TOXICITY OF CYCLOPHOSPHAMIDE DURING DEVELOPMENT

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ABSTRACT

TOXICITY OF CYCLOPHOSPHAMIDE DURING DEVELOPMENT

By

Robert Downs Short Jr.

Mammalian development represents an integrated sequence of growth and differentiation. Agents which interfere with these processes produce abnormal development. The purpose of this study was to examine the molecular basis of cyclophosphamide induced disruption of embryonic and postnatal development in mice.

Cell division, an important aspect of normal growth, was reduced in embryos after the mothers received a teratogenic dose of cyclophosphamide (20 mg/kg i.p.) on day 11 of gestation. The population of dividing cells, which was estimated by measuring the incorporation of ^{14}C-thymidine into DNA, was reduced 12 hours after drug treatment. This treatment produced alkylation of embryonic macromolecules, as measured by the presence of radioactivity in a lipid-extracted acid precipitate, after mothers received ^{14}C-cyclophosphamide. Radioactivity in the acid precipitate declined in the order liver, placenta, and embryo and was present for at least 10 hours after treatment. ^{14}C-cyclophosphamide radioactivity disappeared from the nucleic acid fraction of embryonic tissue more slowly than from the nucleic acid fraction of maternal liver. The population of dividing cells was also reduced in the liver, brain, and carcass of neonatal mice after a dose of

cyclophosphamide (80 mg/kg s.c. at one day of age) which reduced the growth of these tissues. The incorporation of ^{14}C-thymidine into DNA was reduced 1 day after cyclophosphamide to 8, 26, and 25% of control in the liver, brain, and carcass, respectively, and this effect lasted for at least 3 days. The prolonged inhibition of DNA synthesis in neonatal relative to embryonic mice was attributed to a prolonged exposure of these animals to alkylating cyclophosphamide metabolites which were slowly formed from the poorly eliminated parent compound.

The transcription and translation of genetic information are important developmental processes. RNA synthesis, as measured by ^{14}C-uridine incorporation into RNA, was reduced in the liver and brain of mice treated at 1 day of age with 80 mg/kg s.c. cyclophosphamide. Liver RNA synthesis was reduced to 36 and 64% of control at 1 and 5 days after cyclophosphamide, respectively. Brain RNA synthesis was reduced to 78 and 64% of control at 1 and 4 days after treatment, respectively. Protein synthesis, as measured by ^{14}C-leucine incoporation into protein, was not affected in a manner which would indicate that drug treatment altered this process. The qualitative aspects of protein synthesis were studied by comparing the electrophoretic pattern of proteins synthesized *in vitro* by livers from control and cyclophosphamide treated neonates. Drug treatment did not affect the synthesis of specific liver proteins at 1 and 3 days after treatment. Drug treatment, in addition, did not affect the enzymatic activity of glucose-6-phosphatase, which was increased in neonatal liver shortly after birth, at 1 and 3 days after treatment. Drug toxicity, as estimated by these parameters, was not associated with quantitative or qualitative changes in protein synthesis.

The protein content of a tissue is dependent on both the synthesis and degradation of protein. Cyclophosphamide treatment of neonatal mice increased the turnover of liver, brain, and carcass proteins, as measured by the reduced half-life of proteins labeled *in utero* with ^{14}C-guanido-arginine. An increased degradation of radioactive proteins was attributed to the cytotoxicity of cyclophosphamide and catabolic processes associated with cell death.

Doses of cyclophosphamide which disrupted embryonic and postnatal development interfered with biochemical processes essential for normal growth and development. Abnormal development was attributed to a reduced population of proliferating cells, as measured by a reduced incorporation of ^{14}C-thymidine into DNA, and increased cell death, as measured by a reduction in both DNA concentration (mg DNA/g tissue) and DNA content (mg DNA/organ).

TOXICITY OF CYCLOPHOSPHAMIDE DURING DEVELOPMENT

By

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INTRODUCTION

Historical Considerations

Embryology, the branch of science dealing with animal development, studies the ontogenetic development of an individual rather than the phylogenetic development, or evolution, of a species. Embryological studies were conducted as early as 340 B.C. when Aristotle described development of the chick (reviewed by Balinsky, 1970). The first coherent presentation of embryological observation, however, was not made until 1828, when von Baer summarized existing data and reported on his original observations. A purely descriptive approach to embryology, however, failed to answer many of the basic questions concerning developmental processes involved in the transformation of a fertilized egg into an adult. Roux (1850-1924) introduced experimental methodology to embryology in order to resolve opposing theories of development. The principles and techniques of molecular biology have extended the scope of embryology to an understanding of basic processes associated with genomic control of early embryogenesis (reviewed by Davidson, 1968).

Teratology is the study of congenital malformations produced by disruption of normal developmental processes. Embryological principles were applied to the study of malformations in the 1800s as teratologists searched for the causes of anomalies (reviewed by Barrow, 1971). Various physical insults were applied to non-mammalian species in order to gain insight into the mechanisms of normal and abnormal development.

Mammalian experimental teratology did not begin until the late 1940s and 1950s when fetal development was demonstrated to be disrupted by vitamin deficiency, irradiation, hormones and nutritional deprivation. After the thalidomide episode animal screening programs were devised to meet the need of predicting the teratogenic potential of various agents in humans. The greatest disadvantage of extrapolating animal observations to humans, however, is the interspecific variability of responses. The predictive value of animal observations can be improved only by embryological and pharmacological investigations into the mechanisms by which development is disrupted (Tuchmann-Duplessis, 1972).

Phases of Development

The development of an adult organism from a single cell may be divided into six phases (Balinsky, 1970). The first phase of development, gametogenesis, involves the growth and maturation of egg and sperm. The gametes fuse during the second phase of development and the quiescent egg is activated to continue its developmental program. Cleavage, the third phase of development, encompasses a period of rapid cell division without a significant change in embryonic size or cellular differentiation. The embryo, at the end of cleavage, is referred to as a blastula and consists of a layer of cells, the blastoderm, surrounding a cavity, the blastocoele. Gastrulation is the fourth development phase which encompasses the formation of germinal layers endoderm, mesoderm and ectoderm from the blastoderm. Primary organ rudiments are derived from the germinal layers during organogenesis, the fifth phase of development. Whole organ systems are formed from primary organ rudiments which subdivide to produce secondary organ rudiments that give rise to subordinated and simpler organs. The sixth phase of

development is a period of growth and histological differentiation. The organ rudiments grow during this period and acquire the structure and biochemical properties characteristic of adult tissues. Organs grow by increasing both the number and size of cells and tissue specific characteristics are established as a result of differential gene expression.

Processes of Development

Processes involved in the development of an organism include cell division, differential gene expression, changes in the surface properties and shapes of cells, and cellular metabolism. Cell division underlies all the phenomena of multicellular development since cell differentiation usually begins as mitosis ends (Berril, 1971). Cell multiplication, in addition, is an important aspect of morphogenesis. Cell division is one phase of the cell cycle which may be divided into mitosis (M), a period prior to DNA synthesis (G1), a time of DNA synthesis (S), and a period between DNA synthesis and mitosis (G2). DNA synthesis in eukaryotic cells is dependent on chemical substances located in the cytoplasm, protein synthesis, and structural properties of the chromosome (Mitchison, 1971).

The differentiated state is a function of differential gene expression. The production of unique cellular phenotypes from a common genotype occurs either by genetic control mechanisms at the transcription and translation level or epigenetic control mechanisms such as protein modification (Market and Ursprung, 1971). The differentiated state, however, ultimately depends on the transcription of genetic information which determines protein structure and the resulting cellular characteristics (Davidson, 1968).

Changes in the surface properties and shape of cells are an important aspect of differentiation and morphogenesis. The adhesive properties of cell membranes probably determine whether cells remain in a tissue or move about in the body (Markert and Ursprung, 1971). As development progresses cells establish connections which maintain tissue integrity and permit intracellular communication. Changes in cell shapes are important in morphogenic cell movements during gastrulation (reviewed by Wessels *et al.*, 1971).

Development is a dynamic rather than a static process. Cellular metabolism during development, therefore, must provide not only for maintaining the tissue but also must prepare the tissue to conduct adult physiological functions. Cellular metabolism may be subdivided into catabolic and anabolic processes (Lehninger, 1970). Catabolism is the enzymatic degradation of relatively large nutrient molecules, which are derived either from the environment or from the cells' own nutrient storage depots into smaller simpler molecules. Anabolism is the enzymatic synthesis of relatively large molecular components of the cell from simpler precursors.

Principles of Teratology

Congenital malformations have been described in many mammals. There are embryological and non-embryological factors which determine an organism's susceptibility to an agent (Wilson, 1965). Pharmacologic factors include the agent, dose, and maternal physiology. A teratogen may produce specific effects related to metabolic events occurring during animal development. All doses of a teratogen, however, do not disrupt development. Low doses generally permit normal development while higher doses kill all of the embryos and even the mother if

extended high enough. There is a teratogenic zone in which the dose is sufficient to interfere with specific developmental events without destroying the whole embryo. The mother is important not only in maintaining the embryo but also in determining the maternal-embryonic exchange of teratogens. Embryonic factors which influence the action of teratogens are genetic composition and gestational age at treatment. The genotype determines the inherent susceptibility of an embryo to agents at a given time in development. Susceptibility to teratogens varies during the course of gestation. There is generally no teratogenic response to agents administered during cleavage and blastula stages in mammalian embryos. The beginning of teratogenic susceptibility occurs about the time of germ layer formation. Most organs have a period of particular susceptibility to teratogens which probably coincides with the early and critical developmental events for that organ. Agents which disrupt development may initially act at any of the processes essential for normal development, e.g., cell division, differential gene expression, changes in the surface properties and shapes of cells, and general cellular metabolism.

Examples of Agents that Disrupt Development

Inhibitors of Cell Division. Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine), a structural analog of cytidine, inhibited cellular proliferation and DNA synthesis in a variety of systems (reviewed by Chaube and Murphy, 1968). Cytosine arabinoside reduced DNA synthesis either by inhibition of cytidine diphosphate reduction to deoxycytidine diphosphate by ribonucleotide diphosphate reductase (Chu and Fisher, 1962) or by inhibition of DNA polymerization as a result of the

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formation of cytosine arabinoside triphosphate (Furth and Cohen, 1968; Inagak *et al.*, 1969).

Cytosine arabinoside produced anomalies which were dependent on the gestational age (reviewed by Chaube and Murphy, 1968). Teratogenic effects were observed when 20 to 80 mg/kg cytosine arabinoside was administered on gestational days 10 to 12 but not when rats were treated with 5 to 500 mg/kg on days 5 to 9 of gestation. Ritter *et al.* (1971) established a correlation between the degree and duration of inhibited DNA synthesis and severity of malformations in rats. DNA synthesis was reduced in a dose related fashion to less than 25% of control values at 1 hour after treatment with 25, 50, 100 or 200 mg/kg cytosine arabinoside on day 12 of gestation. Recovery to control values occurred by 15 hours after lower doses but remained depressed for at least 27 hours after treatment with 200 mg/kg. An apparent cause and effect relationship between inhibited DNA synthesis and abnormal development was not established since a 25 mg/kg dose of cytosine arabinoside, which produced a cumulative 78% reduction in DNA synthesis over 24 hours, failed to result in anomalies. These results suggested that embryos either had a capacity to repair drug induced damage or that malformations were due to factors other than inhibition of DNA synthesis.

The teratogenicity of hydroxyurea was investigated in order to clarify the role of reduced DNA synthesis in abnormal development (Scott *et al.*, 1971). Hydroxyurea inhibited DNA synthesis in mouse embryo cells and reduced the formation of deoxyribonucleotides by ribonucleotide-diphosphate reductase (Skoog and Nordenskjöld, 1971). Hydroxyurea produced a dose related incidence of malformations in pregnant rats treated with 500, 750 or 1000 mg/kg on day 12 of gestation. Reduced DNA synthesis, which was nearly complete at 5 hours and

lasted for 23 to 29 hours, was correlated with the teratogenic response. A 250 mg/kg dose of hydroxyurea produced a degree of DNA inhibition which did not result in malformations. The effects on DNA synthesis were similar but not identical to those produced by cytosine arabinoside (Ritter *et al.*, 1971). The majority of defects were similar for both agents, e.g., hydrocephalus, micrognathia, ectrodactyly, short kinky tail, cleft, hydroureter, hydronephrosis, fused or wavy ribs, anal atresia, diaphragmatic hernia, and hypoplastic lungs. Hydroxyurea, however, produced defects not observed after cytosine arabinoside, e.g., cardiac and aortic defects, exophthalmia, and cranial dysplasia.

Cytosine arabinoside and hydroxyurea both induced cellular damage in rat embryos (Scott *et al.*, 1971). Cell death, however, was a normal developmental process which influenced both tissue differentiation and organ shaping (reviewed by Menkes *et al.*, 1970). Massive cellular death in the appendages, for example, was involved in the normal formation of the knee joint, elbow, muscles, bones of the hand and foot, digits and general shape of the embryonic limb. Limb malformations, after hydroxyurea, were attributed to extensive death of cells in the forelimb buds (Scott *et al.*, 1971).

Normal cell death differs from drug induced cell death because the former is a regulated specific process while the latter is more generalized. The production of specific types of anomalies by drug treatment, therefore, requires some degree of selectivity in drug action. Agents which inhibit DNA synthesis, for example, may have selective toxicity in rapidly dividing cells. Qualitative aspects of DNA synthesis, in addition, may confer a degree of selectivity to drug action since hydroxyurea had more cytotoxicity in continuously dividing cells than in cells induced to divide (Farber and Baserga, 1969). Rapidly

proliferating tissues, therefore, may show the largest amount of drug-induced cellular death which may result in malformations depending on the regenerative capacity of the tissue.

Cytosine arabinoside and hydroxyurea have been proposed to disrupt normal development by reducing the proliferating cell populations of specific tissues (Scott *et al.*, 1971). These tissues at later stages of development may, therefore, have an insufficient number or type of cells to conduct the genetically prescribed morphogenetic role of the affected tissue or primordium. The proposal, however, fails to provide an explanation as to how cell death and reduced rates of proliferation lead to malformations.

Inhibitors of Gene Expression. Actinomycin D, an antibiotic which reduced the transcription of RNA from DNA (Wheeler and Bennet, 1962), was teratogenic in a variety of species (reviewed by Chaube and Murphy, 1968). Developmental defects were produced in the echinoderm, acetabularia, chick, frog and rat. Actinomycin D was teratogenic in rats during the early period of organogenesis. Rats treated with actinomycin D from day 2 to 6 of gestation had an increased incidence of resorptions but no fetal anomalies. Anomalies were produced, however, in rats treated on gestational days 7 to 9 but not 10 or 11 with acinomycin D in doses of 50 to 75 mg/kg and 0.25 to 5.0 mg/kg, respectively (Tuchmann-Duplessis, 1969; Chaube and Murphy, 1968).

Sea urchin development continued normally in the presence of actinomycin D to the blastula stage when a drug induced inhibition of synthesis of specific proteins was detected (Terman, 1970). Normal development continued to the blastula stage as a result of sustained protein synthesis which was directed by maternally formed long-lived

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messenger RNA. Cleavage and early blastular morphogenesis during mammalian development, however, were dependent on the early activity of the embryonic genome (Mintz, 1964). Mammalian development, in contrast to sea urchin development, was disrupted at earlier stages by actinomycin D.

Autoradiographic studies in rats demonstrated that ^{3}H-actinomycin D, administered on gestational days 7 through 11, was bound to embryonic DNA and inhibited RNA synthesis (Jordan and Wilson, 1970). The inhibition of RNA synthesis was postulated to disrupt closely timed developmental events which occurred during the period of germ layer differentiation and early organogenesis. Since the teratogenic effects of acinomycin D occurred relatively early during organogenesis drug treatment may affect the synthesis of messenger RNA molecules which directed protein synthesis at later stages of development. The actual events associated with the disruption of both cell function and normal development, however, are unknown.

Glucocorticoids have been postulated to produce cleft palate in mice by an action at the transcription level which interfered with gene expression (Zimmerman *et al.*, 1970). The frequency of cortisone induced cleft palate was dependent on the genotype, dose, and gestational age (Kalter, 1954). These teratogenic properties of cortisone may be explained, in part, by strain differences in drug metabolism and time of palate formation.

Triamcinolone acetonide, administered at a dose of 5 mg/kg on day 11.5 of gestation, produced 100% incidence of cleft palate in A/J, 40% in C3H, and 0% in CBA mice (Zimmerman and Bowen, 1972a). The increased resistance of CBA over A/J and C3H mice to drug induced cleft palate was attributed to a greater maternal metabolism of triamcinolone

by this strain. The CBA embryos, as a result, were exposed to lower levels of the parent compound which was shown to be the teratogenic form of triamcinolone (Zimmerman and Bowen, 1972a, 1972b). The greater resistance of C3H over A/J mice to drug-induced cleft palate formation, however, was probably related to a developmental rather than metabolic factor. The sensitivity of A/J mice, in contrast to C3H mice, may be due to a more delayed palate formation which has been correlated with increased teratogenic susceptibility (Zimmerman and Bowen, 1972a; Trasler, 1965).

