolites of cyclophosphamide did not possess the same potency or spectrum of teratogenic effects as cyclophosphamide.

Cyclophosphamide was recently reported (Nordlinder, 1969) to produce toxic effects in developing mice treated 1 day after birth with a single dose of the drug. The treated mice showed increased lethality, lower adult body weights, delayed development of hair and abnormal morphology in comparison to controls. Animals that received cyclophosphamide 1 day after birth had microencephaly and short snout, ears, and tail at maturity (40 days). These observations suggest that cyclophosphamide was able to produce toxic effects in the neonates in the absence of activation since the perinatal period is characterized by a reduced capacity of the mixed function oxidase system (Jondorf *et al.*, 1959). The neonatal mouse, therefore, may provide a system, in addition to the mouse embryo, that shows susceptibility to the non-alkylating form of cyclophosphamide.

The present investigations were undertaken to further characterize the form of cyclophosphamide responsible for developmental toxicity and to define biochemical lesions responsible for its teratogenic action. The form of cyclophosphamide responsible for developmental toxicity was evaluated by 2 methods. The first method involved the confirmation and extension of data concerning the effects of cyclophosphamide in day-old mice. The effect of different drug doses was determined in order to establish dose related effects. The effect of alkylating activity on the development of mice was studied by treating day-old mice with

nor-nitrogen mustard. The validity of this comparison was tested by determining the pharmacokinetic properties of the 2 agents. The second method involved a determination of the ontogeny of cyclophosphamide activation by various perinatal tissues sensitive to its toxic effects. These observations permitted a correlation to be made between perinatal toxicity and the ability of the organism to metabolize the parent compound.

The synthesis of DNA, RNA, and protein are important developmental processes. The disruption of these pathways may produce dramatic effects on development. The ability of cyclophosphamide to disrupt these pathways in embryos was studied in an attempt to define drug induced biochemical lesions.

II. METHODS

A. Animals

Virgin Swiss Webster mice were obtained from Spartan Research Animals (Haslett, Mich.) and housed at 70-75° F in stainless steel cages with wire mesh bottoms. The animals were maintained on a 12-hour light-dark cycle in order to synchronize estrus cycles. The cycle began at 8 a.m. when the lights were turned on. The animals were given free access to food and tap water.

Timed pregnancies were obtained by the daily mating of 150-200 females. One male was placed in a cage containing 10 females at 8 a.m. and the females were examined 1 hour later for signs of copulation as indicated by the presence of vaginal plugs. Approximately 4% of the females are bred each day by this procedure. Mice were isolated and identified as being on day 1 of gestation if plugs were found. Ovulation occurs independently of copulation in mice and there is about a 5 hour delay between ovulation and fertilization.

B. Postnatal Toxicity

The effects of cyclophosphamide and nor-nitrogen mustard on postnatal development were studied in litters obtained from the previously described breeding program. The mice were allowed to deliver spontaneously and drugs were administered 1 day later. The litters were housed on San-i-cell (Paxton Processing Co.) for 2 to 4 weeks.

Six litters, born on the same day, were assigned to each drug treatment group. The group was subdivided into 3 treated and 3 control litters on the basis of litter size and average body weight. The drugs were administered at doses of 45 and 80 mg/kg for cyclophosphamide treated litters and 54 mg/kg for nor-nitrogen mustard treated litters. The doses were calculated on the basis of the average mouse weight for each litter. The drugs were prepared immediately before use and injected subcutaneously in a volume of 50 µl of normal saline (Gibson and Becker, 1967). Mice in control litters received an equivalent volume of the vehicle. The average body weights were measured at the indicated times after birth by determining the total litter weight and size.

Urine and plasma levels of nor-nitrogen mustard and cyclophosphamide were measured by the alkylating analysis (see below). After drug administration one-day-old mice were either isolated (0-4 hours) or returned to the mother (8 hours) prior to sacrifice. Blood was obtained by decapitation and exsanguination and urine was collected from excised bladders. The samples from cyclophosphamide treated animals were hydrolyzed in 0.5 ml of 1N HCl for 5 minutes on a boiling water bath to convert the parent compound to products that can be detected in the alkylating analysis. As a result of this treatment the analysis measures total drug in blood or urine.

C. *In vitro* Cyclophosphamide Activation

The ability of mice to activate cyclophosphamide was studied as a function of sex and age in an *in vitro* system. The effects of inducers and inhibitors of this system were studied, in addition, by pretreating virgin females with either phenobarbital (100 mg/kg for 3 days) or SKF 525-A (32 mg/kg one hour before sacrifice).

1. Animals

Mature and 4-week-old mice were purchased from Spartan Research Animals prior to use. The remaining animals were obtained from the breeding program described above.

2. Tissue Preparation

All tissue samples were obtained between 9 and 11 am. Mature and 4-week-old mice were sacrificed by cervical dislocation. Livers of these animals were perfused with 1.15% KCl in 0.5M tris (hydroxymethyl) aminomethane (tris, Sigma) buffer pH 7.4 (KCl-tris) prior to removal. The livers of a whole litter were pooled for single determinations. When determinations were made on the ability of the neonatal body to activate cyclophosphamide the liver, bladder, and viscera containing ingested milk were removed. The bladder and milk were removed in an effort to exclude variable factors that could affect the activation reaction. In prenatal studies the mother was killed by cervical dislocation and a laparotomy was performed. The uterine wall was exposed and cut to permit removal of the conceptus and its placenta. The tissues were maintained in an ice bath for up to 2 hours prior to being weighed and homogenized in 2 ml KCl-tris per gram tissue. The tissues were homogenized in a loosely fitting, motor driven, teflon-glass Potter-Elvehjem homogenizer. The tissue fraction for incubation was obtained by centrifuging the homogenate at 9000 g for 30 minutes in an International centrifuge model B-20.

3. Incubation Protocol

All incubations were conducted in a Dubnoff metabolic shaking apparatus at 100 oscillations/min and 37° C in room air. The incubation media consisted of glucose-6-phosphate (G6P, Sigma), nicotinamide adenine

dinucleotide phosphate (NADP, Sigma), magnesium sulfate ($MgSO_4$), cyclophosphamide, 0.5 ml of 0.5 M tris buffer pH 7.4, and 0.5 ml of the 9000 g supernatant for a total volume of 2.5 ml. The components were added to a 50 ml beaker and a marble was included to insure adequate agitation (Fouts, 1970). The amounts of the components added are indicated in the results section. Since the reaction did not proceed in the presence of 0.5 ml of 95% ethanol it was decided to use this relatively mild technique for terminating the reaction. The media was centrifuged briefly, after the addition of ethanol, to remove precipitable material. A sample of the supernatant was analyzed for alkylating metabolites (see below). The results were quantified with a standard curve prepared by hydrolyzing cyclophosphamide in 1N HCl for 30 minutes on a boiling water bath. An estimation of the non-enzymatic formation of alkylating metabolites was obtained from an incubation media that consisted of cyclophosphamide, tris buffer, and distilled water. The amount of alkylating material determined in this system was subtracted from the amount of alkylating material formed by the various tissue samples. Two incubations were conducted for each sample and the results were averaged to represent a single determination. Protein determinations were made on the 9000 g supernatant by the method of Lowry (Lowry *et al.*, 1951) and crystalline bovine serum albumin (Armour) served as a standard.

The product formed was expressed either as mμmoles of alkylating activity/mg protein or μmoles of alkylating activity/gram tissue. The kinetics of activation were determined by the double reciprocal plot of Lineweaver and Burk and a least squares regression analysis (Steel and Torrie, 1960).

4. Alkylating Analysis

The alkylating analysis of Friedman and Boger (1961) as modified by Gibson and Becker (1968b) was used in this study. This method depends on the ability of an alkylating agent to react with nitrobenzyl pyridine (NBP) to form a quaternary pyridinium ion that is highly colored in an alkaline solution. A 0.5 ml sample of the supernatant was diluted to 3.0 ml with distilled water. The tubes were placed in an ice bath and 1.0 ml of cold 0.2 M acetate buffer pH 4.6 was added to each sample. The NBP reagent (0.4 ml of 5% NBP in acetone) was added and the tubes were shaken. All tubes were heated for 20 minutes on a boiling water bath. The tubes were removed at the end of this period, cooled in ice, and shaken. The color was developed by adding 2.0 ml acetone, 5.0 ml ethyl acetate, and 1.5 ml 0.25 N sodium hydroxide to each tube. The color was extracted into the organic phase by shaking 10 times, adding approximately 200 mg sodium chloride, and then shaking 10 more times. The optical density of the organic layer was determined at 540 mμ using a Bausch and Lomb Spectronic 20.

D. Biosynthesis of Macromolecules

L-Leucine (UL)-^{14}C (197-240 mC/mM), orotic acid-6-^{14}C (3.17 mC/mM), uridine-2-^{14}C (38.8 mC/mM) and thymidine-2-^{14}C (55.7 mC/mM) were purchased from ICN Tracer Lab (Irvine, Calif.)

Cyclophosphamide was administered at a teratogenic dose of 20 mg/kg on day 11 of gestation (Gibson and Becker, 1968a). Control animals were injected at the same time with distilled water. The drug was injected in a volume of 0.1 ml/10 g body weight. The precursors were administered at various intervals after cyclophosphamide and the animals were sacrificed at the indicated times. Embryos and placentae were removed by previously described techniques, weighed, and homogenized in 10% tri-

chloroacetic acid (TCA). Maternal liver and kidney were also removed in some experiments and processed by the same techniques. The supernatant was saved, after centrifugation, for the determination of the acid soluble precursor pool.

1. Amino Acid Incorporation

^{14}C-leucine was administered to pregnant mice at a dose of 5 μC/mouse to measure protein synthetic rates. After the initial centrifugation the precipitate was washed with successive 5 ml portions of 10% TCA containing 20 mg/ml L-leucine, 10% TCA, and 25% potassium acetate in 95% ethanol. The precipitate was washed with TCA containing the non-radioactive precursor in order to minimize the binding of the radioactive precursor to acid insoluble material. The precipitate was then heated at 60° C for 5 minutes in an alcohol-ether solution (3:1) to extract lipids. After centrifugation the precipitate was heated in 10% TCA at 90° C for 15 minutes. The insoluble material was then treated with successive 5 ml washes of 25% potassium acetate in 95% ethanol, alcohol-ether (3:1) and finally 2 ether washes. A portion of the precipitate was transferred to a tared counting vial, dried, weighed, and solubilized in 1 ml of Soluene 100 (Packard) at 40° C for at least 8 hours. The radioactivity was determined in a Beckman LS-100 liquid scintillation system after the addition of 15 ml of a toluene base counting solution (5 g 2,5-diphenyloxazole (PPO), 200 mg 1,4 bis [2-(4-methyl-5-phenyloxazolyl)]benzene (POPOP), and 1 liter toluene) to the vial containing the protein. The samples were counted for a sufficient time to give results with a maximum of 5% error. The counts/min were converted to distintegrations/min with ^{14}C-toluene and internal standardization techniques. The data were expressed as dpm/mg protein.