Palate formation in mice involved both movement of the shelves of the secondary palate from a vertical position beside the tongue to a horizontal position above the tongue and fusion at the midline (Walker and Fraser, 1956). Glucocorticoids, which delayed shelf movement, produced cleft palate by a desynchronization of developmental events (Walker and Fraser, 1957). If shelf movement was delayed sufficiently then head growth would have carried the shelves too far apart to meet and fuse at the midline.

Palatal shelves changed their orientation between day 14.5 and 15.5 of gestation in mice (Walker and Fraser, 1956). There was, therefore, about a three day latent period between the time of optimal drug sensitivity and palate closure. The delay between drug administration and effect led to the proposal that glucocorticoid-induced cleft palate was produced by a reduced synthesis of messenger RNA molecules which directed the synthesis of proteins required for palate closure (Zimmerman *et al.*, 1970). Triamcinolone, in support of this theory, inhibited total embryonic RNA synthesis 6 to 24 hours after treatment. The coadministration of cycloheximide, in addition, partially reversed the tendency toward cleft palate formation. These observations suggested

that triamcinolone disrupted the normal pattern of RNA and protein synthesis which subsequently delayed the movement of palatal shelves from a vertical to a horizontal position.

Glucocorticoids, in addition to disrupting development during the phase of organogenesis, also affected the subsequent postnatal phase of growth and histological differentiation. Rats that received 1 mg of cortisol at 2 days of age grew at a reduced rate, were more nervous and developed a blotchy skin with areas of hyperpigmentation and sparse hair (Schapiro, 1965). Corticosterone administered to mice at doses of 0.08 to 0.6 mg/day between 2 and 7 days of age produced a significant reduction in liver, brain, and body weights at the end of treatment (Howard, 1965). Corticosterone treatment, in addition, reduced the accumulation of total liver and brain DNA (Howard, 1965, 1968). These effects were evident by 7 days and persisted for at least 8 months. A pellet of corticosterone, 1.3 mg/g body weight, implanted in 2-day-old rats, produced a reduced body weight with no change in pituitary and liver weight relative to body weight or liver DNA and RNA/DNA ratios (Taylor and Howard, 1971). Treated animals, in addition, had a short snout and a 4 to 5% reduction in nose to anus length. Cortisol treatment at doses of 0.2 mg/day for the first 4 days after birth reduced the incorporation of ^{14}C-thymidine into brain DNA but did not change DNA concentration or RNA/DNA and protein/DNA ratios (Cotterrell, 1972). The corticosterone induced biochemical lesions were associated with impaired brain function and behavior (Howard and Granoff, 1968a). Treated mice, for example, had a permanent impairment of fine adjustment mechanisms of motor control.

The effect of cortisol on the nervous system was age dependent (Vernadakis and Woodbury, 1971). Acute or chronic drug treatment in

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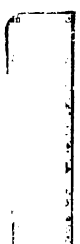
rats between 1 and 7 days after birth produced a delayed maturation of the nervous system and reduced brain excitability. These parameters, on the other hand, were increased when cortisol was administered after the 8th postnatal day. These effects were accompanied by changes in oxygen consumption and intracellular to extracellular potassium concentrations. Rats treated with glucocorticoids at 2 days of age had impaired glucose homeostasis and suppressed circadian periodicity of plasma corticosteroids at maturity (Taylor and Howard, 1971; Krieger, 1972). Treatment at 12 to 14 days of age, on the other hand, produced no effect on circadian variations of corticosteroids. These observations indicated that postnatal development in the brain progressed through various developmental stages which differed in susceptibility to drug treatment.

Agents that Affect Cell Surfaces and Shapes. Changes in the properties of cell surfaces represent an important aspect of embryonic differentiation and morphogenesis (Market and Ursprung, 1971). Agents which affect cell surfaces have the potential to disrupt development, although there is little teratological data available for this class of compounds. Lectins are protein molecules that specifically bind to various carbohydrates on the cell surface and cause agglutination (reviewed by Sharon and Lis, 1972). Concanavalin A is a powerful non-specific lectin which forms a precipitate with numerous polysaccharides. The concanavalin A binding properties of embryonic and adult cells have been shown to be different (Moscona, 1971). Dissociated embryonic cells were readily agglutinated by concanavalin A while adult cells required an unmasking of receptor sites by trypsin digestion before agglutination occurred. The presence of exposed concanavalin A binding sites on embryonic cell surfaces suggested that these sites functioned



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in morphogenetic cell contacts, cell mobility, and tissue organization during embryonic development. Agents which block these receptors during normal embryonic development may contribute to the production of anomalies.

Microfilaments are contractile elements of cells which influence cell shape (reviewed by Wessells *et al.*, 1971). These structures have been shown to play important roles in morphogenic cell movements during tubular gland formation in the oviduct, formation of salivary epithelium and invagination during gastrulation. Cytochalasin B disrupted developmental events which depended on the integrity of the microfilament system. Agents which affect the function of the microfilaments, therefore, may have teratogenic effects.

Agents that Affect Cellular Metabolism. The integrity of cellular metabolism is important not only in maintaining the viability of cells but also in permitting phenotypic expression of cellular characteristics. Agents which disrupt metabolic functions, therefore, possess a teratogenic potential. A glucose antimetabolite, 2-deoxy-D-glucose (2-DG), disrupted chick development as evidenced by a reduced hatchability without an increased incidence of anomalies (Landauer and Clark, 1962). Since glucose treatment reversed these effects it was proposed that 2-DG interfered with the utilization of glucose. Rat embryos, in support of this proposal, treated with 2-DG had a reduced incorporation of radioactivity from ^{14}C-U-glucose into both protein and nucleic acids (Tanimura and Shepard, 1970).

An adequate diet is an essential requirement for normal development. Diets deficient in vitamins A, riboflavin, B_{12}, pantothenic acid, pteroylglutamic acid, or E produced an increased incidence of anomalies in rats (reviewed by Johnson, 1965). The critical period of sensitivity

to the teratogenic diet, as revealed by antimetabolite induced vitamin deficiency, was the period of differentiation and organogenesis.

Malnutrition, induced by food deprivation, contributed to an increased incidence of congenital defects. Mice deprived of food for 24 or 30 hours on days 7 to 10 of gestation had vertebral, rib and cranial defects (Runner and Miller, 1956). The highest incidence of anomalies was produced by fasts on day 9 of gestation. Forty-hour fasts, in addition, were usually incompatible with pregnancy.

Nutritional deprivation of neonatal animals affected subsequent growth and differentiation. Mice separated from their mothers for 16 out of 24 hours from 3 to 7 days of age had reduced body, brain, and liver weights at 7 days of age (Howard, 1965). Brain growth, relative to liver and body growth, was spared during this period since the brain to body weight ratio increased while the liver to body weight ratio was not affected. The concept of a brain sparing effect during malnutrition was observed by others (reviewed by Dobbin, 1970). Reduced cerebral and cerebellar weights and total brain DNA was observed at 9 months of age in mice isolated overnight from their mothers between 2 and 16 days of age (Howard and Granoff, 1968b). There was no impairment in voluntary running, maze learning, or visual discrimination in isolated mice despite impaired brain growth. Malnutrition was reported in other studies to delay behavioral development and affect learning ability (reviewed by Dobbing, 1970).

The magnitude of nutritional deprivation and temporal aspects of its administration determined both the extent of damage and recoverability of tissue following the initiation of an adequate diet (Winick and Noble, 1966). Organ damage and recoverability, after malnutrition, were dependent on the growth phase of organs at the time of malnutrition.

Developmental growth may be divided into three phases which occurred in characteristic patterns for each organ (Winick and Noble, 1965). During the first phase, a period of rapid DNA synthesis, cell number increased while cell size remained constant (hyperplasia). As the rate of cell division slowed down, during the second phase, the size of individual cells increased and organs grew by increased cell number and cell size (hyperplasia and hypertrophy). During the third phase organs grew as a result of the enlargement of individual cells (hypertrophy). Organs from undernourished rats had a reduced weight, protein, RNA, and DNA content, which was not restored by refeeding, when food was restricted during the period of rapid DNA synthesis (Winick and Noble, 1966). The effect of malnutrition on brain development, for example, was reversed by feeding when food deprivation occurred both at 21 to 41 and 64 to 86 days of age but not at 0 to 21 days of age when the organs grew by hyperplasia.

The developmental increase in protein synthesis, which occurred during hypertrophy, may be regulated, in part, both by hormones and by changes in the intracellular amino acid pools. Enzymic differentiation referred to the processes involved in prenatal and early postnatal development whereby the organs of an animal acquired their characteristic quantitative pattern of enzymes (reviewed by Greengard, 1969). The accumulation of various liver enzymes (e.g., glucose-6-phosphatase) immediately after birth may be initiated by glucagon and epinephrine which may act through the formation of cyclic AMP. Changes in the intracellular amino acid pool were proposed to regulate the developmental increase in protein synthesis (Miller, 1970). An increase in the amino acid pool, for example, may increase polysomal stability and the binding of ribosomes to the endoplasmic reticulum. Since growth

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retardation produced by malnutrition was correlated with a protein, rather than caloric deficiency, a reduced amino acid pool may have contributed to impaired organ growth.

Cyclophosphamide

Background. Cyclphosphamide (N,N-bis-(2-chloroethyl)-N',O-propylene phosphoric acid ester diamine monohydrate, Cytoxan^{R}, Endoxan^{R}, NSC 26271) is a clinically useful antineoplastic agent of the alkylating type with potent toxic properties in developing systems (see below). The molecule, which consists of nor-nitrogen mustard to which a cyclic phosphoramide ring structure has been attached, was designed by Arnold and Bourseaux (1958) to require metabolic activation for the production of alkylating metabolites. The parent compound was proposed to be activated primarily within tumors by the hydrolytic cleavage of the P-N bond between nitrogen mustard and the phosphamide ring by phosphatases and phosphamidases.

Metabolism. The requirement of bioactivation for the production of cytotoxicity was demonstrated by Foley *et al.* (1961). Cell growth was inhibited when tissue cultures were treated with sera from rats administered cyclophosphamide but not by cyclophosphamide added directly to the sera. Homogenates of livers but not neoplastic tissue from cyclophosphamide treated rats produced cytotoxicity in tissue culture. Alkylating cyclophosphamide metabolites were demonstrated in serum, urine, and bile of animals and humans by means of the nitrobenzyl pyridine (NBP) colorimetric assay (Brock and Hohorst, 1963). The maximum NBP activity in rat serum was reached 15 minutes after cyclophosphamide (1 g/kg) and dropped to 50% of maximum after 4 hours, and NBP activity was not detectable 24 hours after treatment.

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Cyclophosphamide activation occurred primarily in the liver and, to a lesser extent, in the adrenal cortex and lung (Brock and Hohorst, 1963). Liver fractionation studies indicated that the cyclophosphamide activating system was located primarily in the microsomal fraction and required NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) and molecular oxygen. The *in vivo* conversion of cyclophosphamide to NBP-alkylating metabolites, in addition, was decreased by SKF 525-A (beta- diethylaminoethyl diphenylpropylacetate) and increased by phenobarbital pretreatment (Rauen and Kramer, 1964). These studies suggested that the metabolic activation of cyclophosphamide occurred primarily in the same mixed function oxidase system of liver microsomes described by Conney (1967) for the metabolism of many drugs and steroids.

These conclusions concerning cyclophosphamide activation have been confirmed and extended. Activation occurred primarily, if not exclusively, in rat liver microsomes and required NADPH and molecular oxygen (Cohen and Jao, 1970). This process was competitively inhibited *in vitro* by compounds such as hexobarbital and steroids which were metabolized by the mixed function oxidase system. Cyclophosphamide metabolism, in addition, was inhibited by carbon monoxide, which bound to the microsomal P-450 hemoprotein as well as p-hydroxymercuribenzoate and cytochrome C which disrupted the flow of charge along the microsomal electron transport chain. Sladek (1971), in addition, demonstrated that cyclophosphamide combined with the microsomal P-450 hemoprotein to produce a Type I spectral interaction (λ max. in the range of 385-390 mμ and a λ min. in the range 418-247 mμ).

Arnold and Bourseaux (1958) originally proposed that cyclophosphamide was hydrolyzed by tumor phosphatases and phosphamidases to produce nor-nitrogen mustard. Studies have reported that nor-nitrogen

mustard was a cyclophosphamide metabolite present in rat urine (Rauen and Dirschka, 1964) and generated *in vitro* by a drug metabolizing system (Rauen and Norpoth, 1965). Hohorst *et al.* (1966), on the other hand, was unable to detect nor-nitrogen mustard as a metabolite of cyclophosphamide. Friedman (1967) confirmed that free nor-nitrogen mustard was not detectable in rat tissue at various times after drug treatment.

Hill *et al.* (1972) proposed a pathway for the metabolic activation of cyclophosphamide in mouse liver. The carbon adjacent to the ring nitrogen was oxidized by the cytochrome P-450 linked enzyme to form 4-hydroxycyclophosphamide which was subsequently oxidized to products containing carbonyl groups. Aldophosphamide, generated by the microsomal system, inhibited clone formation of human epidermoid carcinoma No. 2 cells and produced toxicity in L1210 cells. Carboxyphosphamide was produced from aldophosphamide by a soluble enzyme which had the properties of aldehyde oxidase. This metabolite produced little toxicity in either human epidermoid carcinoma No. 2 cells or L1210 cells. The spontaneous cyclization of carboxyphosphamide produced 4-ketocyclophosphamide which had weak cytotoxic effects against human epidermoid carcinoma No. 2 cells and L1210 cells (Struck *et al.*, 1971).

Struck *et al.* (1971) used infrared spectroscopy, nuclear magnetic resonance and mass spectroscopy to identify carboxyphosphamide and 4-ketocyclophosphamide in urine from dogs. They estimated that 40 to 50% and 25 to 30% of the total 24 hour urine radioactivity in dogs and humans, respectively, after ^{14}C-cyclophosphamide was due to carboxyphosphamide. Unmetabolized cyclophosphamide in urine from dogs and humans, on the other hand, was 7% and 16%, respectively, of the administered dose and approximately 11% and 27% of the urinary radioactivity.

Bakke *et al.* (1972) identified eight cyclophsophamide metabolites in the urine of sheep after a single oral dose of radioactive cyclophosphamide. Cyclophosphamide was labeled either with ^{14}C at the 4 position in the ring or with ^{3}H in the side chain on the carbons having chlorine substitution. Metabolites represented approximately 95% of the urinary radioactivity from ring labeled cyclophosphamide and 88% of the radioactivity from side chain labeled cyclophosphamide. There was, in addition, no evidence that nor-nitrogen mustard was a urinary metabolite of cyclophosphamide. These observations suggested that the ring portion of the molecule was not cleaved from the alkylating portion during metabolic activation in sheep.

Pharmacological Considerations. The antineoplastic activity of cyclophosphamide was evident in various animal tumor systems. Drug therapy in rodents was beneficial in the treatment of leukemia (Lane, 1959; Burchenal *et al.*, 1960), lymphosarcoma (Lane, 1960), mammary carcinoma (Lane, 1960), and sarcomas (Burchenal *et al.*, 1960). Since cyclophosphamide resistant L1210 leukemia cells were also resistant to other alkylating agents but not antimetabolites, it was concluded that cytotoxicity was produced by an alkylating mechanism (Lane, 1967). The antitumor activity of cyclophosphamide, however, was not altered either by SKF 525-A pretreatment of mice bearing L1210 leukemia cells (Hart and Adamson, 1969) or by phenobarbital pretreatment of rats with Walker 256 carcinoma cells (Sladek, 1972). Phenobarbital and SKF 525-A pretreatment, in addition, did not produce any therapeutic advantage over cyclophosphamide alone in the treatment of mice bearing L1210 leukemia cells or Lewis lung carcinoma (Field *et al.*, 1972). The failure of drug pretreatments to provide a correlation between cytotoxicity and alkylating metabolites may be due to their failure to significantly

alter the total exposure of cancerous cells to alkylating metabolites. Phenobarbital pretreatment, for example, produced only a slight change in the total recovery of a cyclophosphamide dose as urinary metabolites (Jao *et al.*, 1972).

Cyclophosphamide has found wide clinical use in the treatment of a wide variety of malignant and non-malignant diseases in humans. Drug therapy was effective in the treatment of acute childhood leukemia resistant to antimetabolite therapy (Brubaker *et al.*, 1962; Fernbach *et al.*, 1962), chronic lymphatic leukemia (Hall, 1963), recurrent and metastatic ovarian cancer (Decker *et al.*, 1967; Beck and Boyes, 1968) and advanced lung cancer (Bergsagel *et al.*, 1968). Cyclophosphamide, in addition, has been used as an immunosuppressive agent in lipoid nephrosis (West *et al.*, 1966), nephrotic syndrome in children (Drummond *et al.*, 1968), and whole organ transplantation (Starzl *et al.*, 1971). Cyclophosphamide is administered in doses of 2 to 8 mg/kg orally or intravenously for 6 days followed by maintenance doses of 50 to 300 mg daily or 5 mg/kg intravenously twice weekly. High dose cyclophosphamide therapy (1 to 6 doses of 60 and 120 mg/kg intravenously) with marrow infusion has been successfully employed in the treatment of a variety of malignant diseases (Buckner *et al.*, 1972).