2. Nucleic Acid Precursor Incorporation

^{14}C-labeled precursors of RNA (orotic acid and uridine) and DNA (thymidine) were administered to pregnant mice at a dose of 100 μC/kg to determine nucleic acid synthetic rates. After removal of the TCA soluble radioactivity the precipitate was washed with 10% TCA containing the appropriate non-radioactive precursor in an effort to remove unincorporated radioactivity. The precipitate from uridine and thymidine treated animals was washed with an additional portion of 10% TCA. All the precipitates were heated for 5 minutes at 60° C in an alcohol-ether solution (3:1) to extract lipids. The precipitate was heated for 15 minutes at 90° C in 5 ml of 5% TCA to solubilize the nucleic acids. The supernatant was saved for the determination of radioactivity and nucleic acids. If the yield of radioactivity was expected to be low, then a wash of the precipitate was omitted. The radioactivity in 1.0 ml of the TCA extract was determined in 15 ml of a dioxane base counting solution (60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml absolute methanol, 20 ml ethylene glycol and dioxane for a final volume of 1 liter) using a ^{137}Cs external standard in a Beckman LS-100 system. The calibrated channel ratios from the external standard were used to compute dpm from the observed count rate. The samples were counted to at least 5% error and the results were expressed as dpm/ml TCA extract.

3. Determination of RNA and DNA

RNA was estimated in the TCA extract by the orcinol procedure for determining ribose as recommended by Ceriotti (1955). Yeast RNA (Sigma) was used as the standard to obtain quantitative results. The orcinol reagent was prepared daily by mixing 200 mg orcinol, 10 ml of 4mM $CuCl_2$ in concentrated HCl, and a sufficient volume of concentrated

HCl to give 100 ml of the reagent. A sample of the TCA extract was brought to a volume of 5 ml by the addition of 5% TCA and 5 ml of the orcinol reagent was added. The solutions were heated for 40 minutes on a boiling water bath and then cooled. The color was extracted with 5 ml of isoamyl alcohol and the optical density of the organic layer was determined at 675 mμ. The results were expressed as mg RNA/ml of the TCA extract.

DNA was estimated by the reaction of deoxyribose with diphenylamine using Dischés' method as modified by Burton (1956). The diphenylamine reagent was prepared by dissolving 1.5 g diphenylamine in 100 ml of glacial acetic acid and adding 1.5 ml of concentrated sulfuric acid. The solution was placed in small dark bottles and stored in the refrigerator until needed. Acetaldehyde (0.1 ml of a 16 mg/ml solution) was added to the diphenylamine reagent prior to use. A sample of the nucleic acid extract was brought to a volume of 1.0 ml by the addition of 5% TCA and 2.0 ml of the diphenylamine reagent was added. The color was developed for 16-20 hours at room temperature and the optical density of the solution was determined at 600 mμ. The DNA was quantified with a standard curve prepared from calf thymus DNA (Sigma). The data were expressed as mg DNA/ml TCA extract.

4. Estimation of Pool Size

A 1 ml portion of the acid soluble supernatant was added to 15 ml of the Dioxane base counting solution previously described. The radioactivity was determined on a Beckman LS-100 using either internal (amino acid pool) or external (nucleic acid pool) standardization techniques. The samples were counted to a maximum error of 5% and the data were expressed as dpm/g of tissue.

E. Statistical Analysis

Statistical analysis was performed by Student's t test (Steel and Torrie, 1960). The level of significance was selected as $P < 0.05$.

III. RESULTS

A. Postnatal Toxicity

The data in Figure 1 show that day-old mice treated with cyclophosphamide have a reduced growth rate and lower adult body weights than controls. The data in Figure 2 and Table 1 compare the effect of cyclophosphamide (45 and 80 mg/kg) and nor-nitrogen mustard on weight gains and mortality of litters treated 1 day after birth. The dose of nor-nitrogen mustard used in this study was calculated to be equimolar with the higher dose of cyclophosphamide. The data indicate that there was a dose related effect of cyclophosphamide in reducing the growth rates and increasing the mortality of treated litters. Mice treated with nor-nitrogen mustard, on the other hand, were similar to controls in regards to weight gain and mortality. These animals, in addition, did not exhibit any gross morphologic defects. The cyclophosphamide treated animals, however, demonstrated many of the morphologic abnormalities reported by Nordlinder (1969); for example, delayed development of hair, microcephaly, and short nose, ears, and tail. Mice that received the lower dose of cyclophosphamide did not exhibit morphologic defects, at maturity, to the same degree as mice receiving the higher dose.

The pharmacokinetic properties of these 2 agents in day-old mice were determined. However, a combination of factors such as sample size, dose, and sensitivity of the assay did not permit quantitative determinations. The data did, however, permit qualitative conclusions concerning

Figure 1. Growth curves of litters treated with either cyclophosphamide or normal saline at 24–48 hours after birth. The treated group received the subcutaneous administration of 45 mg/kg cyclophosphamide and the control group received an equal volume of the vehicle. The values plotted represent the mean $\pm$ S. E. of the ratio of the body weights, at a given age, to the average adult control body weight at the end of 6-7 weeks. The value used in calculating this ratio was 28.6 grams. The closed circles and solid line correspond to the control litters and the open triangles and dashed line correspond to the treated group. The values represent the mean for 3 litters.

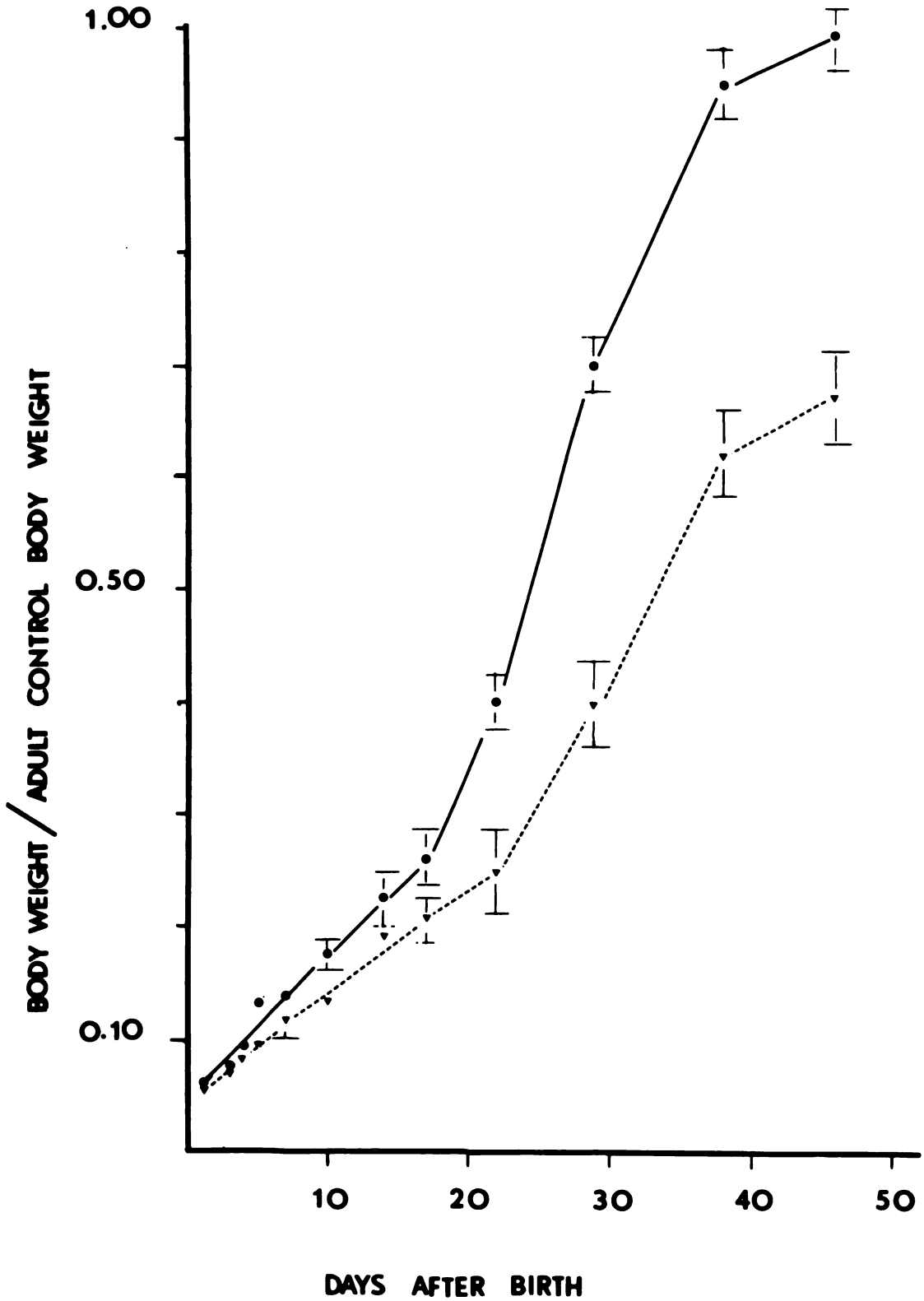
1.00
0.50
0.10
BODY WEIGHT / ADULT CONTROL BODY WEIGHT
10
20
30
40
50
DAYS AFTER BIRTH

Figure 1

Figure 2. Growth curves of cyclophosphamide and nor-nitrogen mustard treated litters. The values plotted were determined by the process described in Figure 1. The growth curves were obtained from litters that received either nor-nitrogen mustard 54 mg/kg, A; cyclophosphamide 45 mg/kg, B; or cyclophosphamide 80 mg/kg, C. The individual control growth curves have been omitted. The growth curves for nor-nitrogen mustard and its control were identical. Each curve was determined with 3 treated and 3 control litters and values earlier than 10 days have been omitted for clarity.

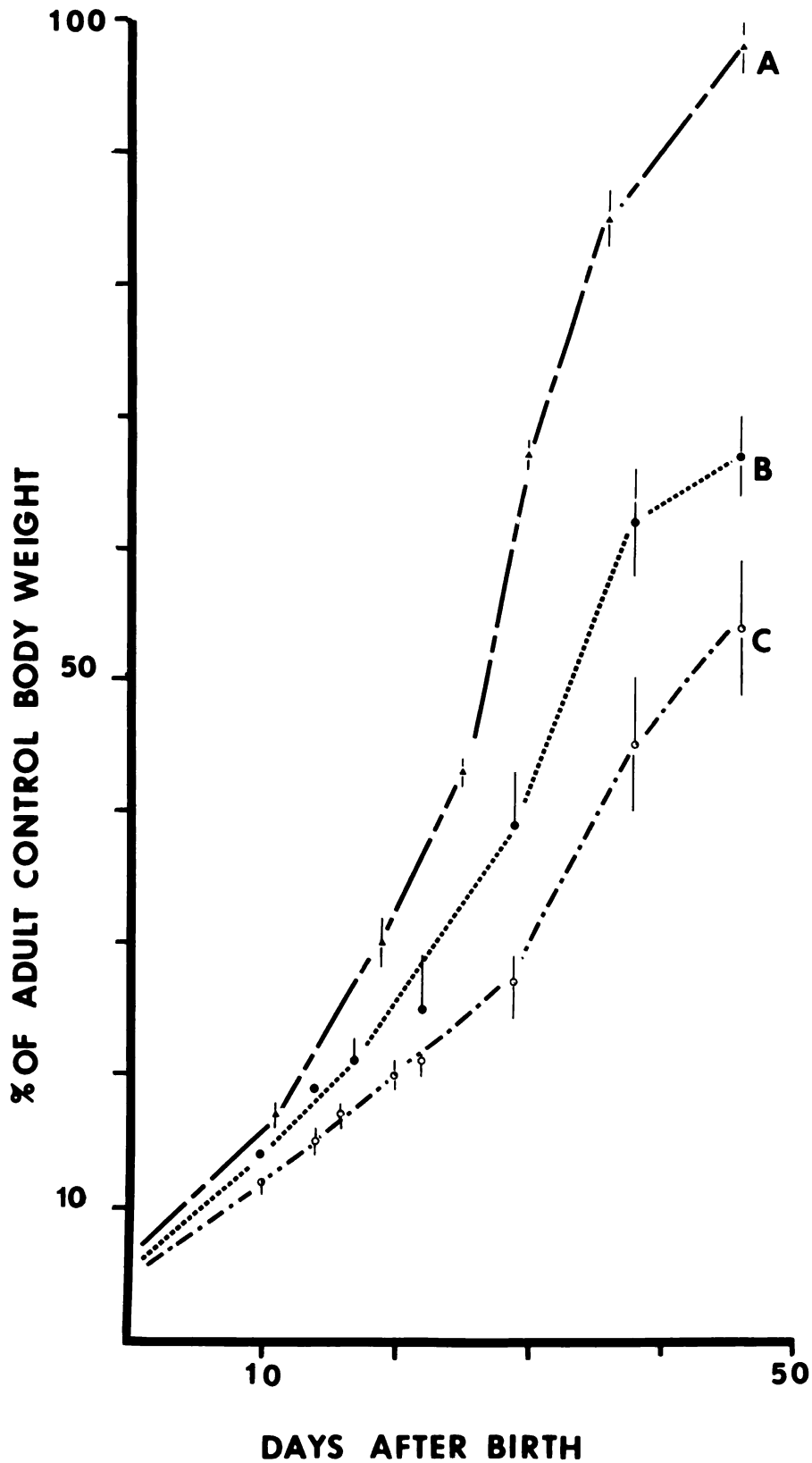
100
50
10
% OF ADULT CONTROL BODY WEIGHT
A
B
C
10
50
DAYS AFTER BIRTH

Figure 2

Table 1. Cumulative litter mortality produced by nor-nitrogen mustard or cyclophosphamide administration to day-old mice

Treatment	% Mortality	
	Control	Treated
Cyclophosphamide 45 mg/kg	0[a]	22[b] ±15
Cyclophosphamide 80 mg/kg	15 ±14	44[b] ±14
Nor-nitrogen mustard 54 mg/kg	8 ±4	11 ± 5

[a]The values represent the average percent mortality ± S. E. for 3 litters 46 days after birth.

[b]Significantly different from control at $P < 0.05$ (Student's *t* test).

absorption and elimination. Since both drugs were present in the urine it was concluded that the agents had been absorbed, to some degree, from the subcutaneous injection site. Nor-nitrogen mustard could not be detected in the plasma 1 hour after injection. Cyclophosphamide, on the other hand, was detected in both plasma and urine at the end of 4 hours but only in the urine at the end of 8 hours. These observations suggest that the 2 agents may have different pharmacokinetic properties.

B. *In vitro* Cyclophosphamide Activation

The effect of a pH range of 6.7 to 8.4 was evaluated on the ability of the 9000 g supernatant from mature virgin females to activate 12.8 mM cyclophosphamide after a 1 hour incubation. Maximum activity was observed at pH 7.4 (Table 2) and this pH was used in subsequent incubations. The effect of varying concentration of G6P, $MgSO_4$, and NADP on the ability of mature female livers to activate 12.8 mM cyclophosphamide after a 1 hour incubation was studied. The addition of 12.5 µmoles G6P, 12.5 µmoles $MgSO_4$ and 1.5 mg. NADP to the media enabled the reaction to proceed independently of these cofactors (Table 3). Subsequent incubations were conducted using these amounts of cofactors.

The kinetics of activation were determined using the 9000 g supernatant of liver from mature females and a substrate concentration range of 0.4 to 12.8 mM. The apparent Km was 0.92 mM and the Vmax was 1.7 µmoles of alkylating activity/min/mg protein (Figure 3). The calculated line had a regression coefficient equal to 0.92. As a result of the kinetic analysis subsequent incubations were conducted using a substrate concentration of 12.8 mM and a 10 minute incubation unless otherwise indicated.

Table 2. Optimization of pH for the *in vitro* activation of cyclophosphamide[a]

	Cyclophosphamide alkylating activity relative to the activity at pH 7.4[b]			
	6.7	7.4	7.9	8.4
Relative activity	0.67[c] ±0.07	1.0	0.73 ±0.08	0.47 ±0.08

Table 3. Optimization of cofactors for the *in vitro* activation of cyclophosphamide[a]

mg NADP added	Cyclophosphamide alkylating activity relative to the activity produced by the addition of 1.5 mg NADP and 25 µmoles G6P[b]		
	µmoles of G6P and $MgSO_4$ added		
	6.25	25	37.5
1.0	0.56±0.06[c]	0.56±0.10	---
1.5	0.78±0.18	1.0±0.21	0.93±0.06
2.0	1.0 ± 0.14	0.86±0.18	0.71±0.14
2.5	0.71±0.14	0.73±0.10	0.71±0.04

[a] 9000 g supernatant of liver from mature virgin female.

[b] One hour incubation.

[c] Mean ± S. E.

Figure 3. The kinetics of cyclophosphamide activation. The kinetics of activation were determined in the optimized system using the 9000 g supernatant of liver from virgin females and a substrate concentration range of 0.4 to 12.8 mM.

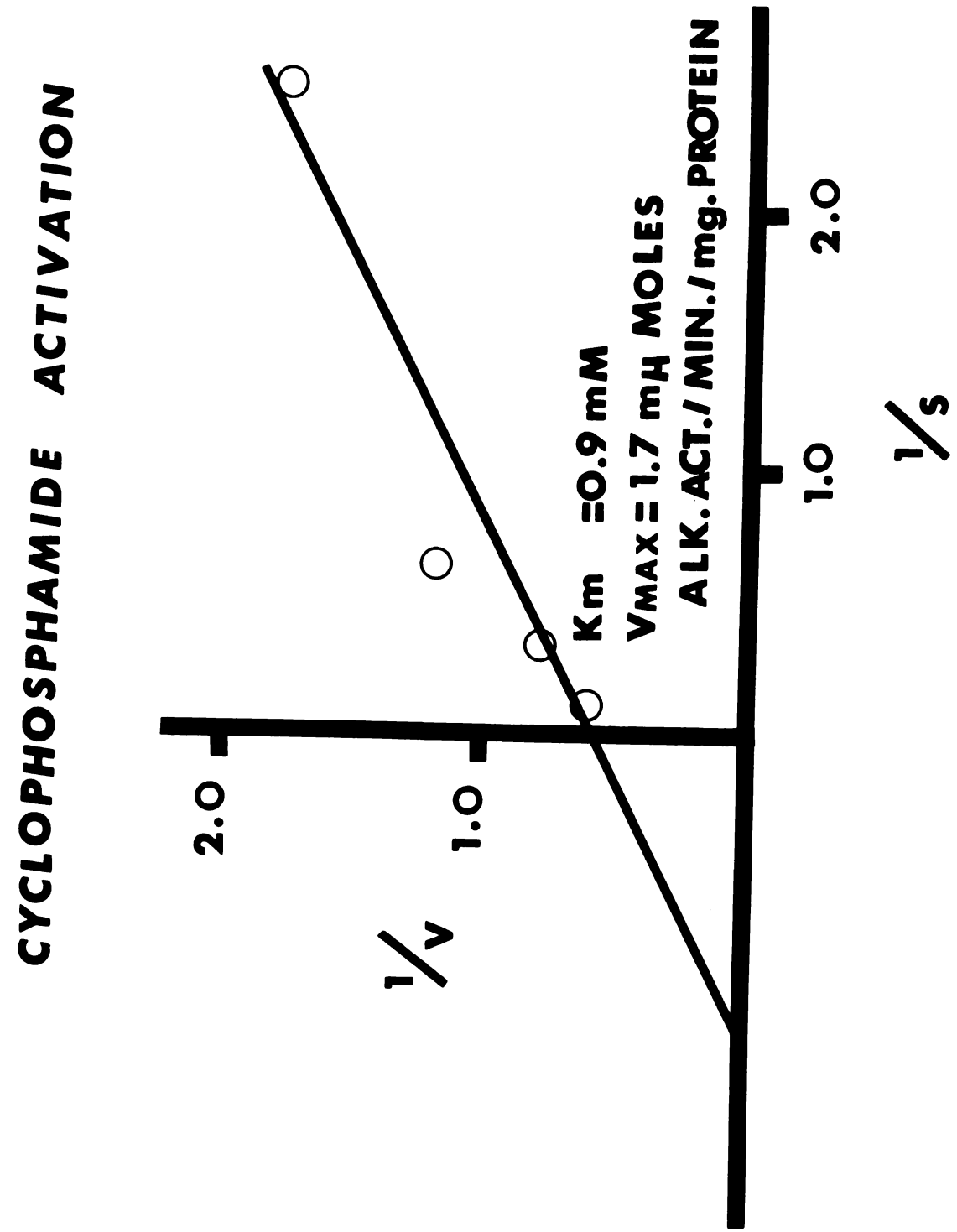
CYCLOPHOSPHAMIDE ACTIVATION
2.0
1.0
1/v
Km =0.9 mM
VMAX = 1.7 mH MOLES
ALK. ACT./MIN./mg. PROTEIN
1.0
2.0
1/s

Figure 3

The activating ability of livers from phenobarbital and SKF 525-A pretreated female mice was investigated in this system. These experiments were conducted to provide an *in vitro* confirmation of the *in vivo* observations of Gibson and Becker (1968b) which showed that these pretreatments altered the maternal metabolism of cyclophosphamide. A 5 minute incubation was used in an effort to maintain a linear reaction rate for livers from phenobarbital pretreated mice. Table 4 shows that phenobarbital pretreated animals had only 35% of control activity. The phenobarbital pretreated mice, in addition, had a significantly increased liver/body weight ratio. It was concluded from these observations that the *in vivo* alteration of cyclophosphamide metabolism could be duplicated using *in vitro* conditions and, therefore, was due to a direct action of the pretreatment drugs on the liver mixed function oxidase system.