Cyclophosphamide did not have selective toxicity for tumor tissues. Rats had a 30 day oral LD_{50} of 170 mg/kg for cyclophosphamide (Dixon, 1968). Mice, on the other hand, had a 28 day subcutaneous LD_{50} of about 400 mg/kg (Hart and Adamson, 1969). Agents which inhibited metabolic activation reduced cyclophosphamide lethality in both mice (Hart and Adamson, 1969) and rats (Dixon, 1968). Increased metabolic activation, on the other hand, was associated with a hastened onset of lethality but no change in the LD_{50} (Hart and Adamson, 1969).

Cyclophosphamide produced a progressive wasting in rats treated with single subcutaneous doses of 65, 85 or 110 mg/kg (Stenram and Nordlinder, 1968). The animals died primarily between 45 and 91 days after treatment presumably as a result of toxic effects on the hematopoietic system. Drug treatment produced bone marrow depression (Lane, 1959) with minimal effects on platelets (Coggins *et al.*, 1960; Eisman *et al.*, 1961). Cyclophosphamide induced leukopenia was correlated with the presence of alkylating metabolites in mice pretreated with agents to increase or decrease metabolic activation (Hayes *et al.*, 1972). Cyclophosphamide, in addition, produced hemorrhagic cystitis (Kaufman, 1963; George, 1963; Specter *et al.*, 1965), alopecia (Coggins *et al.*, 1960; Eisman *et al.*, 1961), and urinary bladder epithelial necrosis which was more pronounced in male rats (Koss and Lavin, 1970).

Toxicity of Cyclophosphamide During Development

Gametogenesis. Cyclophosphamide disrupted various phases of normal ontogenetic development. Humans who received 50 to 100 mg/day of cyclophosphamide had low sperm counts in their seminal fluid (Fairley *et al.*, 1972). The sperm count fell to zero in some patients after 4 months of treatment and all patients who received 6 months of therapy showed azoospermia. The germinal epithelia of mice treated with 97 mg/kg of cyclophosphamide showed a shift in metaphase ratios and an increased incidence of chromosomal aberrations (Rathenberg and Müller, 1972). These observations suggested that cyclophosphamide was potentially mutagenic. The mutagenicity of cyclophosphamide was confirmed by the dominant lethal test in mice (Epstein *et al.*, 1972).

Prenatal Development. Alkylating agents disrupted normal embryonic development in a variety of species. Nitrogen mustard, for example, produced anomalies in amphibians (Gillette and Bodenstein, 1946), mice (Danforth and Center, 1954; Nishimura and Takagaki, 1959), and rats (Haskin, 1948). Fetuses from rats treated with nitrogen mustard at doses of 0.5 or 1.0 mg/kg on days 12, 13, 14 or 15 of gestation were reduced in size and weight and had cranial faults, underdeveloped jaws, cleft palates, fusion of digits on front and hind limbs, abnormal posture of hind limbs, and shortness of the tail. Murphy and Karnofsky (1956) confirmed these results and showed that increased embryonic and fetal lethality was produced by treatment earlier in gestation. Five polyfunctional alkylating agents and X-rays were found to produce similar teratogenic effects in rats (Murphy, 1959).

Cyclophosphamide was teratogenic in a variety of mammalian species. Rabbits treated subcutaneously with 50 mg of cyclophosphamide on days 10 through 13 of gestation produced fetuses with reduced size and increased incidences of resorptions, cleft palates, and hypognathus (Gerlinger, 1964; Gerlinger and Clavert, 1965a). Higher doses of cyclophosphamide produced complete fetal resorption and lethality in one dam. A 50 mg/rabbit dose of cyclophosphamide on day 10 or 13 of gestation produced abnormal gonadal differentiation which was more pronounced in females (Gerlinger and Clavert, 1956b). Cyclophosphamide embryotoxicity was evident in early rabbit embryonic development while teratogenicity was confined to the later period of organogenesis (Fritz and Hess, 1971).

Cyclophosphamide teratogenicity was studied in the rat by Brock and Kreybig (1964) and extended by Kreybig (1965). Pregnant rats treated intraperitoneally with single doses of 20 to 40 mg/kg

cyclophosphamide on days 10 through 15 of gestation produced fetuses with exencephaly, microcephaly, syndactyly, polydactyly, and missing ribs. The critical period for teratogenicity was days 12 and 13 while resorptions were observed on days 11 through 14 of gestation. Genetic differences in the teratogenic response to cyclophosphamide were not evident in the anomalies produced in six strains of rats (Kreybig, 1965). The *in utero* cytological and histological effects of cyclophosphamide were studied in rats treated on day 13 (Kreybig and Schmidt, 1966) and days 15 and 16 (Kreybig and Schmidt, 1967) of gestation. General cellular damage such as karyorrhexis, karyopyknosis, hyperchromatosis, and abnormal immigration of embryonic cells were evident 24 hours after treatment. Necrotic substances diminished and repair occurred during the subsequent 24 hour period.

Mice, in addition, were susceptible to the teratogenic effects of cyclophosphamide. Embryonic development of DM/MK mice was disrupted by cyclophosphamide, but this strain appeared to be more resistant than other previously studied species (Shoji and Ohzu, 1965). Swiss Webster mice, on the other hand, were more susceptible to cyclophosphamide teratogenicity (Gibson and Becker, 1968a). Cyclophosphamide administered intraperitoneally at single dose of 5 and 10 mg/kg on days 10 through 15 of gestation produced decreased growth and increased resorptions. Single dose of 20 mg/kg administered on the same schedule produced a variety of teratogenic effects in addition to a reduction in growth and an increase in lethality. The anomalies produced included gross defects (cleft palate, exencephaly, digital defects and kinky tail), skeletal anomalies (polydactyly, syndactyly, ectrodactyly, adactyly, fusion of the long bones, curvature of the long bones, and missing ribs), and soft tissue malformations (open eyes, aphakia, microphakia, hydronephrosis,

and hydrocephalus). The maximum teratogenic response was produced by treating mice intraperitoneally with 20 mg/kg cyclophosphamide on day 11 of gestation.

The role of metabolic activation in the production of cyclophosphamide teratogenicity was investigated in Swiss-Webster mice pretreated with either SKF 525-A or phenobarbital (Gibson and Becker, 1968b). SKF 525-A pretreatment significantly increased fetal weight loss and the incidence of resorptions, open eyes, polydactyly, aphakia, hydronephrosis, absence or non-ossification of ribs, bone fusion, and bone curvature. Phenobarbital pretreatment, on the other hand, significantly reversed the cyclophosphamide induced weight loss as well as the incidence of limb defects, hydrocephalus, exencephaly, cleft palate, aphakia, and hydronephrosis. As a result of these studies it was proposed (Gibson and Becker, 1968b) that cyclophosphamide teratogenicity was associated with the parent compound rather than with alkylating metabolites.

The proposal that the cyclophosphamide parent compound had toxic properties during development received support from additional studies. The placental transport of ring labeled ^{14}C-cyclophosphamide, under conditions of altered metabolic activation, showed that both teratogenicity and embryonic levels of radioactivity were decreased by phenobarbital and increased by SKF 525-A pretreatment (Gibson and Becker, 1971a). These observations indicated that there was a placental barrier to the transport of cyclophosphamide metabolites and suggested that teratogenicity was associated with the parent compound. Embryos and placentas, in addition, had a poorly developed *in vitro* ability to metabolically activate the parent compound (Short and Gibson, 1971a). Gibson and Becker (1971b) studied the teratogenicity of structural

analogs of cyclophosphamide which were proposed as metabolites or degradation products (Foley *et al.*, 1961). Minimal teratogenic doses, which also produced signs of maternal toxicity, failed to parallel the response to a teratogenic dose of cyclophosphamide. The above observations supported the proposal (Gibson and Becker, 1968b) that the parent compound had unique toxic properties during embryonic development.

Recent evidence, however, has implicated cyclophosphamide metabolites and the alkylation of DNA in the disruption of normal embryonic development (Murthy *et al.*, 1971). The binding of ^{3}H-cyclophosphamide to embryonic DNA, but not RNA or protein, was correlated with teratogenicity by measuring drug binding to macromolecules after various doses of cyclophosphamide and modification of its metabolic activation. Jusko (1972), as a result of an analysis of the dose embryolethality data of Gibson and Becker (1968a), classified cyclophosphamide as a Type 1 teratogen. Type 1 teratogens had no minimal embryopathic dose while Type 2 teratogens had minimal embryopathic doses. Since nitrogen mustard and cyclophosphamide were Type 1 teratogens this may indicate that embryos lack a sufficient capability to repair alkylated DNA.

Postnatal Development. Cyclophosphamide disrupted development during the postnatal phase of growth and differentiation. Mice treated with cyclophosphamide 24 to 48 hours after birth had delayed development of hair, short snouts, ears and tails, and reduced body weights at maturity (Nordlinder, 1969). Cyclophosphamide neonatal toxicity, in addition, was dose related and not duplicated by equimolar doses of nor-nitrogen mustard, an agent with inherent alkylating activity (Short and Gibson, 1971b). Since alkylating activity per se did not disrupt development and cyclophosphamide neonatal toxicity occurred at a time

when mice had a poorly developed activating ability (Short and Gibson, 1971a), it was proposed that the parent compound may have toxic properties during postnatal as well as embryonic development. Recent evidence, however, indicated that neonatal toxicity was produced by an alkylating mechanism (Bus *et al.*, 1973). Phenobarbital pretreatment increased and SKF 525-A decreased various signs of cyclophosphamide toxicity. The slow elimination of radioactivity from plasma following treatment of neonatal mice with ^{14}C-cyclophosphamide suggested that the poorly developed activating system (Short and Gibson, 1971a) possessed a sufficient capacity to generate alkylating metabolites over a prolonged period of time.

Cytotoxicity of Alkylating Agents. Alkylating agents are highly reactive electrophilic compounds which are capable of substituting an alkyl group for a hydrogen ion on nucleophilic centers of molecules. Nucleophilic centers which are reactive with alkylating agents include organic and inorganic anions, amino groups, sulfhydryl groups, and sulfide groups (Ross, 1958).

Alkylating agents have been shown to react with protein (Alexander and Cousens, 1958). Nitrogen mustards, esters of methane sulfonic acid, and epoxides reacted with carbonyl and histidine groups, in bovine serum albumin although only a fraction of the groups were accessible to the reagents. The most reactive group to all of the alkylating agents was the sulfhydryl group in denatured egg albumin. These groups, however, were not available for reaction in native protein.

Alkylating agents react with nucleic acids. Alkylation of DNA occurred primarily at the N7 position of guanine (Lawley and Brookes, 1967). The cytotoxicity of alkylating agents was greater for

difunctional than monofunctional alkylating agents (Brookes and Lawley, 1961). Since the extent and duration of DNA alkylation has been correlated with toxicity in *Escherichia coli* (Lawley and Brookes, 1965), the increased toxicity of difunctional alkylating agents may be due to the nature of their interaction with DNA. Monofunctional alkylating agents yielded 7 alkylguanines while difunctional agents produced, in addition, di(guanin-7-yl) derivatives (Lawley and Brookes, 1967). Approximately 25% of sulfur mustard that reacted with DNA gave the diguaninyl product which was suggestive of an interstrand cross linking. The increased cytotoxicity of difunctional alkylating agents, therefore, may be due to the formation of interstrand cross-links in the DNA duplex.

A variety of systems have the ability to remove alkylated bases from DNA and restore DNA integrity. Venitt (1968) demonstrated strain differences in *E. coli* sensitivity to sulfur mustard which were dependent on the cells' ability to remove alkylated bases from DNA. DNA repair has also been demonstrated in the HeLa and Chinese hamster cells following treatment with a variety of alkylating agents (Roberts *et al.*, 1971a, 1971b).

Extensive biochemical lesions occurred in cells as a result of damage produced by alkylating agents. The primary lesion produced by sulfur mustard was an inhibition of DNA, but not RNA or protein, synthesis in HeLa cells (Roberts *et al.*, 1968). Nitrogen mustard, on the other hand, reduced both DNA and RNA synthesis in hamster plasmacytomas (Wheeler and Alexander, 1969). The template activity of calf thymus DNA for *E. coli* DNA and RNA polymerase as well as the coding capacity of artificial messenger RNA was reduced by nor-nitrogen mustard treatment (Johnson and Ruddon, 1967; Ruddon and Johnson, 1968). A reduced

replication of cellular DNA induced by alkylating agents led to a mitotic delay as a result of cross-linking of the DNA duplex which prevented strand separation during replication (Roberts *et al.*, 1968). Cells continued to synthesize RNA and protein at normal rates, however, and grew into giant cells with abnormal amounts of RNA and protein.

Radioactivity after the administration of ring-labeled cyclophosphamide to hamsters bearing plasmacytomas was located in the nuclear, mitochondrial, and microsomal fraction of the plasmacytoma (Wheeler and Alexander, 1964). Single doses of cyclophosphamide inhibited the *in vivo* synthesis of DNA and RNA 24 to 48 hours after treatment in the same tumor system (Wheeler and Alexander, 1969). The reduction in DNA synthesis was accompanied by an inhibition of *in vitro* DNA synthesis in a system which contained denatured salmon sperm DNA and soluble plasmacytoma enzymes. Several studies suggested that cyclophosphamide damaged DNA was repaired. Dose fractionation studies in mice with L1210 leukemia cells indicated that sublethal damage was repaired between 3 and 12 hours (DeWys and Kight, 1969). An increased priming capacity of plasmacytoma DNA for DNA synthesis, in addition, was attributed to a cellular response of attempted DNA repair (Tomisek *et al.*, 1971).

Purpose

The purpose of this study was to examine the molecular basis of drug-induced disruption of development in mice. Cyclophosphamide induced disruption of embryonic and neonatal development of mice was the model system studied.

METHODS

Animals

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

Timed pregnancies were obtained by the daily mating of 150 to 200 females. One male was placed in a cage with 5 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 were 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.

Neonatal Swiss-Webster mice were obtained from timed pregnancies started in this laboratory. Pregnant mice were individually housed prior to delivery in clear plastic shoe-box cages that contained a base of Bed-O-Cobs (Anderson Cob Mill, Maumee, Ohio). The mothers delivered spontaneously and were given free access to food and water. The litters were weaned at 3 to 4 weeks of age.

Biosynthesis of DNA by Embryonic Mice

Cyclophosphamide was administered intraperitoneally (i.p.) at a teratogenic dose of 20 mg/kg on day 11 of gestation (Gibson and Becker, 1968a). Treated mice received the drug in a volume of 10 ml distilled water/kg body weight and controls received an equal volume of the vehicle. Thymidine-2-^{14}C (55.7 to 58.9 mc/mM, ICN Tracer Lab, Irvine, Calif.) was administered at a dose of 100 µc/kg at various times after cyclophosphamide. Mice were sacrificed 1 hour after ^{14}C-thymidine by cervical dislocation and a laparotomy was performed. The uterus was removed and the embryos and placentae were obtained through incisions in the uterine wall. ^{14}C-thymidine incorporation into a DNA fraction obtained from maternal liver, placentae, and embryos by method A or B was used as an estimation of DNA synthesis.

Method A (3 to 24 hours after cyclophosphamide). The tissues were removed, weighed, and homogenized in 5 ml of 0.2N perchloric acid (PCA). The supernatant was neutralized with potassium hydroxide and saved for liquid scintillation counting. The precipitate was washed with successive 5 ml portions of 0.2N PCA, which contained 4 mM non-radioactive thymidine to reduce the level of unincorporated radioactivity, 0.2N PCA, 95% ethanol saturated with sodium acetate, a 3:1 ethanol-ether solution, and ether. The precipitate was heated in 4 ml of 0.3N potassium hydroxide for 1 hour, acidified with 3 ml of 30% trichloroacetic acid (TCA), stored at 4°C for at least 8 hours, washed with 5% TCA and heated for 20 minutes at 90°C in 5 ml of 5% TCA. The hot TCA extract was combined with an additional 5% TCA wash of the precipitate and this solution was used for the determination of radioactivity and DNA. The specific activity of this fraction was expressed as disintegrations per minute (DPM)/mg DNA.

Radioactivity in 1 ml of the PCA or TCA extract was measured in 15 ml of a dioxane base counting solution (60 g naphthalene, 4 g 2,5-diphenyloxazole (PPO), 200 mg 1,4 bis [2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP), 100 ml absolute methanol, 20 ml ethylene glycol, and dioxane to a final volume of 1 liter) using a Beckman LS-100 system with a ^{137}Cs external standard.

DNA was measured in the TCA extract by the diphenylamine method described 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. Acetaldehyde (0.1 ml of a 16 mg/ml solution) was added to 20 ml of the diphenylamine reagent prior to use. A sample of the DNA extract was brought to a volume of 1 ml with 5% TCA prior to the addition of 2 ml of the diphenylamine reagent. The colored product, which was formed by the reaction of deoxyribose with diphenylamine, developed for 16 to 20 hours at room temperature. The optical density of the solution was determined at 600 nm and DNA was quantified with a standard curve prepared from calf thymus DNA (Sigma, St. Louis, Mo.).

Method B (24 to 72 hours after cyclophosphamide). Tissues were removed, weighed and homogenized in 5 ml of 10% TCA. The precipitate was washed with 10% TCA that contained 4 mM non-radioactive thymidine, 10% TCA, and heated for 5 min at 60°C in a 3:1 alcohol-ether solution. The precipitate was heated for 15 minutes at 90°C in 5 ml of 5% TCA. The supernatant was saved and combined with one additional TCA wash. Radioactivity and DNA were measured in the TCA extract as described in method A. This method was used to show that cyclophosphamide did not affect embryonic DNA synthesis at 24, 48 or 72 hours after treatment

(Short, 1971). The methodology and results of that study are included in this thesis for completeness.

Alkylation of Embryonic Macromolecules

Radioactive ring labeled cyclophosphamide -5-^{14}C (7.6 to 8.2 mc/mM, New England Nuclear, Boston, Mass.) was diluted with non-radioactive cyclophosphamide to give 250 µc/10 mg cyclophosphamide (6.5 µc/mM). The radioactive purity of an aqueous solution for injection was verified by thin-layer chromatography on activated silica-gel plates with a solvent system of n-butanol-acetic acid-H_2O (6:2:2). Radioactivity in fractions of the silica gel plates was measured in PCS (Amersham/Searle, Arlington Heights, Ill.) after the addition of 1 ml water using a Packard Liquid Scintillation Spectrometer Model 3380 equipped with an Absolute Activity Analyzer.

A teratogenic 20 mg/kg dose of cyclophosphamide, which contained 500 µc/kg of ^{14}C-cyclophosphamide, was administered i.p. in a volume of 10 ml water/kg to pregnant mice on day 11 of gestation (Gibson and Becker, 1968a). Mice were sacrificed by cervical dislocation at various times after treatment. Radioactivity bound to maternal liver, placenta, and embryonic macromolecules, which were isolated by one of two methods, was used as a measure of cyclophosphamide alkylation.

Method A. DNA, RNA, and protein fractions were isolated from the various tissues by a modified phenol extraction (Caldwell and Henderson, 1970). All tissues were homogenized in 5 volumes of buffer A [154 mM sodium chloride, 10 mM sodium phosphate pH 7.4, and 1 mM ethylenediaminetetraacetic acid, disodium salt (EDTA)] in a loosely fitting, motor driven, teflon-glass Potter-Elvehjem homogenizer. The homogenate was shaken (280 oscillations/minute) with an equal volume of freshly

distilled buffer A saturated phenol for 10 minutes at room temperature. The emulsion was centrifuged for 20 minutes at 25,000 g and the aqueous upper layer was saved. An equal volume of buffer A was added to the lower phase plus interphase and the mixture was shaken and centrifuged as before. The two aqueous phases were combined and re-extracted with freshly distilled phenol and residual phenol was removed with four ether extractions. Ether was removed under a stream of nitrogen gas.

RNA was precipitated from the aqueous solution as an insoluble salt after the addition of cetyltrimethylamonium bromide (CTMA) to a final concentration of 6 mg CTMA/ml. The precipitate was extrated three times with 0.1M sodium acetate in 70% ethanol and dissolved in 150 mM sodium chloride 15 mM sodium citrate pH 7 (SSC). RNA was precipitated with CTMA, washed three times with ethanolic sodium acetate, and dissolved in SSC.

The lower phase and interphase were mixed with 1 ml of 0.1M sodium citrate in 2.5M sodium chloride and 4 ml of 0.25% sodium dodecylsulfate and shaken for 10 minutes prior to centrifugation. The viscous aqueous layer was extracted with ether and residual ether was removed with a stream of nitrogen gas. DNA and bound RNA were precipitated by CTMA and dissolved in SSC.

The remaining interfacial material was mixed with 0.6N perchloric acid (PCA) and heated for 30 minutes at 70°C. The precipitate was washed twice with PCA, 95% ethanol saturated with sodium acetate, and ether. The acid precipitate was dissolved in 0.1N sodium hydroxide overnight at 40°C.

Radioactivity in 2 or 0.1 ml aliquots of the nucleic acid or protein fraction was measured by liquid scintillation counting (Packard Model 3380) after the addition of 15 ml of PCS (Amersham/Searle). The

radioactivity in each fraction was expressed as DPM/g wet tissue weight.

The yield of nucleic acids by the phenol extraction was compared to the yield obtained following an acid precipitation. Liver, placental, and embryonic tissue were homogenized in 5 volumes of buffer A and divided into two equal portions. DNA and RNA were extracted from one portion by the previously described phenol method. The other portion was acidified with an equal volume of 0.6N PCA. RNA was extracted by heating the precipitate at 40°C for 1 hour in 0.3N sodium hydroxide. The alkaline solution was acidified with 30% trichloroacetic acid (TCA) and the precipitate was washed with 5% TCA. DNA was extracted by heating the precipitate for 20 minutes in 5% TCA on a boiling water bath.

DNA was measured by the previously described method of Burton. RNA was measured by the orcinol procedure (Ceriotti, 1955). The orcinol reagent was freshly prepared by mixing 200 mg orcinol, 10 ml of 4 mM copper chloride ($CuCl_2$) in concentrated hydrochloric acid, and a sufficient volume of concentrated hydrochloric acid to give 100 ml of the reagent. A sample of the TCA extract was added to an equal volume of the orcinol reagent, heated for 40 minutes on a boiling water bath, and cooled. The optical density of the solution was measured at 675 nm and RNA was quantified using yeast RNA (Sigma).

Method B. Approximately 100 to 300 mg of tissue was homogenized in 5 ml of 0.2N PCA that contained 1 mM non-radioactive cyclophosphamide to minimize reversible binding. The precipitate was washed with 3 successive 5 ml portions of 0.2N PCA. Lipids were extracted from the precipitate with 5 ml washes of 1M sodium acetate in absolute methanol, methanol:chloroform (2:1), absolute ethanol, and two ether washes.

Nucleic acids were extracted by heating the precipitate in 5 ml of 0.6N PCA for 30 minutes at 70°C and the precipitate was washed with 0.2N PCA and dissolved in 0.1N sodium hydroxide overnight at 40°C.

Radioactivity in the initial acid soluble, hot acid extract, and protein fraction were measured by liquid scintillation counting (Packard Model 3380) after the addition of PCS (Amersham/Searle). A portion of the ether washed precipitate was transferred to a tared counting vial, dried, weighed, and solubilized in 1 ml of Soluene (Packard, Downers Grove, Ill.) at 40°C for at least 8 hours. Radioactivity was measured after the addition of a toluene base counting solution (5 g PPO, 200 mg POPOP, and 1 liter toluene). DNA was estimated by the previously described method of Burton (1956). Protein was measured by the Lowry method (1951). A 0.5 ml aliquot of the protein solution was mixed with 2.5 ml of reagent C (0.5 ml of 2% $CuSO_4 \cdot 5H_2O$, 0.5 ml 2% NaK tartrate and 50 ml 2% Na_2CO_3 in 0.1N NaOH) and allowed to stand for 10 minutes. Phenol reagent (0.25 ml of a 1N solution) was rapidly added and the solution was mixed and allowed to stand for 30 minutes. The optical density of the solution was measured at 500 or 700 nm and the protein was quantified using bovine serum albumin (Sigma) as the standard. A nomograph (California Corporation for Biochemical Research, Los Angeles, Calif.) of the optical density at 260 and 280 was used to measure total nucleic acids and protein in the nucleic acid extract. Acid soluble radioactivity was expressed in terms of wet tissue weight and bound radioactivity was expressed in terms of the amount of acid precipitate, nucleic acid, DNA, or protein present.

Postnatal Toxicity

Neonatal Swiss-Webster mice were obtained from timed pregnancies started in this laboratory. Cyclophosphamide was administered subcutaneously (s.c.) 24 to 48 hours after birth at a dose of 80 mg/kg in a volume of 20 ml normal saline/kg. This treatment significantly reduced the growth rate and increased lethality (Short and Gibson, 1971b). Control animals received an equal volume of normal saline. The animals were allowed to develop and some were weaned at 3 to 4 weeks of age. Body and organ weights were measured at various times after treatment.

Biosynthesis of Macromolecules by Neonatal Mice

Drug induced biochemical lesions were studied in neonatal mice treated, as previously described, with 80 mg/kg s.c. cyclophosphamide 24 to 48 hours after birth. ^{14}C-labelled precursors of DNA, RNA or protein were administered s.c. at a dose of 100 μc/kg in a volume of 10 ml/kg at various intervals after cyclophosphamide and the animals were sacrificed by decapitation at the indicated times. The liver and brain from 1 to 3 mice in a litter were removed and pooled to represent a single experimental unit. The tissue was weighed and homogenized in 5 ml of 0.2N perchloric acid (PCA). The bodies were homogenized in distilled water with a Polytron homogenizer (Brinkmann, Westbury, N.Y.). A portion of the homogenate was made 0.3N in PCA and stored at least 15 minutes on ice.

^{14}C-Thymidine Incorporation. Thymidine-2-^{14}C (50 mc/mM, ICN Tracer Lab) was administered 1 hour prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5 ml washes of 0.2N PCA containing 4 mM non-radioactive thymidine, 0.2N PCA, 95% ethanol saturated with sodium acetate, ethanol:ether (3:1) and ether.

The precipitate was heated at 37°C for 1 hour in 4 ml of 0.3N sodium hydroxide, acidified with 3 ml of 30% trichloroacetic acid (TCA), and stored on ice for 10 to 15 minutes. The precipitate was washed twice with 5 ml of 5% TCA and then heated twice in 5 ml of 5% TCA for 15 minutes on a boiling water bath. The supernatants, after heating, were saved for the measurement of radioactivity and DNA. Radioactivity was measured in the previously described dioxane-base counting solution using the Packard 3380 Liquid Scintillation Spectrometer. DNA was measured by the diphenylamine method of Burton (1956). The specific activity was expressed as DPM/mg DNA.

^{14}C-Uridine Incorporation. Uridine-2-^{14}C (50.8 mc/mM, ICN Tracer Lab) was administered 1 hour prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5 ml portions of 0.2N PCA, 0.2N PCA containing 4 mM non-radioactive uridine, two 0.2N PCA washes, 95% ethanol saturated with sodium acetate, ethanol:ether (3:1), and ether. The precipitate was heated at 37°C for 1 hour in 4 ml of 0.3N sodium hydroxide, acidified with 3 ml of 30% TCA and stored on ice for 10 to 15 minutes. The precipitate was washed with 2.5 ml of 10% TCA and the supernatant was combined with the supernatant after alkaline hydrolysis for the measurement of radioactivity and RNA. Radioactivity was measured in the dioxane-base counting solution using the Packard 3380. RNA was measured by the orcinol procedure (Ceriotti, 1955). The results were expressed as DPM/mg RNA.

^{14}C-Leucine Incorporation. L-Leucine (UL)^{14}C (197 mc/mM ICN Tracer Lab) was administered 30 minutes prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5 ml washes of 0.2N PCA containing 40 mM leucine and 0.2N PCA. The

precipitate was washed with successive 5 ml portions of 95% ethanol saturated with sodium acetate, ethanol:ether (3:1) and ether. The precipitate was dissolved in 0.3N sodium hydroxide overnight at 40°C prior to the measurement of protein and radioactivity. An aliquot of the alkaline protein solution was dissolved in Hyamine Hydroxide (Packard) and heated for at least 8 hours at 40°C prior to the addition of the dioxane-base counting solution. Protein was measured by the Lowry method (1951). The specific activity was expressed as DPM/mg protein.

Protein Turnover in Neonatal Mice

Proteins in neonatal mice were labeled with ^{14}C-arginine prior to cyclophosphamide treatment by administering the radioactive precursor to pregnant mice prior to parturition. Pregnant Swiss-Webster mice received 100 µc/kg i.p. of L-arginine (guanido-^{14}C) (59 mc/mM, ICN Tracer Labs) at 10 p.m. on day 18 and at 10 a.m. and 10 p.m. on day 19 of gestation. Litters were delivered spontaneously and received either 80 mg/kg cyclophosphamide or normal saline one day after birth. This treatment significantly affected subsequent growth and development (Short and Gibson, 1971b).

Neonatal mice were sacrificed at 1, 3, 5, and 10 days after cyclophosphamide by decapitation. The liver and brain from 1 to 2 mice were weighed and homogenized in 4 volumes of 154 mM potassium chloride and 10 mM tris(hydroxymethyl)aminomethane (TRIS, Sigma) pH 7.3 (KCl-TRIS) using a loosely fitting, motor driven, teflon-glass Potter-Elvehjem homogenizer. The bodies were homogenized in 4 volumes of KCl-TRIS with a Polytron homogenizer (Brinkmann). The homogenates were separated into a soluble and particulate fraction by centrifugation at 40,000 revolutions per minute in a 50 titanium rotor (Beckman) for 1 hour (105,000 g

x 1 hour) in a Beckman Model L3-50 ultracentrifuge. The supernatant was saved and the precipitate was resuspended in 5 ml of KCl-TRIS and centrifuged at 105,000 g for 1 hour. The supernatants were combined and made 0.3N with concentrated perchloric acid (PCA). The acid precipitate was washed with successive 5 ml portions of 0.2N PCA, 0.2N PCA, 95% ethanol saturated with sodium acetate, ethanol:ether (3:1) and ether. The precipitate was finally dissolved in 5 ml of 0.3N sodium hydroxide. The pellet and walls of the centrifuge tube were carefully washed with distilled water after the second centrifugation and the pellet was dissolved in 5 ml of 0.3N sodium hydroxide. Radioactivity in the alkaline protein solution was measured by liquid scintillation counting in a Packard 3380 after the addition of PCS-solubilizer (Amersham/Searle). Protein was measured by the Lowry method (1951). The specific activity was expressed as DPM/mg protein.

Qualitative Changes in Neonatal Protein Synthesis

Electrophoretic Analysis of Protein Synthesis. Litters of day-old Swiss-Webster mice re-eived either 80 mg/kg cyclophosphamide or normal saline subcutaneously in a volume of 20 ml/kg. Mice were sacrificed by decapitation and livers and brains were removed and transferred directly to Hank's solution (Altman and Dittmer, 1964) which was maintained at 37°C in a glass stoppered Erlenmeyer flask. Hank's solution contained either L-leucine (UL)^{14}C (1 µc/ml) or L-leucine (4,5)^{3}H (2 µc/ml). The flasks were gased with 95% O_2:5% CO_2 for 2 minutes and incubated for 1 hour at 37°C. Tissues labeled with ^{3}H and ^{14}C-leucine were removed from the incubation media, mixed, and homogenized in 0.01M TRIS pH 7.3 using a Polytron homogenizer. The homogenate was centrifuged at 20,000 g for 30 minutes in a Sorvall RC2-B centrifuge. The

supernatant was made 1% in regard to both sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and heated at 60°C for 10 minutes. The supernatant was cooled to room temperature and made 0.3M with iodoacetamide. The mixture was dialyzed overnight at room temperature with two changes of 0.01M TRIS and 0.1% SDS buffer. Dialysis tubing was boiled in 0.01M ethylenediaminetetraacetic acid (tetrasodium salt) and 0.1M sodium carbonate for 30 minute periods until the solution was clear, washed in distilled water, and stored at 0°C in 50% ethanol before use. The protein was concentrated by dialyzing the sample for 2 hours against 40% sucrose that contained 0.1% SDS and 0.01M TRIS pH 7.3.

Polyacrylamide gel electrophoresis was conducted in a discontinuous system modified from one described by Umbreit *et al.* (1972). The modifications were reducing the running gel acrylamide concentration to 5% and including 0.1% SDS in both the buffer reservoir and gels. Gel solutions were deaerated under vacuum for 10 seconds, added to tubes (0.6 cm i.d.), and photopolymerized under fluorescent light for 30 minutes. The stacking gel was 0.6 cm and consisted of 2.5% acrylamide. The running gel was 6 cm and contained 5% acrylamide. The upper and lower buffer reservoir was a TRIS-glycine buffer pH 8.3. Aliquots of the protein sample (20 to 50 µl) were electrophoresed at 2 mA/tube for 2 to 3 hours at room temperatures using an ISCO polyacrylamide electrophoresis apparatus.

The gels, after electrophoresis, were preserved in 10% TCA. Gels were frozen in a bath of dry ice and acetone and sliced with a hand-held razor blade cutter. The fractions were solubilized in Soluene (Packard) according to the method developed by Terman (1970). Radioactivity from ^{3}H and ^{14}C was measured simultaneously in a Packard 3380 liquid

scintillation spectrometer after the addition of the toluene base counting solution previously described.