In order to investigate the ontogeny of cyclophosphamide activation it was necessary to determine if a sex difference in the activation reaction would be a complicating factor. The data in Table 5 indicated that there was no significant sex difference in cyclophosphamide activation at 4 weeks of age or at maturity. Since there was no sex difference in cyclophosphamide activation it was possible to pool litters without separating the mice on the basis of sex.

The data presented in Table 6 indicated that the whole fetus on days 15, 17, or 19 of gestation was not able to activate the parent compound to the same degree as the maternal liver. The corresponding placentae, in addition, did not possess activity equivalent to the maternal liver. The range of values for mg protein/incubate were: maternal liver (21-31), placenta (9-14), and fetus (8-12).

Table 4. Effect of phenobarbital and SKF 525-A on cyclophosphamide activation and liver/body weight ratio

Pretreatment	mμmoles Alk. Act.[a] / mg protein	g liver / 100 g body
Control	9.0 ± 0.9 (6)[b]	4.5 ± 0.2 (6)
Phenobarbital	21.0 ± 2.3 (5)[c]	5.6 ± 0.2 (6)[c]
SKF 525-A	3.2 ± 0.6 (6)[c]	4.3 ± 0.1 (6)

[a]Five min incubation. Alk. Act. = alkylating activity.

[b]Mean ± S. E. (number of observations).

[c]Significantly different from control ($P < 0.05$).

Table 5. Activation of cyclophosphamide by the 9000 g supernatant of livers from mature and 4-week-old mice

Animal age	mμmoles alkylating activity/mg protein[a]	
	female	male
4 week	16.0 ± 2.2 (6)[b]	20.0 ± 1.8 (6)
mature	18.0 ± 1.6 (8)	18.0 ± 3.2 (8)

[a] Ten min incubation.

[b] The values represent the mean ± S. E. (number of determinations).

Table 6. Activation of cyclophosphamide by the 9000 g supernatant of tissues obtained from pregnant mice

Day of gestation	mμmoles alkylating activity per mg protein[a]		
	Maternal liver	Placenta	Fetus
15	21.0 ± 4.4 (3)[b]	1.7 ± 0.2 (3)	1.8 ± 1.0 (4)
17	14.0 ± 1.7 (6)	7.5 ± 1.9 (6)	3.0 ± 3.0 (4)
19	15.0 ± 0.8 (7)	4.6 ± 1.9 (7)	1.0 ± 1.0 (5)

[a]Ten min incubation.

[b]The values represent the mean ± S. E. (number of determinations).

The data presented in Figure 4 show that the neonatal liver was not able to activate cyclophosphamide as readily as adult virgin female liver for at least 2 weeks after birth. There was an increase in activity between 14 and 21 days of age and activity equivalent to adult values was reached by 21-day-old mice. There was, in addition, a significant difference in activity between control and nursing mothers at 3, 7, 14 and 21 days after giving birth. The range of values for mg protein/incubate were: maternal liver (22-39), mature female liver (21-33) and neonatal liver (11-20).

The data in Table 7 show activation expressed in terms of tissue weight. This presentation of the data provides a more obvious basis for comparing the abilities of various tissues to metabolize cyclophosphamide. This expression of the data does not alter the relationship between maternal and control females at 14 and 21 days postpartum. The neonatal livers at 21 days of age, however, have significantly lower activity than adults. The table shows, in addition, that the neonatal body, without the liver, is a relatively insignificant site of activation for at least 5 days after birth.

C. Biosynthesis of Macromolecules

1. Protein Synthesis

The data in Figure 5 are a plot of the specific activities of total embryonic protein, after a 30-minute pulse of ^{14}C-leucine, as a function of time after cyclophosphamide. Cyclophosphamide was administered on day 11 of gestation. As a result of this treatment the embryos had a significantly reduced capacity to incorporate the precursor at 72 and 96 hours after drug administration. There were, however, no significant differences in the specific activities of control and drug treated proteins

Figure 4. The ontogeny of cyclophosphamide activation. The *in vitro* cyclophosphamide activating activity in neonatal mice and maternal livers was expressed as a percent of the activity found in livers from mature females. Livers from whole litters were pooled. The values for the female liver were determined simultaneously with neonatal and maternal livers. The range of values for adult females, after averaging a group, was 16-20 mμmoles alkylating activity/mg protein after a 10 minute incubation. The combined average for all females was 18 $\pm$ 0.6 mμmoles alkylating activity/mg protein for 39 determinations. Closed circles correspond to values determined for the mother and open triangles correspond to values determined in the neonates. The points are the mean $\pm$ S. E. for 3-9 determinations.

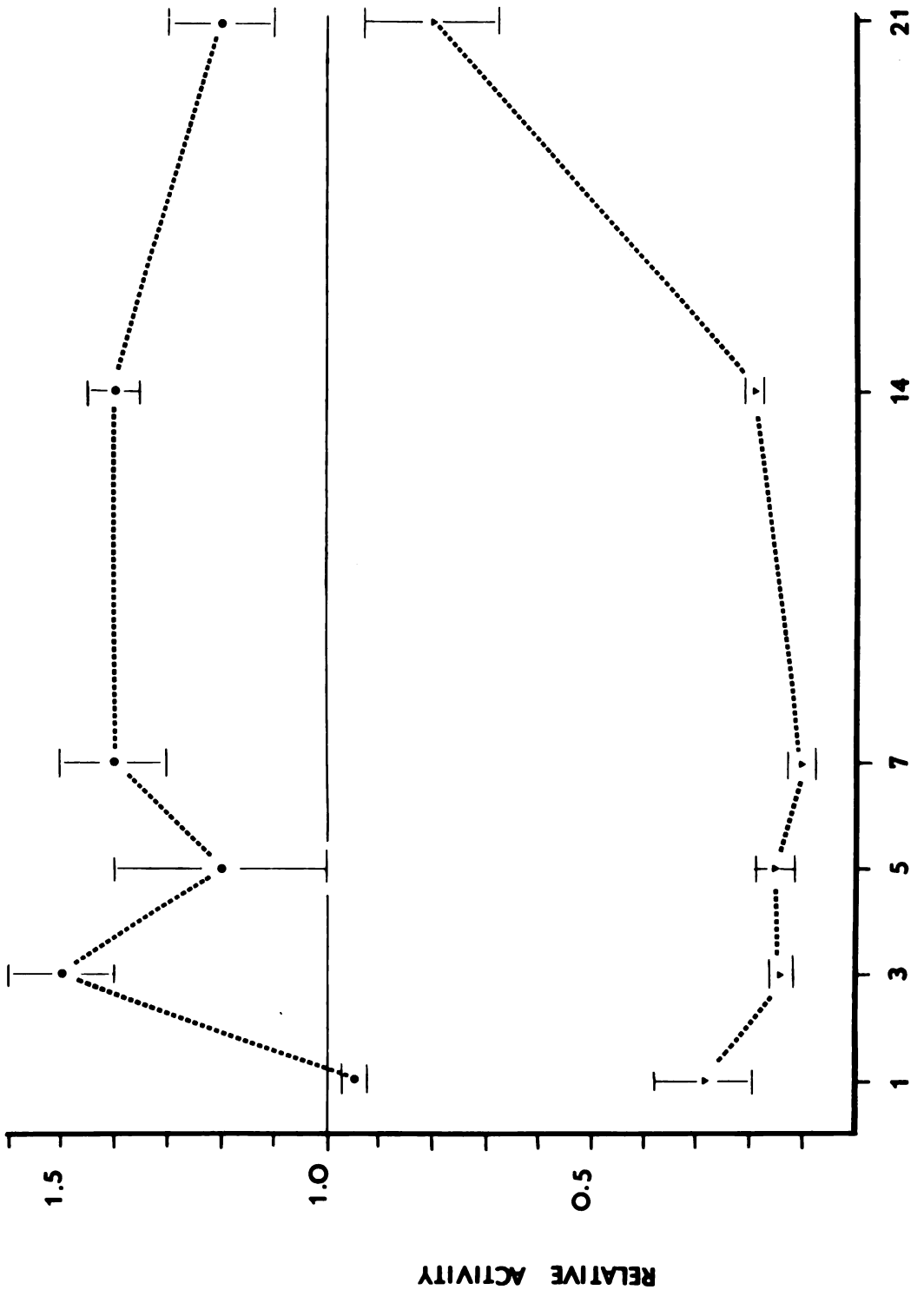

Figure 4

Table 7. Activation of cyclophosphamide by the 9000 g supernatant of perinatal tissue obtained from mice

Days after birth	μmoles alkylating activity per gram tissue x 10^{-2}[a]				
	Maternal liver	Neonatal liver	Neonatal body	Fetus	Placenta
-5	280 ± 21 (6)[b]	---	---	32 ± 14 (6)	31 ± 9 (6)
-1	239 ± 18 (7)	---	---	37 ± 20 (7)	33 ± 14 (7)
+1	270 ± 18 (6)	41 ± 14 (6)	18 ± 10 (5)	---	---
+5	370 ± 54 (5)	27 ± 6 (4)	33 ± 19 (3)	---	---
+14	460 ± 4 (3)	47 ± 3 (3)	---	---	---
+21	400 ± 39 (7)	189±34 (7)	---	---	---

[a]Ten min incubation.

[b]The values represent the mean ± S. E. (number of determinations). The activity in mature virgin females was 300 ± 23 (10).