Developmental Changes in Glucose-6-Phosphatase Activity. Litters of day-old Swiss-Webster mice received either 80 mg/kg cyclophosphamide or normal saline s.c. in a volume of 20 ml/kg. Glucose-6-phosphatase activity was assayed by the method described by Greengard *et al.* (1967) which was used to show developmental changes in enzyme activity in rat liver. Neonatal and adult mice were sacrificed by decapitation and cervical dislocation, respectively. Livers were removed, weighed, and homogenized in 10 volumes of 0.25M sucrose. All incubations were conducted for 10 minutes at 37°C in room air using a Dubnoff metabolic shaking apparatus. The incubation media consisted of 0.25 ml of homogenate in 20 mM glucose-6-phosphate and 50 mM TRIS-maleate buffer pH 6.5 for a final volume of 1 ml. Reactions were stopped by the addition of 1 ml of 1.2N perchloric acid (PCA). Inorganic phosphate was measured in the supernatant as described below and protein was measured (Lowry *et al.*, 1951) in the acid precipitate which was solubilized in sodium hydroxide. All incubations were performed in duplicate and blank values were obtained by adding the homogenate to media that was acidified at the end of a 10 minute incubation.

Inorganic phosphate was measured in 1 ml of the supernatant by the addition of 1.5 ml of sodium molybdate solution. The yellow color was extracted into 5 ml of butyl acetate and the absorbance was measured at 390 nm. The values were quantified using a standard curve prepared from potassium phosphate. The sodium molybdate solution was prepared by adding 14.5 g of sodium molybdate to a solution of 210 ml of 70% PCA and 90 g of sodium hydroxide which was titrated to a cresol red:

thymol blue (25 mg:150 mg in 100 ml water) light blue and adding sufficient water to give a final volume of 1 liter.

Recommendations

Various methods were used in these studies to measure the incorporation into or binding of radioactive molecules to macromolecules. The review by Munro and Fleck (1966) provided a valuable source of information for devising and improving the methodology used to isolate DNA, RNA, and protein fractions. The procedure used to measure the biosynthesis of macromolecules by neonatal mice is a modification of a procedure suggested by Munro and Fleck (1966) and is recommended as a suitable method for further studies.

Statistics

Statistical evaluation of the data was performed by Student's t-test (Steel and Torrie, 1960). Equations for lines were calculated by linear regression analysis (Mendenhall, 1971). The level of significance was chosen as $P<0.05$.

RESULTS

Biosynthesis of DNA by Embryonic Mice

DNA synthesis and cell multiplication are important aspects of normal development. DNA synthesis, as measured by ^{14}C-thymidine incorporation into DNA, was greater in embryos and placentae than in maternal liver (Table 1). The more rapid rate of DNA synthesis in developing tissue indicated that an increase in cell number in contrast to an increase in cell size was an important factor in growth. A dose of cyclophosphamide, which disrupted normal embryonic development, also reduced DNA synthesis in mouse embryos. Cyclophosphamide-treated embryos had a significantly impaired ability to incorporate ^{14}C-thymidine into DNA 12 hours after treatment (Figure 1). A reduced DNA concentration (mg DNA/g wet weight of embryo) was subsequently observed at 12, 24, 48, and 72 hours after drug treatment (Table 1). DNA synthesis was reduced in the placenta at 24, 48, and 72 hours after cyclophosphamide (Table 1) but the concentration of placental DNA was reduced only at 12, 24, and 48 hours after treatment (Table 2). The specific activity of DNA, after a pulse of ^{14}C-thymidine, was significantly reduced in maternal livers at 6, 12, and 72 hours after cyclophosphamide (Table 1) and the DNA concentration was reduced at 24 and 48 hours after treatment (Table 2). The initial acid soluble radioactivity of tissues from treated mice was equal to or greater than the same fraction from control tissue. Cyclophosphamide, therefore, did not affect the placental transport of radioactivity and thereby

Table 1. ^{14}C-thymidine incorporation after cyclophosphamide treatment

Hours after cyclophosphamide[b]	Method of measuring incorporation[c]	DPM/mg DNA x 10^{-3}[a]					
		Liver		Placenta		Embryo	
		Cont.	Treat	Cont.	Treat	Cont.	Treat
3	A	5.2[d] ±0.7	4.5 ±0.3	29 ±3.9	25 ±2.0	27 ±5.1	25 ±2.3
6	A	5.1 ±0.4	3.5[e] ±0.6	36 ±7.3	29 ±5.6	32 ±7.3	23 ±1.6
12	A	5.1 ±0.6	3.3[e] ±0.4	23 ±2.2	19 ±1.3	26 ±1.9	17[e] ±1.8
24	A	10 ±3.3	5.6 ±0.9	44 ±3.0	30[e] ±2.4	28 ±4.8	27 ±3.1
24	B	4.9 ±0.4	6.0 ±0.8	43 ±2.6	31[e] ±1.8	19 ±1.9	26 ±4.3
48	B	4.8 ±1.3	2.6 ±0.5	27 ±2.0	15[e] ±3.6	18 ±2.1	18 ±4.0
72	B	3.9 ±0.3	2.0[e] ±0.4	22 ±2.0	14[e] ±1.2	19 ±1.2	17 ±3.6

[a]One-hour pulse (100 μc/kg) ^{14}C-thymidine.

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

[c]See Methods.

[d]Mean ± SE for 3-6 observations.

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

Figure 1. ^{14}C-thymidine incorporation into embryonic DNA as a function of time after cyclophosphamide. Pregnant mice were treated with cyclophosphamide (20 mg/kg i.p.) on day 11 of gestation and a 1 hour pulse of ^{14}C-thymidine (100 μc/kg i.p.) was administered at various intervals after drug treatment. The values are the mean ± SE of the specific activity of embryonic DNA for 3-6 determinations. An asterisk indicates a significant difference from control ($P<0.05$).

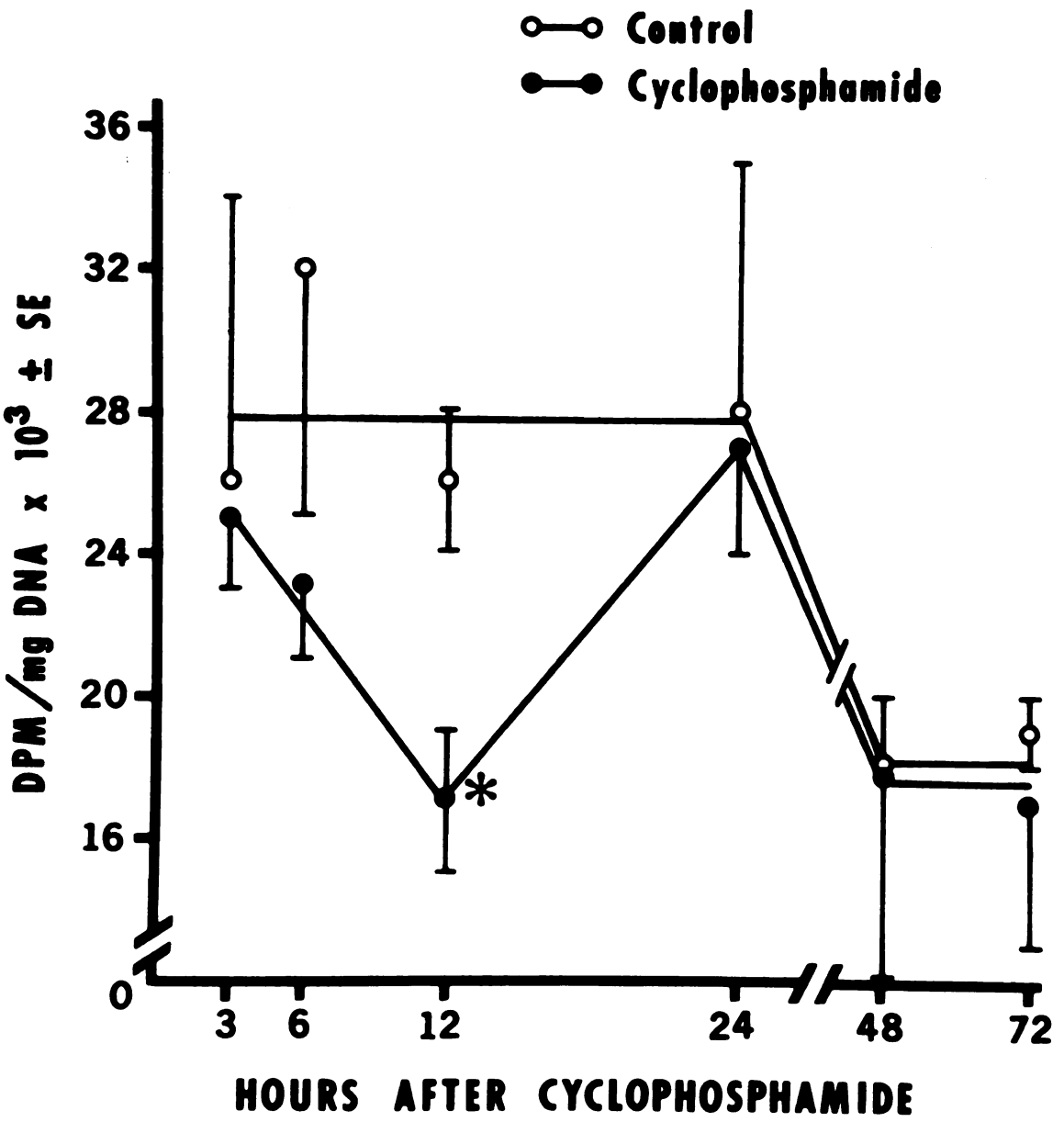
Control
Cyclophosphamide
DPM/mg DNA x 10^3 ± SE
36
32
28
24
20
16
0
3
6
12
24
48
72
*
HOURS AFTER CYCLOPHOSPHAMIDE

Figure 1

Table 2. DNA content after cyclophosphamide treatment

Hours after cyclophosphamide[a]	Method of isolating DNA[b]	mg DNA per g wet weight tissue					
		Liver		Placenta		Embryo	
		Cont.	Treat	Cont.	Treat	Cont.	Treat
3	A	2.4[c] ±0.2	2.5 ±0.3	3.1 ±0.2	3.1 ±0.3	4.9 ±0.9	4.5 ±0.5
6	A	3.2 ±0.2	3.0 ±0.2	3.2 ±0.2	2.9 ±0.1	3.9 ±0.4	4.3 ±0.2
12	A	3.4 ±0.2	3.3 ±0.1	3.6 ±0.1	3.1[d] ±0.2	4.3 ±0.2	3.7[d] ±0.1
24	A	3.4 ±0.2	3.0 ±0.1	3.7 ±0.1	3.3[d] ±0.1	5.1 ±0.3	3.3[d] ±0.2
24	B	2.9 ±0	2.3[d] ±0.2	4.0 ±0.4	3.7 ±0.3	5.8 ±0.8	3.7[d] ±0.2
48	B	2.6 ±0.2	1.6[d] ±0.1	4.1 ±0.1	3.4[d] ±0.2	6.3 ±0.2	4.3[d] ±0.2
72	B	2.6 ±0.1	2.9 ±0.2	2.9 ±0.1	3.0 ±0.2	6.4 ±0.2	5.5[d] ±0.3

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

[b]See Methods.

[c]Mean ± SE for 3-6 observations.

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

limit precursor availability for incorporation. These observations indicated that cyclophosphamide significantly reduced DNA synthesis, as measured by ^{14}C-thymidine incorporation, in both embryonic and adult tissues.

Alkylation of Embryonic Macromolecules

Drug binding to macromolecules was studied to determine if a teratogenic dose of cyclophosphamide produced alkylation of embryonic macromolecules. The temporal aspects of drug binding were measured to evaluate changes in this parameter with time. Alkylation in both embryonic and adult tissues was compared because the adult had a proven ability to form alkylating metabolites from the parent compound. Drug binding to total macromolecules rather than subcellular fractions was measrued to avoid the requirement of obtaining high yields of pure subcellular fractions from a heterogeneous mixture of embryonic tissues.

Radioactivity in DNA, RNA, and protein fractions from maternal liver, placenta, and embryo, after ^{14}C-cyclophosphamide, are shown in Table 3. Negligible amounts of radioactivity were observed in the DNA and RNA fractions relative to radioactivity in the protein fraction. As a result of these low levels of radioactivity the yield of macromolecules was measured in order to determine how accurately this observation reflected cyclophosphamide binding to DNA and RNA. The phenol method used in the present study was reported to yield 75 to 80% of the DNA, 70 to 80% of total RNA, and 90 to 95% of the protein in Ehrlich ascites tumor cells (Caldwell and Henderson, 1969). The yield of nucleic acids from maternal liver, placenta, and embryos by the phenol procedure relative to the yield of DNA and RNA from an acid precipitate were 65 to 80% for RNA but only 25 to 30% for DNA. The phenol procedure used in the present study was concluded to be unsatisfactory for measuring

Table 3. ^{14}C-cyclophosphamide radioactivity in DNA, RNA, and protein fractions from maternal liver, placenta, and embryo

Tissue	DPM/g tissue[a,b]		
	DNA	RNA	Protein x 10^3
Liver	10[c] ±6	36 ±13	242 ±30
Placenta	48 ±25	408 ±123	52 ±16
Embryo	61 ±35	184 ±125	42 ±17

[a]500 µc/kg ^{14}C-cyclophosphamide was injected i.p. on day 11 of gestation.

[b]DNA, and protein fractions were isolated by a phenol method (see Methods).

[c]Mean ± SE for determinations made at 0.5, 1.5, 3, and 5 hours after ^{14}C-cyclophosphamide.

drug binding to nucleic acids in an embryonic system as a result of both the small amount of tissue available and loss of macromolecules during the isolation procedure.

Temporal aspects of the binding of radioactivity to macromolecules from the maternal liver, placenta, and embryo, after ^{14}C-cyclophosphamide, were studied by an acid precipitation procedure. This method provided readily detectable levels of radioactivity. Acid soluble radioactivity was present in all of the tissues studied for at least 10 hours after ^{14}C-cyclophosphamide (Figure 2). The liver had the highest level of acid soluble radioactivity at 0.5, 1.5, 3.0, 5.0 and 10 hours after treatment. The data indicated that radioactivity after a teratogenic dose of ^{14}C-cyclophosphamide crossed the placenta and remained in in acid soluble form in embryos for at least 10 hours after drug treatment. This study, in addition, demonstrated that acid soluble radioactivity was eliminated from all of the tissues studied and the elimination occurred in a biphasic fashion.

The acid washed-lipid extracted acid precipitate of maternal liver, placenta, and embryo contained radioactivity after ^{14}C-cyclophosphamide treatment (Figure 3). The specific activity of the acid precipitate decreased in the order liver, placenta, and embryo at all of the times studied. Radioactivity in the acid precipitate reached maximal values by 1.5 hours after treatment and declined with time. The decline in specific activity appeared to plateau towards the end of the observation period.

Radioactive molecules were solubilized from the acid-washed-lipid-extracted precipitate of tissue by hot acid (Table 4). The acid extract contained both DNA, as measured by the diphenylamine reagent, and protein and nucleic acids, as measured by the optical density at 280

Figure 2. Acid soluble radioactivity in the maternal liver, placenta, and embryo as a function of time after 500 μc/kg (20 mg/kg) ^{14}C-cyclophosphamide. The values plotted are the mean ± SE for at least 4 observations.

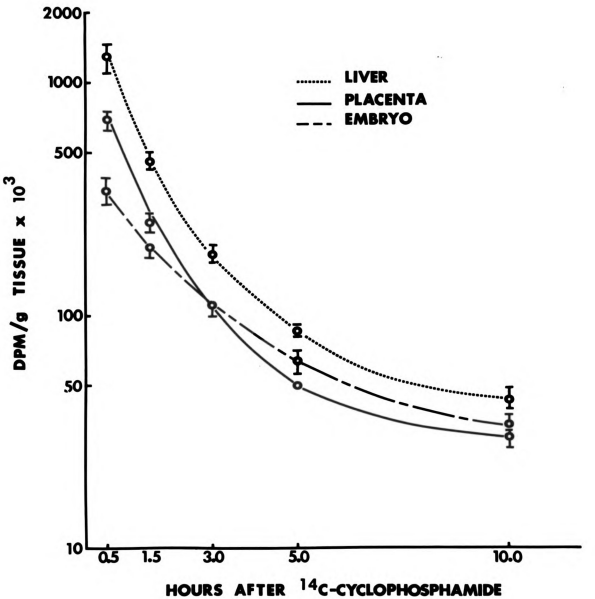
2000
1000
500
100
50
10
LIVER
PLACENTA
EMBRYO
DPM/g TISSUE x 10^3
0.5
1.5
3.0
5.0
10.0
HOURS AFTER ^{14}C-CYCLOPHOSPHAMIDE

Figure 2

Figure 3. Radioactivity in the acid washed lipid extracted acid precipitate from maternal liver, placenta, and embryo as a function of time after 500 μc/kg (20 mg/kg) ^{14}C-cyclophosphamide. The values plotted are the mean ± SE for 3 to 5 observations.

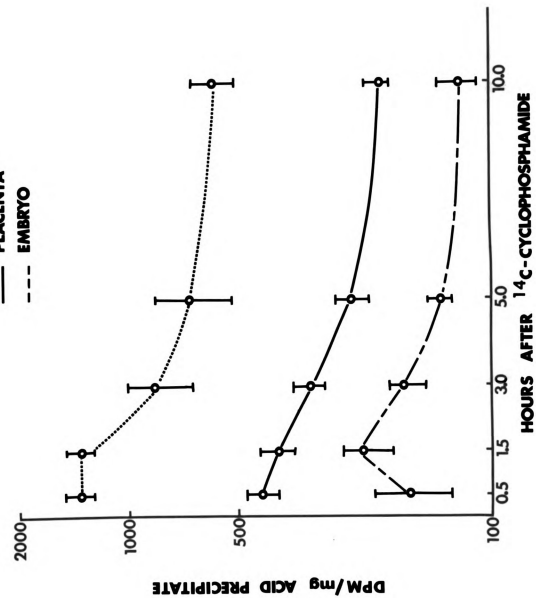
LIVER
PLACENTA
EMBRYO
DPM/mg ACID PRECIPITATE
2000
1000
500
100
0.5
1.5
3.0
5.0
10.0
HOURS AFTER 14C-CYCLOPHOSPHAMIDE

Figure 3

and 260 nm (Tables 4 and 5). The expression of radioactivity in terms of the amount of extracted DNA indicated that the specific activity of this fraction decreased in the order liver, placenta, and embryo at all of the times studied (Table 4). Radioactivity of this fraction expressed in terms of milligrams total nucleic acid was lower than the specific activity of the same fraction expressed in terms of milligrams DNA at all of the times studied in liver and placenta but not embryo. The decline in specific activity of the nucleic acid extract (DPM/mg DNA) between 0.5 and 5.0 hours after ^{14}C-cyclophosphamide expressed as a percent of the initial values was 28, 38, and 83% for the liver, placenta, and embryo, respectively.

^{14}C-cyclophosphamide radioactivity was associated with the protein fraction (Table 5). The specific activity of this fraction decreased in the order liver, placenta, and embryo. Radioactivity in both the protein and nucleic acid fraction, expressed in terms of milligrams protein, indicated that the specific activity was higher in the former. The specific activity of the protein fraction declined to 75, 61, and 66% of the initial values observed in liver, placenta, and embryo, respectively, between 0.5 and 5.0 hours after treatment.

The data in Figure 4 depict the temporal changes in the ratio of acid precipitable to acid soluble radioactivity after ^{14}C-cyclophosphamide in maternal liver, placenta, and embryo. This value is used as a measure of the ratio of bound to free radioactivity. This ratio decreased in the order liver, placenta, and embryo. This observation, in conjunction with previously discussed macromolecule specific activity data, indicated that the alkylation of tissue macromolecules also decreased in the order liver, placenta, and embryo. These differences may be

Table 4. ^{14}C-cyclophosphamide radioactivity in the nucleic acid extract from maternal liver, placenta, and embryo[a]

Hours after ^{14}C-cyclo[b]	DPM/mg DNA[c] x 10^{-2}			DPM/mg NA[d] x 10^{-2}		
	Liver	Placenta	Embryo	Liver	Placenta	Embryo
0.5	126.8[e] ±19.1	27.3 ±5.8	3.0 ±1.5	9.3 ±0.8	3.9 ±0.6	1.9 ±0.5
1.5	77.1 ±25.0	19.0 ±10.8	1.1 ±0.1	6.6 ±1.6	2.4 ±0.3	4.8 ±4.4
3.0	66.8 ±40.1	19.5 ±9.1	2.6 ±0.6	4.0 ±0.8	2.2 ±0.1	3.8 ±2.4
5.0	34.9 ±6.3	10.4 ±2.8	2.5 ±0.6	3.8 ±0.4	2.0 ±0.3	2.7 ±1.2

[a]The nucleic acid extract was obtained by heating a lipid extracted acid precipitate in 0.6N perchloric acid for 30 minutes at 70°C.

[b]500 μc/kg of ^{14}C-cyclophosphamide (20 mg/kg) was administered i.p. on day 11 of gestation.

[c]DNA was measured by the diphenylamine method of Burton (1956).

[d]Total nucleic acids were determined from the optical density at 260 and 280 nm.

[e]Mean ± SE for at least 3 determinations.

Table 5. ^{14}C-cyclophosphamide radioactivity in the protein and nucleic acid fraction from maternal liver, placenta, and embryo

Hours after ^{14}C-cyclo[a]	Protein Fraction[b] DPM/mg protein[c] x 10^{-2}			Nucleic Acid Fraction[d] DPM/mg protein[e] x 10^{-2}		
	Liver	Placenta	Embryo	Liver	Placenta	Embryo
0.5	14.9[f] ±0.8	4.9 ±0.5	2.9 ±0.6	0.80 ±0.07	0.45 ±0.03	0.14 ±0.02
1.5	17.4 ±1.2	4.1 ±0.4	3.3 ±0.6	0.57 ±0.13	0.24 ±0.04	0.09 ±0.04
3.0	13.9 ±1.2	4.5 ±0.8	2.4 ±0.4	0.37 ±0.06	0.21 ±0.01	0.13 ±0.01
5.0	11.2 ±0.7	3.0 ±0.3	1.9 ±0.2	0.34 ±0.04	0.18 ±0.02	0.13 ±0.01

[a] 500 µc/kg of ^{14}C-cyclophosphamide (20 mg/kg) was administered i.p. on day 11 of gestation.

[b] An acid precipitate washed with lipid solvents and heated with 0.6N perchloric acid at 70°C for 30 minutes.

[c] Protein was measured by the Lowry method (1951).

[d] The hot 0.6N perchloric acid extract in b.

[e] Protein was determined from the optical density at 260 and 280 nm.

[f] Mean ± SE for at least 3 determinations.

Figure 4. The ratio of acid precipitable to acid soluble radioactivity in the maternal liver, placenta, and embryo as a function of time after 500 μc/kg (20 mg/kg) ^{14}C-cyclophosphamide. The values plotted are the mean ± SE for at least 3 observations.

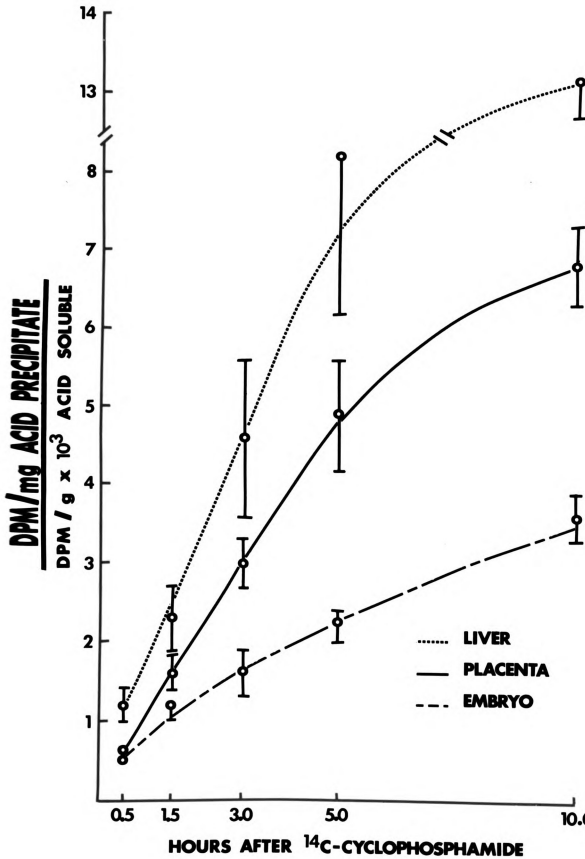
DPM/mg ACID PRECIPITATE
DPM / g x 10^3 ACID SOLUBLE
14
13
8
7
6
5
4
3
2
1
LIVER
PLACENTA
EMBRYO
0.5
1.5
3.0
5.0
10.0
HOURS AFTER ^{14}C-CYCLOPHOSPHAMIDE

Figure 4

attributed to reduced levels of either alkylating metabolites or target sites for alkylation in the placenta and embryo relative to the liver.

Postnatal Toxicity

Neonatal mice treated subcutaneously with 80 mg/kg cyclophosphamide 24 to 48 hours after birth matured into abnormal adults. Treated mice, at 7 weeks of age, were reduced in size and had short snouts, ears, and tails (Figures 5 and 6). Organ weights and organ to body weight ratios were measured at various times after drug treatment for the liver and brain (Table 6). There was no significant change in these values in drug treated mice for at least 1 week. Liver and brain weights were significantly reduced 35 days after treatment when the body weights were only 60% of control. The organ to body weight ratio, however, was changed only for the brain where an increase was observed. Cyclophosphamide treatment, therefore, interfered with body, brain, and liver growth. The liver weight to body weight ratio, however, indicated that drug treatment did not produce a selective reduction in liver growth since body weights were reduced to the same extent as liver weights. Brain growth, relative to body growth, was not as severely affected in cyclophosphamide treated neonates since the brain weight to body weight ratio was increased over control values.

Biosynthesis of Macromolecules by Neonatal Mice

Neonatal mice actively synthesized DNA, RNA, and protein as measured by ^{14}C-precursor incorporation into the respective macromolecules. Neonatal mice, therefore, were actively involved in cell replication and RNA and protein synthesis during the period of drug exposure. Cyclophosphamide induced biochemical lesions were present in neonatal

Figure 5. Adult mice that received either 80 mg/kg cyclophosphamide 24 to 48 hours after birth or normal saline. The mouse on the left was a control and the 2 mice on the right were drug treated.

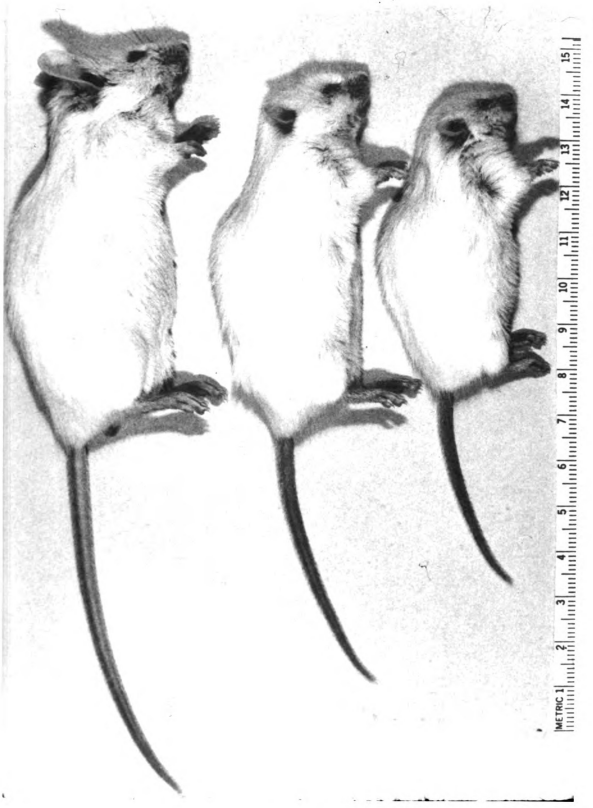

Figure 5

Figure 6. Adult mice that received either 80 mg/kg cyclophosphamide 24 to 48 hours after birth or normal saline. The mouse on the bottom was a control and the mouse on the top was drug treated.

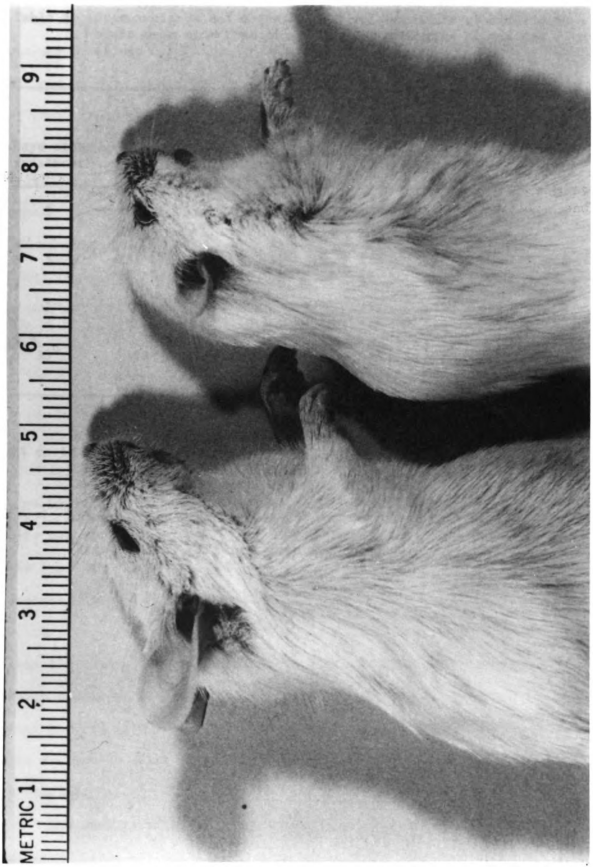

Figure 6

Table 6. Organ weights and organ to body weight ratios of liver and brain from mice treated with cyclophosphamide at one day of age

Days after treatment[a]	Liver				Brain			
	mg tissue/ mouse		g tissue/ 100 g BW		mg tissue/ mouse		g tissue/ 100 g BW	
	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat
3	105[b] ±6	98 ±1	3.6 ±0.2	3.8 ±0.2	152 ±5	135 ±8	5.2 ±0.2	5.4 ±0.7
5	151 ±5	123 ±23	3.7 ±0.2	3.8 ±0.4	189 ±6	177 ±15	4.6 ±0.2	5.6 ±0.3
7	130 ±11	107 ±26	2.8 ±0.4	3.8 ±0.1	217 ±16	179 ±26	4.7 ±0.2	6.8 ±1.0
35[c]	1440 ±11	720[d] ±50	5.6 ±0.2	5.4 ±0	440 ±10	370[d] ±0	1.7 ±0	2.8[d] ±0

[a]Cyclophosphamide (80 mg/kg s.c.) or normal saline was injected 24 to 48 hours after birth.

[b]Mean ± SE for at least 3 determinations.

[c]Treated body weights were 60% of control at this time.

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

mice treated subcutaneously with 80 mg/kg of the drug 24 to 48 hours after birth. DNA synthesis, as measured by ^{14}C-thymidine incorporation, was significantly inhibited in neonatal liver (Figure 7), brain (Figure 8), and carcass (Figure 9) 24 hours after cyclophosphamide. DNA synthesis at this time was 8, 26, and 25% of control, respectively. DNA synthesis remained reduced in the livers and brains from drug treated mice for at least 5 days. ^{14}C-thymidine incorporation into DNA of carcasses from treated mice returned to control values 5 days after treatment. The DNA concentration (mg DNA/g wet weight) and DNA content (mg DNA/organ) were significantly reduced in liver between 1 and 5 days after treatment (Table 7). DNA concentration was reduced in the brain and carcass at 1 and 3 and at 2 and 3 days after treatment, respectively (Tables 8 and 9).

Hydroxyurea, an inhibitor of DNA synthesis, was used as an internal control to distinguish between real incorporation and nonspecific binding of ^{14}C-thymidine to DNA (Morley and Kingdon, 1972). Hydroxyurea was administered subcutaneously at a dose of 500 mg/kg to neonatal mice one hour before the ^{14}C-thymidine pulse. This treatment reduced the specific activity of DNA to less than 1% of the value found in controls and abolished differences between control and treated mice 24 hours after cyclophosphamide (Table 10). This observation indicated that the radioactivity associated with DNA isolated from neonatal mice was a result of incorporation rather than nonspecific binding. Day-old mice treated with 500 mg/kg hydroxyurea appeared normal and grew normally for at least 4 weeks despite the dramatic initial inhibition of DNA synthesis.

The incorporation of ^{14}C-uridine into total RNA was reduced in neonatal liver between 1 and 5 days after cyclophosphamide (Figure 7).

Figure 7. DNA, RNA and protein synthesis in the liver of cyclophosphamide treated animals expressed as a percent of control values. Mice received either 80 mg/kg cyclophosphamide or normal saline one day after birth and 100 μc/kg ^{14}C-labelled thymidine, uridine, or leucine at various times afterwards. Precursor incorporation into DNA, RNA and protein was measured at various times after treatment. The specific activity of macromolecules from treated animals was expressed as a percent of the values simultaneously determined in controls. The average control specific activities were: 39,000 DPM/mg DNA, 4000 DPM/mg RNA, and 1000 DPM/mg protein. The values represent the mean $\pm$ SE for 3 to 6 determinations. Open symbols indicate values that are significantly different from control ($P<0.05$).