Figure 5. ^{14}C-Leucine incorporation into embryonic protein as a function of time after cyclophosphamide. Cyclophosphamide was administered on day 11 of gestation and the precursor was administered to the mother 30 minutes prior to sacrifice and removal of the embryos. Protein was isolated and counted. The values plotted represent the mean $\pm$ S. E. of the specific activities for at least 3 determinations. An asterisk indicates a significant difference from control ($P < 0.05$).

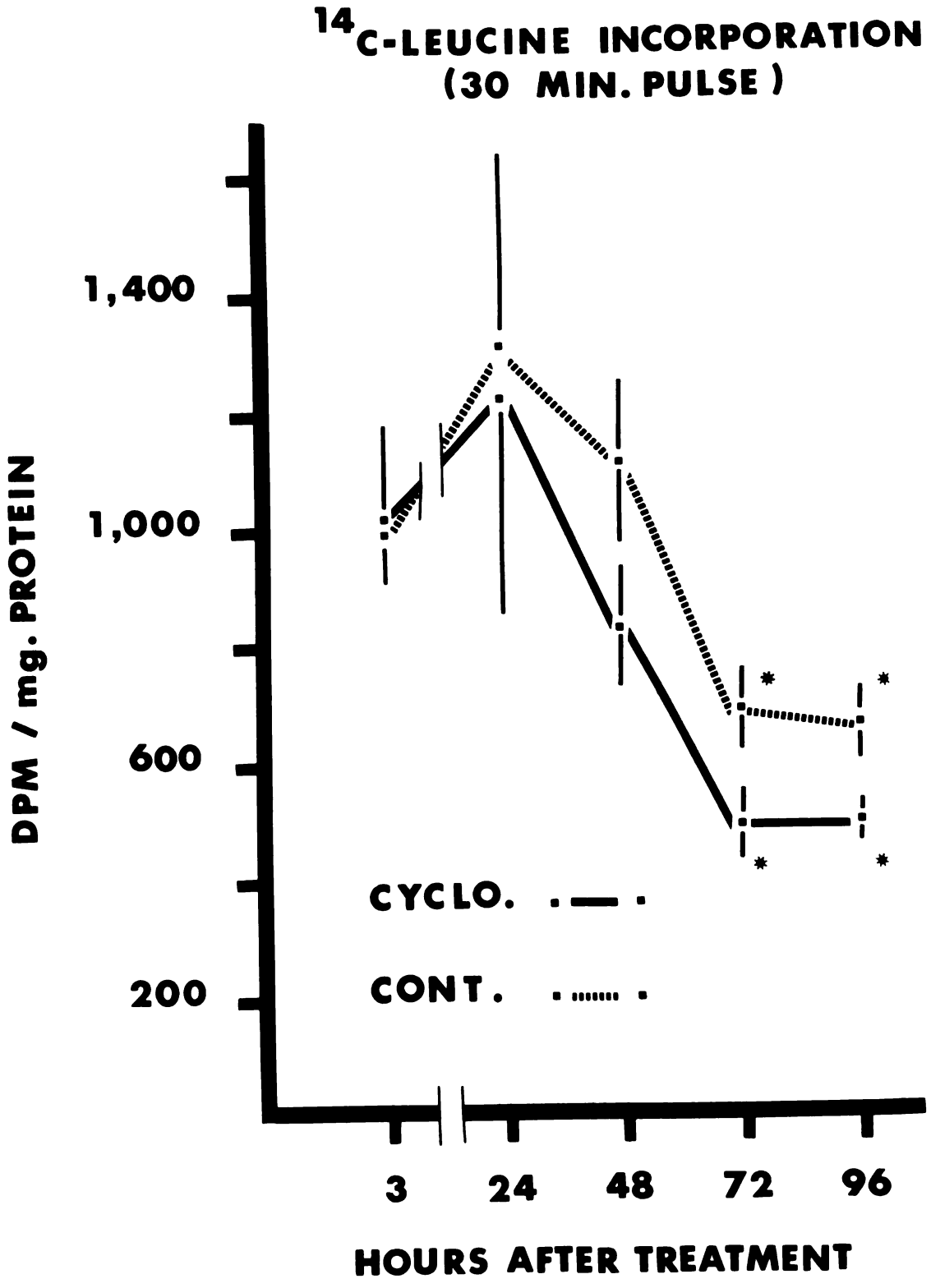
^{14}C-LEUCINE INCORPORATION
(30 MIN. PULSE)
DPM / mg. PROTEIN
1,400
1,000
600
200
CYCLO.
CONT.
3
24
48
72
96
HOURS AFTER TREATMENT

Figure 5

at 3, 24, and 48 hours after treatment. The acid soluble precursor pool from the 2 groups of embryos, in addition, was not significantly different at any of the times studied and, therefore, depletion of the precursor could not account for the decrease in incorporation. Cyclophosphamide treatment did not affect the ability of the placenta to incorporate the precursor at any of the times studied. There was, however, a significant reduction in precursor incorporation in the liver at 96 hours after cyclophosphamide and in the kidney at 72 and 96 hours after treatment.

The duration of the ^{14}C-leucine pulse was varied 72 hours after drug treatment, in order to determine if a 30 minute pulse period was optimum for precursor incorporation by the embryos. The acid soluble pool was maximally labeled by 15 minutes in both groups of embryos. The incorporation of the precursor into proteins by control embryos reached a plateau at 30 minutes (Figure 6). The specific activity of proteins from treated embryos, however, was significantly different from controls at 30 and 60 minutes after precursor administration. The specific activity of protein was not significantly different between the 2 groups at 120 minutes after precursor administration. There were, in addition, no significant differences between either group in the acid soluble precursor pool at any of the times studied. Time dependent differences between the 2 groups in the placental incorporation of precursor, on the other hand, were not detected at any of the times studied.

2. RNA Synthesis

Initial experiments using orotic acid as a precursor of RNA showed a placental barrier to its transport into the embryo. Since a

Figure 6. ^{14}C-Leucine incorporation into embryonic protein as a function of time after precursor. The precursor was administered to the mother 72 hours after cyclophosphamide treatment. The mothers were sacrificed at the indicated times and the embryos removed. The protein was isolated and counted. The values plotted represent the mean $\pm$ S. E. of the specific activities for at least 3 determinations. An asterisk indicates a significant difference from control ($P < 0.05$).

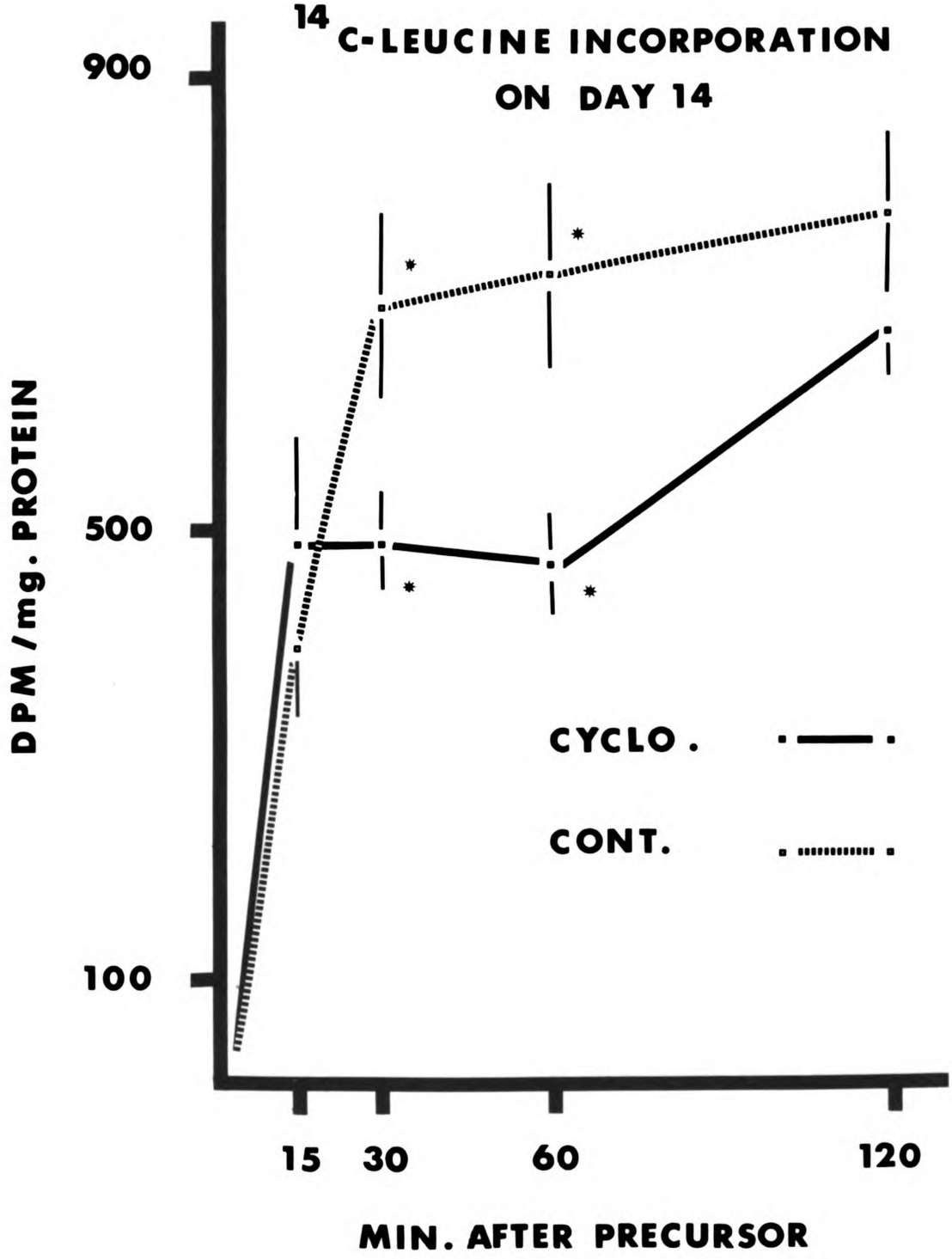
14 C-LEUCINE INCORPORATION
ON DAY 14
900
500
100
DPM / mg. PROTEIN
CYCLO.
CONT.
15
30
60
120
MIN. AFTER PRECURSOR

Figure 6

30 minute pulse did not produce any detectable labeling of RNA in embryos the time course of precursor incorporation was investigated. The mice used in this study were not pretreated and ranged bewteen 12 and 14 days of gestation. Figure 7 shows that both the liver and placenta have a greater ability than the embryo to incorporate the precursor into nucleic acid under the conditions of this study. The reduced incorporation of orotic acid was attributed to a placental barrier because the acid soluble precursor pool in the placenta and liver were approximately 10 and 100 times, respectively, the precursor pool of the embryo. A 4 hour pulse was selected in an effort to obtain satisfactory labeling of RNA in the embryo with a minimum precursor exposure. Using these conditions, it was determined that cyclophosphamide did not significantly affect the ability of the embryo to synthesize RNA 24 hours after treatment.