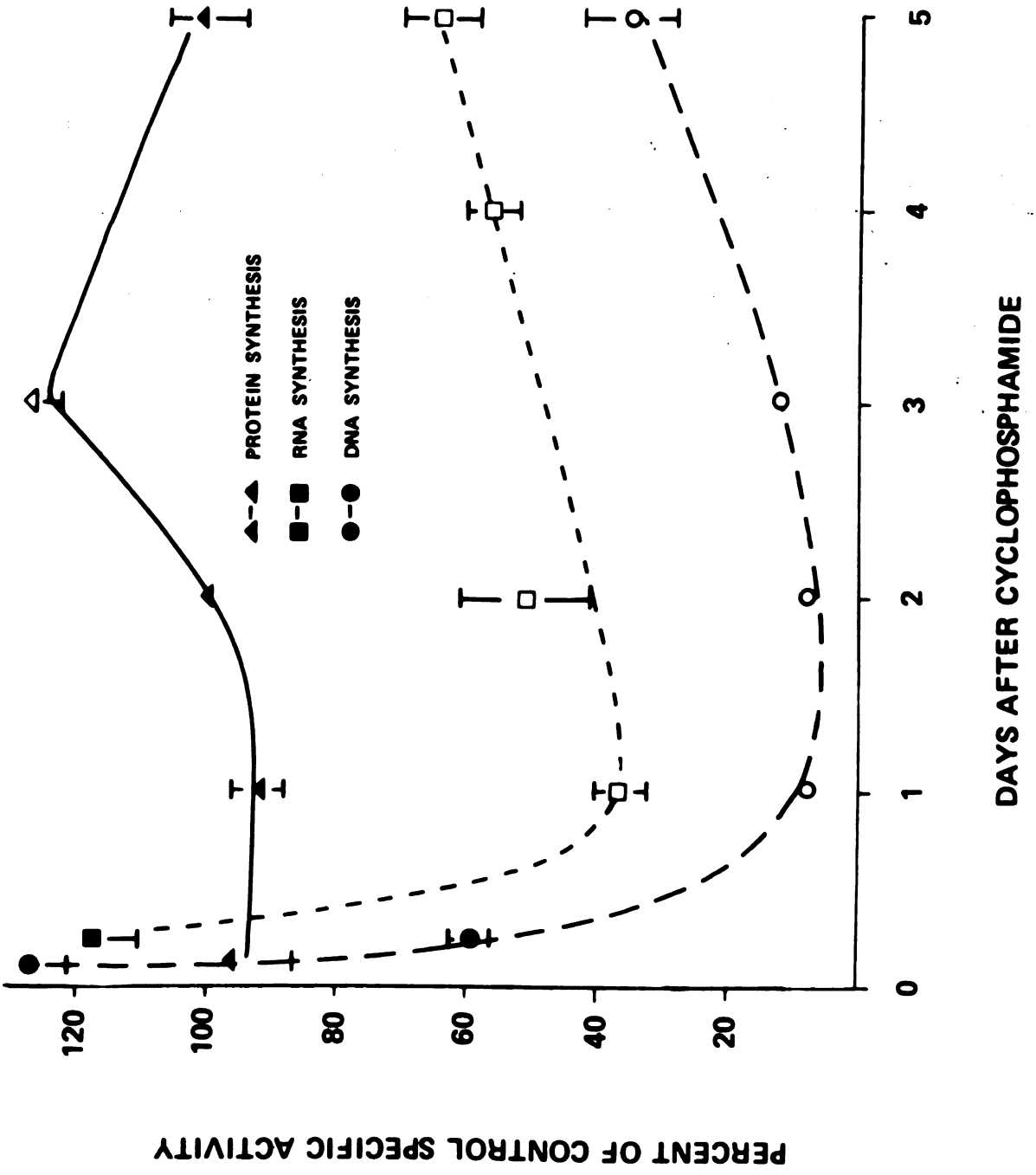
PERCENT OF CONTROL SPECIFIC ACTIVITY
120
100
80
60
40
20
0
1
2
3
4
5
DAYS AFTER CYCLOPHOSPHAMIDE
PROTEIN SYNTHESIS
RNA SYNTHESIS
DNA SYNTHESIS

Figure 7

Figure 8. DNA, RNA and protein synthesis in the brain of cyclophosphamide treated animals expressed as a percent of control values. See legend to Figure 7 for method. The average control specific activities were: 3000 DPM/mg DNA, 1000 DPM/mg RNA, and 500 DPM/mg protein.

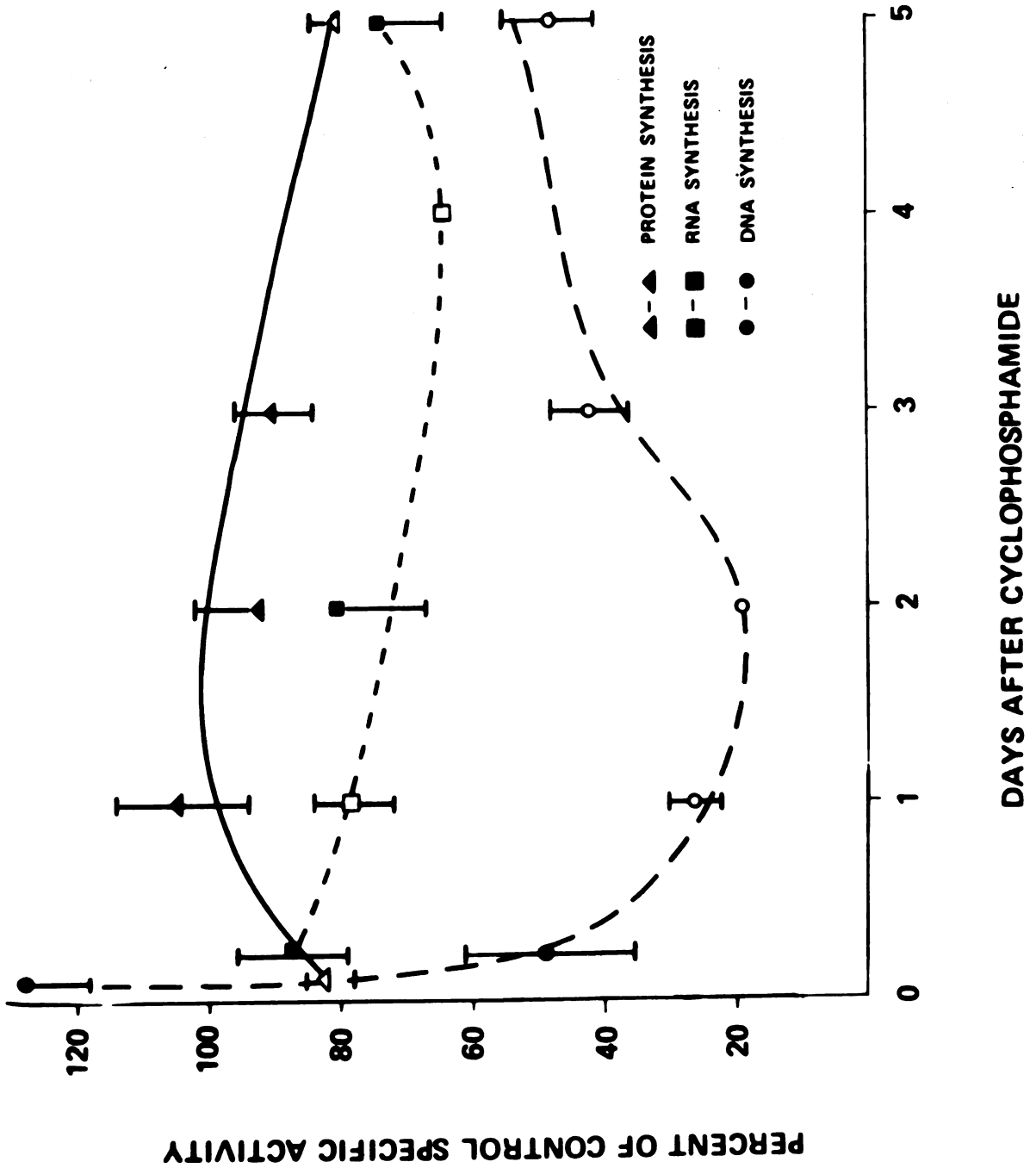
PERCENT OF CONTROL SPECIFIC ACTIVITY
120
100
80
60
40
20
0
1
2
3
4
5
DAYS AFTER CYCLOPHOSPHAMIDE
PROTEIN SYNTHESIS
RNA SYNTHESIS
DNA SYNTHESIS

Figure 8

Figure 9. DNA, RNA and protein synthesis in the carcass of cyclophosphamide treated animals expressed as a percent of control values. See legend to Figure 7 for method. The average control specific activities were: 15,000 DPM/mg DNA, 3000 DPM/mg RNA, and 1000 DPM/mg protein.

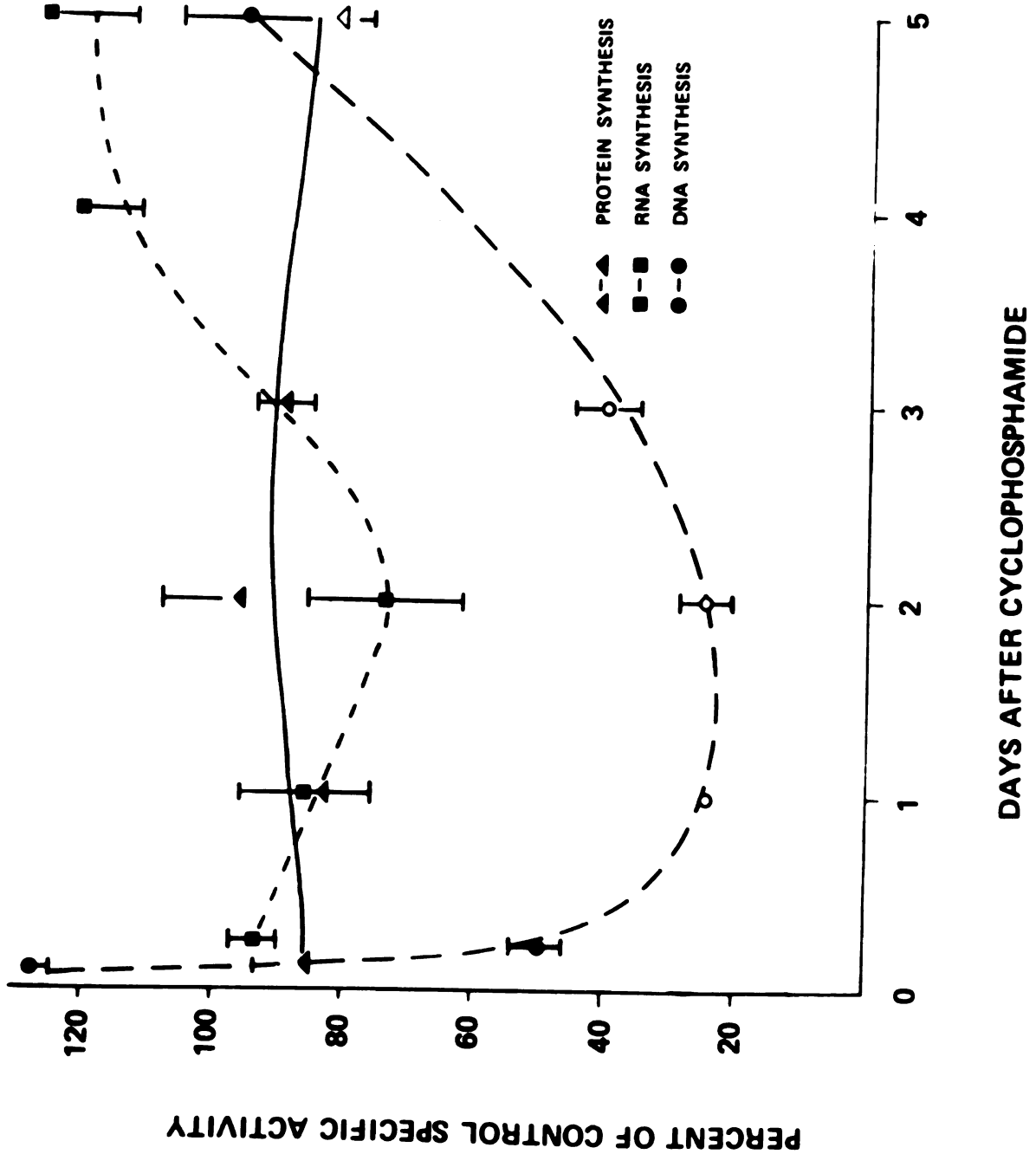
PERCENT OF CONTROL SPECIFIC ACTIVITY
120
100
80
60
40
20
0
1
2
3
4
5
DAYS AFTER CYCLOPHOSPHAMIDE
PROTEIN SYNTHESIS
RNA SYNTHESIS
DNA SYNTHESIS

Figure 9

Table 7. Concentration and content of DNA and RNA in neonatal liver after treatment with cyclophosphamide at one day of age

Time after treatment[a]	Concentration (mean mg/g wet weight ± SE)				Content (mean mg/organ ± SE)			
	DNA		RNA		DNA		RNA	
	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat
3 hr	10.5 ±0.4	9.4 ±0.7	---- ----	---- ----	0.80 ±0.02	0.68 ±0.05	---- ----	---- ----
6 hr	9.6 ±0.2	8.2 ±0.3	14.4 ±0.2	13.5 ±0.4	0.76 ±0.08	0.67 ±0.03	1.2 ±0	1.4 ±0.1
1 day	8.9 ±0.3	5.9[b] ±0.4	12.1 ±1.0	12.0 ±0.3	0.71 ±0.02	0.48[b] ±0.03	1.1 ±0.1	1.0
2 days	9.6 ±0.4	5.4[b] ±0.2	12.6 ±0.5	13.2 ±0.7	0.91 ±0.05	0.40[b] ±0.03	1.2 ±0.1	1.2 ±0.1
3 days	9.6 ±0.6	4.8[b] ±0.3	---- ----	---- ----	0.61 ±0.02	0.31[b] ±0.04	---- ----	---- ----
4 days	---- ----	---- ----	13.6 ±0.2	13.3 ±0.1	---- ----	---- ----	1.4 ±0.1	1.4 ±0.1
5 days	6.4 ±0.4	4.6[b] ±0.2	15.4 ±0.4	15.8 ±0.1	0.82 ±0.10	0.48[b] ±0.05	1.7 ±0.2	1.8 ±0.3

[a]Mice received cyclophosphamide 80 mg/kg or normal saline.

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

Table 8. Concentration and content of DNA and RNA in neonatal brain after treatment with cyclophosphamide at one day of age

Time after treatment[a]	Concentration (mean mg/g wet weight ± SE)				Content (mean mg/organ ± SE)			
	DNA		RNA		DNA		RNA	
	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat
3 hr	4.0 ±0.2	4.2 ±0.1	---- ----	---- ----			---- ----	---- ----
6 hr	4.8 ±0.1	4.6 ±0.1	4.6 ±0	4.5 ±0.2	0.41 ±0.02	0.37 ±0	0.47 ±.03	0.47 ±.03
1 day	3.7 ±0.1	3.5[b] ±0.1	4.0 ±0.2	3.8 ±0.1	0.39 ±0.01	0.38 ±0.01	0.40 ±0	0.37 ±.02
2 days	3.3 ±0.1	3.4 ±0.3	3.9 ±0.1	3.8 ±0.2	0.40 ±0.03	0.38 ±0.02	0.53 ±0.03	0.45 ±0.04
3 days	2.7 ±0.1	2.3[b] ±0.1	---- ----	---- ----	0.23 ±0.02	0.21 ±0.01	---- ----	---- ----
4 days	---- ----	---- ----	3.8 ±0.1	4.3[b] ±0.1	---- ----	---- ----	0.57 ±0.03	0.60 ±0
5 days	2.1 ±0.1	1.9 ±0.1	3.9 ±0.1	3.7 ±0.1	0.42 ±0.02	0.50 ±0.10	0.70 ±0.06	0.67 ±0.06

[a]Mice received cyclophosphamide 80 mg/kg or normal saline.

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

Table 9. Concentration of DNA and RNA in neonatal carcass after treatment with cyclophosphamide at one day of age

Time after treatment[a]	Concentration (mean mg/g wet weight ± SE)			
	DNA		RNA	
	Cont	Treat	Cont	Treat
3 hr	4.7 ±0.1	4.9 ±0.1	---- ----	---- ----
6 hr	2.9 ±0.9	4.8 ±0.1	5.8 ±0.2	6.1 ±0.2
1 day	4.3 ±0.2	4.0 ±0.1	4.6 ±0.1	4.6 ±0.1
2 days	3.4 ±0.3	3.6[b] ±0.2	5.7 ±0.2	5.5 ±0.2
3 days	3.8 ±0.3	2.7[b] ±0.1	---- ----	---- ----
4 days	---- ----	---- ----	6.3 ±0.2	5.3[b] ±0
5 days	4.1 ±0.2	2.8 ±0.1	6.4 ±0.1	5.5[b] ±0.2

[a]Mice received cyclophosphamide 80 mg/kg or normal saline.

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

Table 10. DNA specific activity in neonatal mice after hydroxyurea treatment[a]

Tissue	DNA Specific Activity		
	Relative[b] DPM/mg DNA	DPM/mg DNA	
	Control	Control	Treated[c]
Liver	0.27%[d] ±0.03	106 ±10	156 ±42
Placental	0.83% ±0.27	24 ±8	22 ±4
Embryo	0.54% ±0.05	78 ±7	58 ±12

[a]Day-old mice received 500 mg/kg hydroxyurea s.c. 1 hour before a 1 hour ^{14}C-thymidine pulse.

[b]Relative to mice that did not receive hydroxyurea.

[c]Cyclophosphamide 80 mg/kg s.c. 24 hours before hydroxyurea.

[d]Mean ± SE of 3 determinations.

RNA synthesis ranged from 36 to 64% of control between 1 and 5 days after treatment in the liver of treated mice. Liver RNA concentration and content were not affected by drug treatment (Table 7). RNA synthesis was reduced in brain to 78 and 64% of control on days 1 and 4 after treatment (Figure 8). RNA concentration was increased in brain only at 4 days after treatment (Table 9). RNA synthesis was not changed in carcass but the RNA concentration was reduced 4 and 5 days after treatment.