A 4 hour ^{14}C-uridine pulse was initially used as a result of the previous experience with orotic acid. These experiments indicated that there was no significant difference in the ability of control and cyclophosphamide treated embryos to incorporate ^{14}C-uridine 24 hours after drug treatment. The specific activity of the RNA, after a 4 hour pulse indicated the feasibility of a shorter pulse period. The precursor, therefore, was given for a 1 hour period in subsequent experiments. The ability of embryos to synthesize RNA was not affected at 3 or 72 hours after drug treatment as determined with a 1 hour ^{14}C-uridine pulse.

The data in Figure 8 summarize the incorporation of ^{14}C-orotic acid and ^{14}C-uridine into RNA of cyclophosphamide treated embryos as a percent of the incorporation determined in control embryos. There was an indication that RNA synthesis was depressed; however, the differences between a treated group and its control were not statistically significant. The data in Figure 9 are the values of mg RNA/g of wet weight of embryo.

Figure 7. The incorporation of ^{14}C-orotic acid into RNA. The precursor was administered to mice on days 12-14 of gestation. The mothers were sacrificed at the indicated times after precursor administration. Radioactivity was determined and RNA measured in the hot TCA extract of the acid precipitate from the indicated tissues. The values plotted represent either a mean $\pm$ range for 2 determinations (1 and 2 hours) or the mean $\pm$ S. E. for 3 to 4 determinations.

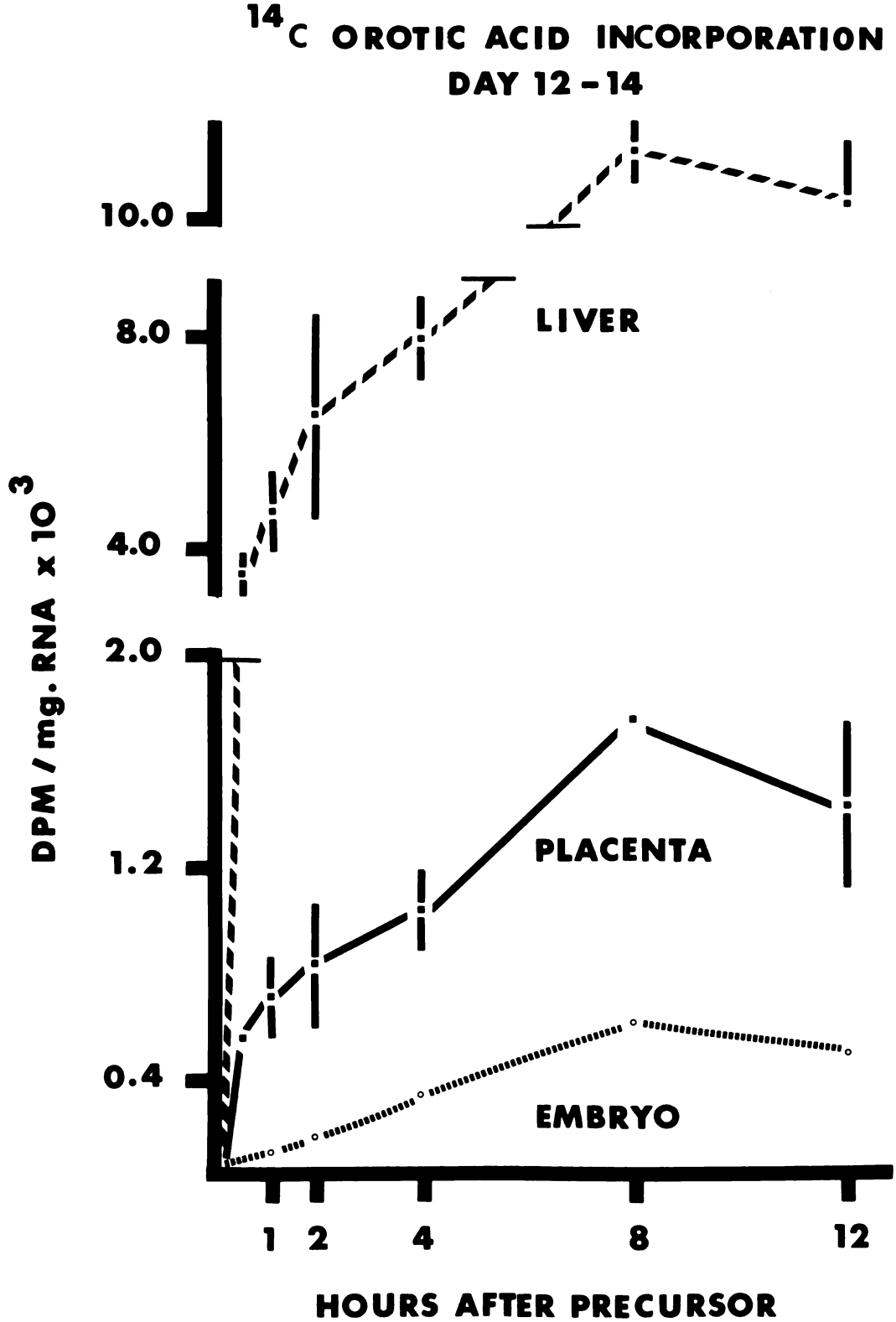
^{14}C OROTIC ACID INCORPORATION
DAY 12 - 14
10.0
8.0
4.0
2.0
1.2
0.4
DPM / mg. RNA x 10^3
LIVER
PLACENTA
EMBRYO
1
2
4
8
12
HOURS AFTER PRECURSOR

Figure 7

Figure 8. The incorporation of either ^{14}C-uridine or ^{14}C-orotic acid into RNA of embryos. Cyclophosphamide was administered on day 11 of gestation and the animals were sacrificed at the indicated times. ^{14}C-orotic acid was given for 4 hours and ^{14}C-uridine was given for either 4 hours (24 hours after cyclophosphamide) or 1 hour (3 and 72 hours after cyclophosphamide) prior to sacrifice. The values correspond to the mean $\pm$ range for 2 determinations or to the mean $\pm$ S. E. for 3 or more determinations. The ratio of treated/control determinations was: 3 hours after treatment (4/2), 24 hours after treatment (4/3 for uridine and 3/3 for orotic acid) and 72 hours after treatment (2/3).

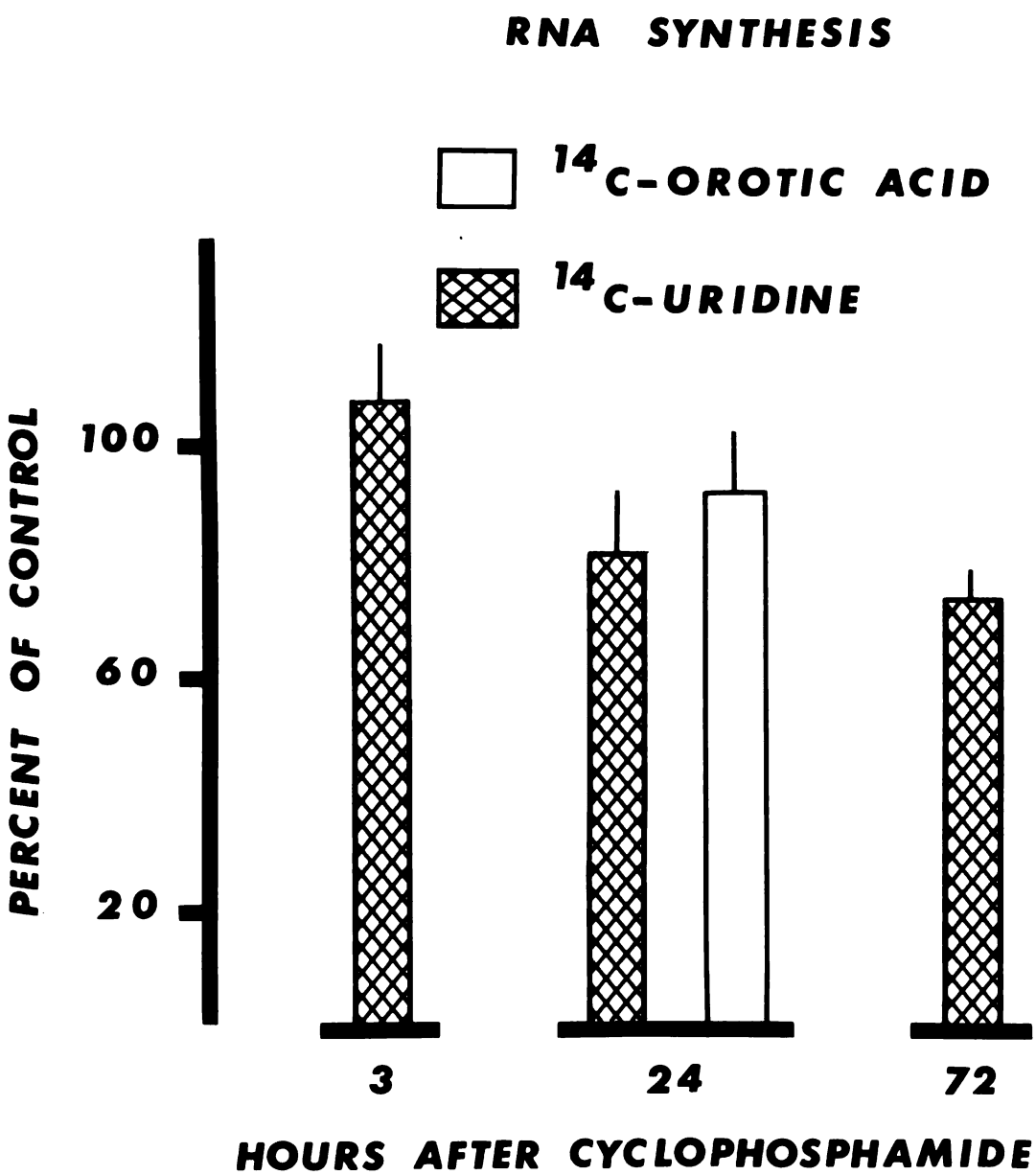
RNA SYNTHESIS
14C-OROTIC ACID
14C-URIDINE
PERCENT OF CONTROL
100
60
20
3
24
72
HOURS AFTER CYCLOPHOSPHAMIDE

Figure 8

Figure 9. Milligrams RNA/g wet weight of embryo as a function of gestational age and treatment. Mice were treated with cyclophosphamide on day 11 of gestation and sacrificed 3 or 24 hours later. RNA was determined in the hot TCA extract by the orcinol method. The values represent the mean $\pm$ S. E. for at least 3 determinations.

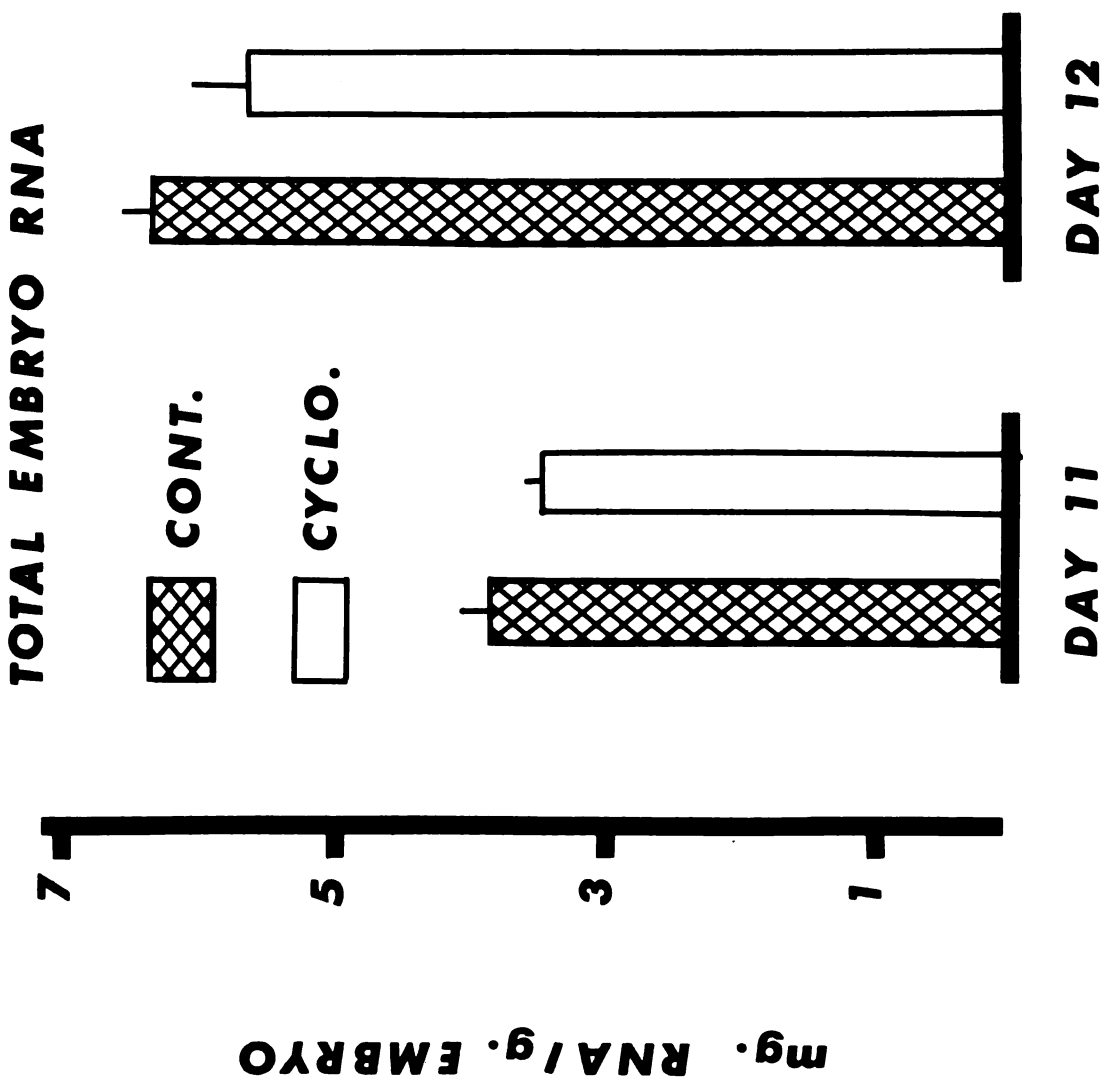

Figure 9

There were no significant differences between control and treated groups on a given day but there was a significant temporal difference within a group. This indicated that the embryo was able to increase the amount of RNA present for at least 24 hours after cyclophosphamide treatment. There was, in addition, no significant cyclophosphamide induced inhibition of RNA synthesis in the liver or placenta detected with either of the precursors or pulse periods.

3. DNA Synthesis

The data in Table 8 are conceptually viewed as representing the average DNA/cell weight ratio. The information in Table 9 is a measurement of the ability of cells to synthesize DNA. The tissue from treated embryos had a reduced amount of DNA for at least 48 hours after treatment (Table 8). These embryos had a greater rate of thymidine incorporation than controls 24 hours after drug treatment. The differences were not statistically significant but correlated with the increasing amounts of DNA in the drug treated embryos (Table 8).

Placental tissue, on the other hand, demonstrated an impaired ability to incorporate thymidine into DNA at all of the times studied (Table 9). There was, in addition, a decreased amount of DNA 48 hours after treatment. The decreased amount of DNA, therefore, may have been due to an inhibition of DNA synthesis. A decreased specific activity and absolute amount of DNA, with gestational age, may reflect a reduced mitotic rate and functional maturation of the cells. The amount of DNA/tissue weight may be decreased, during maturation, by increased amounts of cellular protein, water, etc. The specific activity of treated placental DNA, however, declined at a more rapid rate than control placental DNA. This greater decrease may reflect an impaired ability to synthesize DNA.

Table 8. DNA content after cyclophosphamide treatment

Hours after Cyclophosphamide[a]	mg DNA/g tissue					
	Liver		Placenta		Embryo	
	Cont.	Treat.	Cont.	Treat.	Cont.	Treat.
24	2.9[b] ±0.0	2.3 ±0.2	4.0 ±0.4	3.7 ±0.3	5.8 ±0.8	3.7 ±0.2
48	2.6 ±0.2	1.6[c] ±0.1	4.1 ±0.1	3.4[c] ±0.2	6.3 ±0.2	4.3[c] ±0.2
72	2.6 ±0.1	2.9 ±0.2	2.9 ±0.1	3.0 ±0.2	6.4 ±0.2	5.5 ±0.3

[a]Cyclophosphamide (20 mg/kg) was administered to the mother on day 11 of gestation.

[b]Mean ± S. E. for 3 to 4 observations.

[c]Significantly different from control ($P < 0.05$).

Table 9. DNA synthesis after cyclophosphamide treatment

Hours after Cyclophosphamide[b]	DPM/mg DNA x 10^3[a]					
	Liver		Placenta		Embryo	
	Cont.	Treat.	Cont.	Treat.	Cont.	Treat.
24	4.8[c] ±0.4	6.0 ±0.8	42.9 ± 2.6	31.1[d] ± 1.8	19.4 ± 1.9	26.3 ± 4.3
48	4.8 ±1.3	2.6 ±0.5	27.0 ± 2.0	15.2[d] ± 3.6	18.3 ± 2.1	17.6 ± 4.0
72	3.9 ±0.3	2.0[d] ±0.4	22.2 ± 2.0	13.6[d] ± 1.2	18.6 ± 1.2	16.8 ± 3.6

[a] One hour pulse of ^{14}C-thymidine.

[b] Cyclophosphamide (20 mg/kg) was administered to the mother on day 11 of gestation.

[c] Mean ± S. E. for 3 to 4 observations.

[d] Significantly different from control ($P < 0.05$).

The amount of liver DNA was significantly decreased 48 hours after cyclophosphamide treatment (Table 8). A significant inhibition of DNA synthesis was not detected as early in the liver as it was in the placenta. Since the liver had a lower rate of thymidine incorporation than the placenta it may have required a longer time for an inhibition of liver DNA template activity to become evident.

DISCUSSION

Cyclophosphamide, a clinically useful antineoplastic agent, has toxic effects in both embryonic and neonatal mice. Day-old mice treated with 80 mg/kg cyclophosphamide grew at a reduced rate with increased lethality and abnormal morphology. This was in contrast to adult mice who survived a cyclophosphamide dose of 300 mg/kg for at least 1 month (Hayes *et al.*, 1971).

An effort was made to assess the effect of alkylating activity on the growth and development of neonatal mice by treating day-old mice with nor-nitrogen mustard. The administration of an equimolar dose of nor-nitrogen mustard did not produce any observed effects on growth, lethality, or morphology. The validity of comparing cyclophosphamide and nor-nitrogen mustard was evaluated by the determination of their pharmacokinetic properties. Cyclophosphamide was detected in the plasma and urine of day-old mice for at least 4 hours after treatment. Nor-nitrogen mustard, on the other hand, was detected only in the urine 1 hour after treatment. This observation suggests at least 2 mechanisms to account for the different effects of the 2 agents. The first explanation depends on the observation that day-old mice had an immature system for forming excretable polar metabolites. The parent compound, therefore, may have served as an inactive depot molecule from which toxic molecules were formed at a slower rate than in adults. Cyclophosphamide treated litters, as a result, may have been exposed to

cytoxic agents for a more prolonged period than nor-nitrogen mustard treated neonates. The second explanation is that cyclophosphamide produced its toxic effects on development by a mechanism other than alkylation. The dramatic developmental effects produced by this agent, therefore, may have been due to the prolonged presence of the parent compound in the body.

An *in vitro* system was optimized, using the 9000 g supernatant of liver from adult females, in order to verify that perinatal mice have an underdeveloped capacity to convert the parent compound to alkylating metabolites. The kinetics of activation, determined in this system, were similar to values reported by Sladek (1971). Sladek obtained a Km of 0.7 mM and a Vmax of 9.7 μmoles/g/hr for activation in a microsomal system from mouse liver. A microsomal preparation from male rats, in addition, had a pH optimum equivalent to the mouse system employed in this study. Male rats, in contrast to male mice, had greater cyclophosphamide activation activity than females.

The ontogeny of cyclophosphamide activation was in basic agreement with observations concerning the development of the liver microsomal drug metabolizing system in rabbits (Fouts and Adamson, 1959) and both mice and guinea pigs (Jondorf *et al.*, 1959). Neither the placenta nor the fetus was a major site for the production of alkylating metabolites (Tables 6 and 7) during the period of susceptibility to cyclophosphamide teratogenicity. When the teratogenic effects of cyclophosphamide were increased, as a result of reducing the ability of the maternal liver to metabolize the drug (Gibson and Becker, 1968b and Table 4), it was unlikely that increased maternal plasma levels of the parent compound (Gibson and Becker, 1971a) were activated to any degree by the placenta or fetus. The embryo levels of ^{14}C-cyclophosphamide metabolites (Gibson and Becker,

1971a), in addition, remained relatively constant regardless of the ability of the mother to activate the parent compound. This observation indicated that the embryo-placental unit did not possess a significant *in vivo* ability to form alkylating metabolites.

Neonatal mice showed a poorly developed capacity to activate the parent compound for at least 2 weeks after birth. The neonatal body, in addition, did not have activity greater than the immature liver for at least 5 days after birth. Activity, equivalent to adult activity, was reached after 14 days of age. The poor correlation between cyclophosphamide activation and both its teratogenicity and neonatal toxicity, therefore, is offered in support of the hypothesis attributing developmental toxicity to the parent compound.

An interesting aspect of the *in vitro* determinations of cyclophosphamide activation was the observation that livers from nursing mothers had a significantly greater capacity to perform the conversion than virgin controls. This observation may have important implications in both drug metabolism and cyclophosphamide toxicity. It has been shown, for example, that cyclophosphamide induced lethality (Dixon, 1968) and leucopenia (Hayes *et al.*, 1971) are associated with the production of alkylating metabolites.

The biosynthesis of macromolecules in embryos was investigated in an attempt to define biochemical lesions induced by cyclophosphamide. Protein synthesis was significantly depressed only at 72 and 96 hours after cyclophosphamide treatment. The embryos, however, were visibly deformed by this time. Since there was no temporal dissociation between an inhibition of protein synthesis and abnormal morphology, it was not possible, therefore, to conclude that the primary action of the drug,

responsible for the production of anomalies, was an inhibition of protein synthesis. Since protein synthesis was also depressed at the same time in the maternal system it was not possible to conclude that cyclophosphamide was acting by different mechanisms in the embryo and adult. A temporal study of ^{14}C-leucine incorporation into embryonic protein indicated that the pulse period may be an important factor in detecting cyclophosphamide induced effects. These observations raise the possibility that differences in protein synthesis may have been detected at earlier times after drug treatment if a different pulse period had been used.

The time course of ^{14}C-leucine incorporation into proteins 72 hours after cyclophosphamide suggest that treated embryos may have an impaired ability to initiate protein synthesis. The messenger RNA being read on the ribosomes may incorporate radioactivity into protein at an equal rate in both control and treated embryos. The treated tissue may have an impaired ability to form new messenger RNA-ribosomal complexes and to sustain the existing rate of precursor incorporation. This explanation may account for the temporal differences in ^{14}C-leucine incorporation but do not explain why the incorporation is equivalent at the end of 2 hours.

The decrease in specific activity of protein, with gestational age, indicated that the embryo was not in equilibrium with regards to the synthesis and degradation of protein. Protein synthesis, therefore, predominated over protein degradation and the pool of non-radioactive proteins increased with gestational age. Since this phenomenon was present in both control and treated embryos it appeared unlikely that drug treatment dramatically altered the ratio of protein synthesis to protein degradation.

The observations concerning the biosynthesis of nucleic acids, after cyclophosphamide treatment, lay the groundwork for speculation but do not offer information for firm conclusions concerning the mechanism by which this agent produced its teratogenic effect. The cyclophosphamide induced decrease in embryo DNA content (Table 8) may have been the result of factors such as: (1) the selective death of cells with a high DNA/cell weight ratio (e.g., rapidly dividing or newly formed cells); (2) an inhibition of DNA replication and repair with the result that the degradation of damaged chromosomes predominated over DNA repair and synthesis; (3) an alteration of placental function and reduced availability of essential nutrients. Cyclophosphamide treatment altered DNA synthesis in the liver and placenta but not in the embryo (Table 9). The liver and placenta were both exposed to the maternal circulation and, therefore, cyclophosphamide metabolites. Drug induced effects on DNA synthesis in the liver and placenta, in comparison to the embryo, may, therefore, be temporally dissociated or occur by different mechanisms.

The *in vitro* alteration of DNA template activity with nor-nitrogen mustard indicated that this agent was more effective in the inhibition of RNA synthesis than DNA synthesis (Ruddon and Johnson, 1968). Since DNA template activity for the synthesis of DNA and RNA may be dissociated this may explain why only DNA synthesis was affected in the liver and placenta. A problem with these studies was that they detected only quantitative changes in the biosynthesis of macromolecules. The rate of RNA synthesis, for example, may have been equivalent in both control and treated tissue but contained qualitative differences in the types of RNA produced.

This study, in conclusion, supports the observation that cyclophosphamide teratogenicity may be separated from its alkylating activity and suggests that this dichotomy may be applicable to neonatal toxicity. The present observations, however, do not permit conclusions concerning the drug's mechanism of teratogenic action.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abele, D. C., and Dobson, R. L.: The treatment of mycosis fungoides with a new agent, cyclophosphamide (cytoxan). Arch. Derm., 82: 725-731, 1960.

Arnold, H.,and Bourseaux, F.: Synthese und abbau cytostatisch wirksamer cyclischer N-Phosphamidester des Bis (2-chloroethyl)-amines. Agnew. Chem., 70: 539-544, 1958.

Brock, N., and Hohorst, H. J.: Uber die aktivierung von cyclophosphamid *in vivo* and *in vitro*. Arzneim Forsch., 13: 1021-1301, 1963.

Brock, N., and Hohorst, H. J.: Metabolism of cyclophosphamide. Cancer, 20: 900-904, 1967.

Brubaker, C., Sonley, M., Hyman, C. B., Williams, K., and Hammond, D.: Cyclophosphamide therapy of acute leukemia in children. Clin. Res., 10: 107, 1962.

Burton, K.: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., 62: 315-322, 1956.

Ceriotti, J.: Determination of nucleic acids in animal tissues. J. Biol. Chem., 214: 59-70, 1955.

Cohen, J. L., and Jao, J. Y.: Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat. J. Pharmacol. Exp. Ther., 174: 206-210, 1970.

Dixon, R. L.: Effect of chloramphenicol on the metabolism and lethality of cyclophosphamide in rats. Proc. Soc. Exp. Biol. Med., 127: 1151-1155, 1968.

Drummond, K. N., Hillman, D. A., Marchessault, J. H. V., and Feldman, W.: Cyclophosphamide in the nephrotic syndrome of childhood: Its uses in two groups of patients defined by clinical, light microscope, and immunopathologic findings. Canad. Med. Ass. J., 98: 524-531, 1968.

Foley, G. E., Friedman, O. M., and Drolet, B. P.: Studies on the mechanism of action of cytoxan. Evidence of activation *in vivo* and *in vitro*. Canc. Res., 21: 57-63, 1961.

Fouts, J. R.: Some *in vitro* assay conditions that affect detection and quantitation of phenobarbital induced increases in hepatic microsomal drug-metabolizing enzyme activity. Toxicol. Appl. Pharmacol., 16: 48-65, 1970.

Fouts, J. R., and Adamson, R. H.: Drug metabolism in the newborn rabbit. Science, 129: 897-898, 1959.

Friedman, O. M., and Boger, E.: Colorimetric estimation of nitrogen mustard in aqueous media. Anal. Chem., 33: 906-910, 1961.

Gerlinger, P.: Action due cyclophosphamide injecte a la mere sur la realisation de la forme du corps des embryons de lapin. Compt. Rend. Soc. Biol., 158: 2154-2157, 1964.

Gerlinger, P., Ruch, J. V., and Clavert, J.: Action du cyclophosphamide sur la formation de l'embryon. Compt. Rend. Soc. Biol., 157: 173-176, 1963.

Gibson, J. E., and Becker, B. A.: The administration of drugs to one day old animals. Lab. Animal Care, 17: 524-527, 1967.

Gibson, J. E., and Becker, B. A.: The teratogenicity of cyclophosphamide in mice. Canc. Res., 28: 475-480, 1968a.

Gibson, J. E., and Becker, B. A.: Effect of phenobarbital and SKF 525-A on the teratogenicity of cyclophosphamide in mice. Teratology, 1: 393-398, 1968b.

Gibson, J. E., and Becker, B. A.: Effect of phenobarbital and SKF 525-A on placental transfer of cyclophosphamide in mice. J. Pharmacol., Exptl. Therap., 177: 256-262, 1971.

Gibson, J. E., and Becker, B. A.: Teratology of structural truncates of cyclophosphamide in mice. Teratology, in press (1971).

Gomori, G.: Histochemical demonstration of sites of phosphamidases activity. Proc. Soc. Exp. Biol. Med., 69: 407-409, 1948.

Haskin, D.: Some effects of nitrogen mustard on the development of external body form in the fetal rat. Anat. Rec., 102: 493-509, 1948.

Hayes, F. D., Short, R. D., and Gibson, J. E.: A correlation between cyclophosphamide induced leukopenia in mice and the presence of alkylating metabolites. In preparation.

Johnson, J. M., and Ruddon, R. W.: Interaction of nitrogen mustard with polyribonucleotides, ribosomes, and enzymes involved in protein synthesis in a cell-free system. Mol. Pharmacol., 3: 195-203, 1967.

Jondorf, W. R., Maickel, R. P., and Brodie, B. B.: Inability of newborn mice and guinea pigs to metabolize drugs. Biochem. Pharmacol., 1: 352-353, 1959.

Kreybig, T. von: Die teratogene Wirkung von cyclophosphamide wahrend der embryonales entwicklungsphase bei der ratte. Naunyn. Schmiedebergs Arch. Exp. Path. Pharmacol., 252: 173-195, 1965.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with folin phenol reagent. J. Biol. Chem., 193: 265-275, 1951.

Nordlinder, H.: Malformations after treatment of new-born mice with a single dose of cyclophosphamide. Experientia, 25: 1296, 1969.

Ruddon, R. W., and Johnson, J. M.: The effects of a single dose of nitrogen mustard on DNA template activity in purified DNA and RNA polymerase systems. Molec. Pharmacol., 4: 258-273, 1968.

Sladek, N. E.: Metabolism of cyclophosphamide by rat hepatic microsomes. Canc. Res., in press (1971).

Steel, R. G. D., and Torrie, J. H.: *Principles and Procedures of Statistics*, McGraw-Hill Book Co., Inc., New York, 1960.

Wheeler, G. P., and Alexander, J. A.: Effects of nitrogen mustard and cyclophosphamide upon the synthesis of DNA *in vivo* and in cell-free preparations. Canc. Res., 29: 98-109, 1969.