The incorporation of ^{14}C-leucine into total protein did not reveal a consistent pattern which would indicate that drug treatment altered protein synthesis. Protein synthesis was increased in the liver 3 days after treatment (Figure 7) and reduced in the brain at 3 hours and 5 days after treatment (Figure 8). Protein synthesis, in addition, was not significantly changed in the liver or brain at 7, 9, 12, or 21 days after treatment (Table 11).

Protein Turnover in Neonatal Mice

The differentiated state is ultimately dependent on the acquisition of adult tissue specific proteins. Agents which interfere with this process may disrupt normal development. The protein content of a tissue is dependent both on the synthesis of new proteins and the degradation of existing proteins. Since protein synthesis, as measured by ^{14}C-leucine incorporation into protein, was not affected in cyclophosphamide treated neonatal mice, the possibility remained that drug treatment altered the rate of protein degradation.

The specific activity of neonatal proteins labeled *in utero* with ^{14}C-guanido-L-arginine was measured as a function of time after cyclophosphamide in mice treated with 80 mg/kg s.c. of the drug one day after

Table 11. Protein synthesis in mice treated with cyclophosphamide

Days after treatment[b]	Relative specific activity of protein[a] Liver	Brain
7	120[c] ±20	86 ±7
9	82 ±7	74 ±13
12	106 ±7	102 ±3
21	132 ±25	118 ±11

[a]30 minute pulse of 100 μc/kg ^{14}C-leucine.

[b]Cyclophosphamide 80 mg/kg 24 to 48 hours after birth.

[c]Mean ± SE (2 to 3 observations) of protein specific activity expressed as a percent of control.

birth. Protein turnover, as measured by the decline in protein specific activity, refers to the combined synthesis and degradation of tissue constituents (Arias *et al.*, 1969). Cyclophosphamide significantly affected protein turnover in neonatal mice (Tables 12 and 13). The half-life of total soluble protein, which was the time required for the protein specific activity one day after treatment to decline by 50%, was significantly decreased in the liver, brain, and carcass of drug treated neonates (Table 13). A reduced half-life of particulate protein was measured in the liver and brain after cyclophosphamide treatment.

Isotope re-utilization which occurs as a result of protein degradation is a problem in estimating protein half-life. This problem was minimized in protein turnover studies by the use of ^{14}C-guanido-L-arginine which was metabolized by arginase to give ^{14}C-urea with the result that the label was not re-utilized for protein synthesis (Arias *et al.*, 1969). The half-life of total organ protein radioactivity, therefore, is an estimation of protein degradation assuming minimum re-utilization of the isotope. Cyclophosphamide significantly affected protein degradation in neonatal mice (Tables 14 and 15). The half-life of soluble and particulate protein was decreased in the liver but not the brain after drug treatment.

Protein half-lifes as calculated from the decay in protein specific activity and total organ protein radioactivity are summarized in Table 16. The temporal decrease in protein specific activity is attributed both to the synthesis of new proteins and the degradation of existing proteins. The decrease in total organ protein radioactivity, on the other hand, is due to protein degradation. The difference between these two calculations of half-life are dramatically illustrated in the liver.

Table 12. Equations for the calculated least squares regression line of log protein specific activity as a function of days after cyclophosphamide

Protein Fraction[c]	Equation[d]		r[e]	
	Control	Treated	Control	Treated
Liver				
Soluble	y=3.10-0.13x	y=3.41-0.19x	0.98	0.89
Particulate	y=3.17-0.12x	y=3.50-0.19x	0.98	0.86
Brain				
Soluble	y=3.28-0.09x	y=3.53-0.13x	0.98	0.82
Particulate	y=3.28-0.08x	y=3.59-0.12x	0.98	0.77
Carcass				
Soluble	y=3.29-0.13x	y=3.58-0.17x	0.99	0.86
Particulate	y=3.53-0.14x	y=3.70-0.17x	0.98	0.78

[a] ^{14}C-guanido-L-arginine was administered i.p. to the mother prior to birth.

[b] Cyclophosphamide (80 mg/kg s.c.) or normal saline was administered to neonatal mice 1 day after birth.

[c] Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet. The specific activity (DPM/mg protein) of each fraction was measured as a function of days after cyclophosphamide treatment.

[d] The equations were calculated by least squares regression analysis for 3 observations made at 1, 3, 5 and 10 days for control liver and brain (N=12) or 1, 3 and 5 days for carcasses and treated liver and brain (N=9).

[e] Regression coefficient.

Table 13. Half-life of protein from neonatal mice after cyclophosphamide as estimated by the change in protein specific activity[a,b]

Protein Fraction[c]	Half-life (days) Control	Treated	95% Confidence Interval of Slope[d] Control	Treated
Liver				
Soluble	2.4	1.5	-0.125 to -0.135	-0.162 to -0.221
Particulate	2.6	1.6	-0.113 to -0.122	-0.156 to -0.225
Brain				
Soluble	3.7	2.3	-0.092 to -0.099	-0.113 to -0.156
Particulate	4.1	2.5	-0.074 to -0.080	-0.095 to -0.156
Carcass				
Soluble	2.4	1.7	-0.124 to -0.136	-0.143 to -0.203
Particulate	2.2	1.9	-0.127 to -0.144	-0.127 to -0.206

[a]Protein half-life was estimated from the least squares regression line of log protein specific activity vs. days after treatment (Table 12).

[b]Day-old mice received either 80 mg/kg s.c. cyclophosphamide or normal saline.

[c]Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet.

[d]Equations for the lines are given in Table 12.

Table 14. Equations for the calculated least squares regression line of log protein radioactivity per organ vs. days after treatment[a,b]

Protein Fraction[c]	Equation[d]		r[e]	
	Control	Treated	Control	Treated
Liver				
Soluble	y=3.70-0.06x	y=4.06-0.16x	0.80	0.86
Particulate	y=4.05-0.05x	y=4.34-0.15x	0.80	0.84
Brain				
Soluble	y=3.79-0.07x	y=4.03-0.10x	0.76	0.73
Particulate	y=4.43-0.13x	y=4.33-0.09x	0.62	0.68

[a]14-guanido-L-arginine was administered i.p. to the mother prior to birth.

[b]Cyclophosphamide (80 mg/kg s.c.) or normal saline was administered to neonatal mice 1 day after birth.

[c]Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet. Incorporated radioactivity per organ was measured as a function of days after cyclophosphamide treatment.

[d]The equations were calculated by least squares regression analysis for 3 observations made at 1, 3 and 5 days (N=9) after treatment.

[e]Regression coefficient.

Table 15. Half-life of protein from neonatal mice after cyclophosphamide as estimated by the change in organ protein radioactivity[a,b]

Protein Fraction[c]	Half-life (days) Control	Half-life (days) Treated	95% Confidence Interval of Slope Control	95% Confidence Interval of Slope Treated
Liver				
Soluble	6.0	1.8	-0.0426 to -0.0672	-0.1317 to -0.1866
Particulate	6.4	2.0	-0.0377 to -0.0589	-0.1512 to -0.2090
Brain				
Soluble	4.0	4.0	0.0541 to 0.0905	-0.0704 to -0.1256
Particulate	2.2	2.2	0.0834 to 0.1853	-0.0587 to -0.1146

[a]Protein half-life was estimated from the least squares regression line of log organ radioactivity vs. days after treatment (Table 14).

[b]Day-old mice received either 80 mg/kg s.c. cyclophosphamide or normal saline.

[c]Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet.

[d]Equations for the lines are given in Table 14.

Table 16. Half-life (days) of proteins from neonatal mice after cyclophosphamide

Tissue Fraction[a]	Protein Specific Activity[b]		Organ Protein Radioactivity[c]	
	Control	Treated	Control	Treated
Liver				
Soluble	2.4	1.5[d]	6.0	1.8[d]
Particulate	2.6	1.6[d]	6.4	2.0[d]
Brain				
Soluble	3.7	2.3[d]	4.0	4.0
Particulate	4.1	2.5[d]	2.2	2.2
Carcass				
Soluble	2.4	1.7[d]	---	---
Particulate	2.2	1.9	---	---

[a]Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet.

[b]Table 13.

[c]Table 15.

[d]95% confidence limits for the slope of the least squares regression line for control and treated observations do not overlap.

The half-life of liver soluble protein in control mice was increased from 2.4 to 6.0 days when protein synthesis was excluded from the calculations.

The yield of soluble and particulate protein from the liver and brains of neonatal mice were measured as a function of days after treatment during the half-life studies (Table 17). There was a significant reduction in both soluble and particulate total organ protein in the liver at 5 but not at 1 or 3 days after cyclophosphamide. There was no significant change in brain total organ protein or the amount of protein per unit tissue weight in the liver or brain at any of the times studied. Cyclophosphamide treatment, therefore, affected the amount of protein present in neonatal mice but the detection of the effect was dependent on both the tissue and the expression of the data.

Qualitative Changes in Neonatal Protein Synthesis

Differential gene expression, which results in characteristic tissue specific protein patterns, is an important developmental process. Agents which disrupt the developmentally programmed expression of genetic information may produce abnormal development. Although cyclophosphamide did not affect protein synthesis quantitatively, as measured by ^{14}C-leucine incorporation into total protein, drug treatment may have affected the synthesis of specific proteins. Drug induced qualitative changes in protein synthesis, as a result, may have been obscured when protein synthesis was examined quantitatively.

Electrophoretic Analysis of Protein Synthesis. The proposal that postnatal development was disrupted as a result of a change in the synthesis of specific proteins was evaluated by double label isotope

Table 17. Soluble and particulate protein in the liver and brain of neonatal mice after cyclophosphamide

Tissue[a] Fraction	Days after Treat[b]	mg Protein/Organ Control	mg Protein/Organ Treated	mg Protein/g Tissue Control	mg Protein/g Tissue Treated
Liver					
Soluble	1	4.7 ± 0.6[c]	4.7 ± 0.4	59 ± 2	62 ± 2
	3	7.6 ± 0.4	6.0 ± 0.4[d]	64 ± 4	66 ± 5
	5	12.2 ± 1.3	6.4 ± 0.5[d]	67 ± 2	74 ± 3
Particulate	1	8.0 ± 0.8	7.0 ± 0.3	99 ± 3	92 ± 2
	3	12.6 ± 0.5	10.5 ± 1.0[d]	106 ± 4	114 ± 3
	5	19.7 ± 1.4	10.2 ± 0.5[d]	109 ± 1	120 ± 9
Brain					
Soluble	1	3.1 ± 0.2	3.5 ± 0.3	28 ± 1	30 ± 2
	3	4.2 ± 0.2	4.1 ± 0.1	27 ± 1	28 ± 1
	5	5.0 ± 1.0	4.9 ± 0.2	31 ± 1	29 ± 1
Particulate	1	5.9 ± 0.3	6.2 ± 0.2	54 ± 3	54 ± 1
	3	8.5 ± 0.8	6.7 ± 0.3	54 ± 5	47 ± 1
	5	8.9 ± 1.4	8.9 ± 0.5	57 ± 3	54 ± 2

[a]Soluble protein was obtained from the 105,000 g supernatant and particulate protein was obtained from the pellet.

[b]Day-old mice received either 80 mg/kg s.c. cyclophosphamide or normal saline.

[c]Mean ± SE for 3 observations.

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

techniques and polyacrylamide gel electrophoresis. An *in vitro* incubation was used to label the newly synthesized proteins since preliminary experiments indicated that proteins from mice treated with 100 μc/kg of ^{14}C(UL)-L-leucine (197 mc/mMole), 100 μc/kg of ^{14}C(UL)-L-amino acid mixture (1.1 mc/mg), or 500 μc/kg ^{3}H-4,5-L-leucine (50 μc/mMole) for 1 hour periods did not have sufficient specific activity for polyacrylamide gel electrophoresis.

The ability of the methodology employed in this study to detect qualitative changes in protein synthesis was evaluated by comparing protein synthesis in neonatal brains and livers. The distribution of the percent of total ^{3}H and ^{14}C incorporated into protein in the gel fraction indicated that different proteins were synthesized in the liver and brain (Figure 10). These differences were more apparent when the ^{3}H/^{14}C ratio was plotted for each fraction. The magnitude of the differences observed in comparing liver and brain protein synthesis were used as a guide to evaluate possible qualitative changes in protein synthesis produced by cyclophosphamide. The distribution of gel radioactivity from liver protein of control and cyclophosphamide treated mice were similar at 1 and 3 days after treatment (Figures 11 and 12). Apparent differences in the distribution of protein radioactivity were not maintained when the tissue was incubated with the other isotope. Qualitative changes in protein synthesis, as measured by the ^{3}H/^{14}C ratio observed when liver and brain protein synthesis was compared, were not evident in livers from cyclophosphamide treated mice. Cyclophosphamide treatment therefore did not affect the synthesis of specific liver proteins as measured within the resolving power of the methodology employed in this study.

Figure 10. Electrophoretic analysis of proteins synthesized *in vitro* by neonatal brain (^{3}H-leucine) and liver (^{14}C-leucine). The $^{3}H/^{14}C$ ratio was calculated on the basis of percent of total radioactivity present in each fraction.

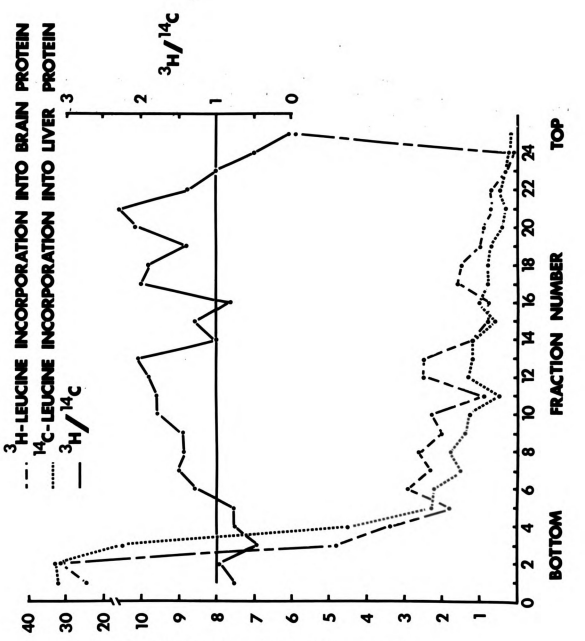
^{3}H-LEUCINE INCORPORATION INTO BRAIN PROTEIN
^{14}C-LEUCINE INCORPORATION INTO LIVER PROTEIN
^{3}H/^{14}C
^{3}H/^{14}C
PERCENT OF TOTAL RADIOACTIVITY
FRACTION NUMBER
BOTTOM
TOP

Figure 10

Figure 11. Electrophoretic analysis of proteins synthesized *in vitro* by livers from cyclophosphamide treated mice (^{3}H-leucine) or livers from control mice (^{14}C-leucine) one day after treatment. The $^{3}H/^{14}C$ ratio was calculated on the basis of percent of total radioactivity present in each fraction.

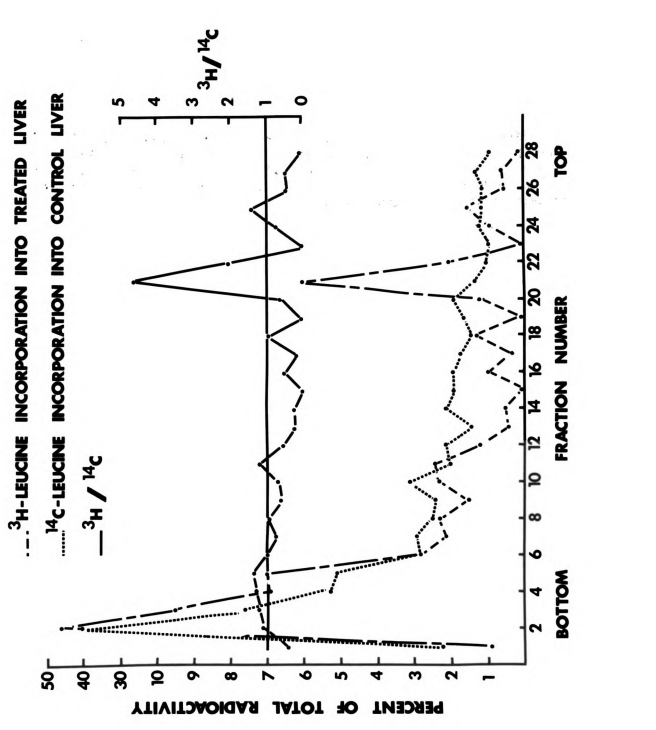
^{3}H-LEUCINE INCORPORATION INTO TREATED LIVER
^{14}C-LEUCINE INCORPORATION INTO CONTROL LIVER
^{3}H / ^{14}C
PERCENT OF TOTAL RADIOACTIVITY
^{3}H/^{14}C
BOTTOM
FRACTION NUMBER
TOP

Figure 11

Figure 12. Electrophoretic analysis of proteins synthesized *in vitro* by livers from control (^{3}H-leucine) or cyclophosphamide treated (^{14}C-leucine) mice 3 days after treatment. The $^{3}H/^{14}C$ ratio was calculated on the basis of percent of total radioactivity present in each fraction.

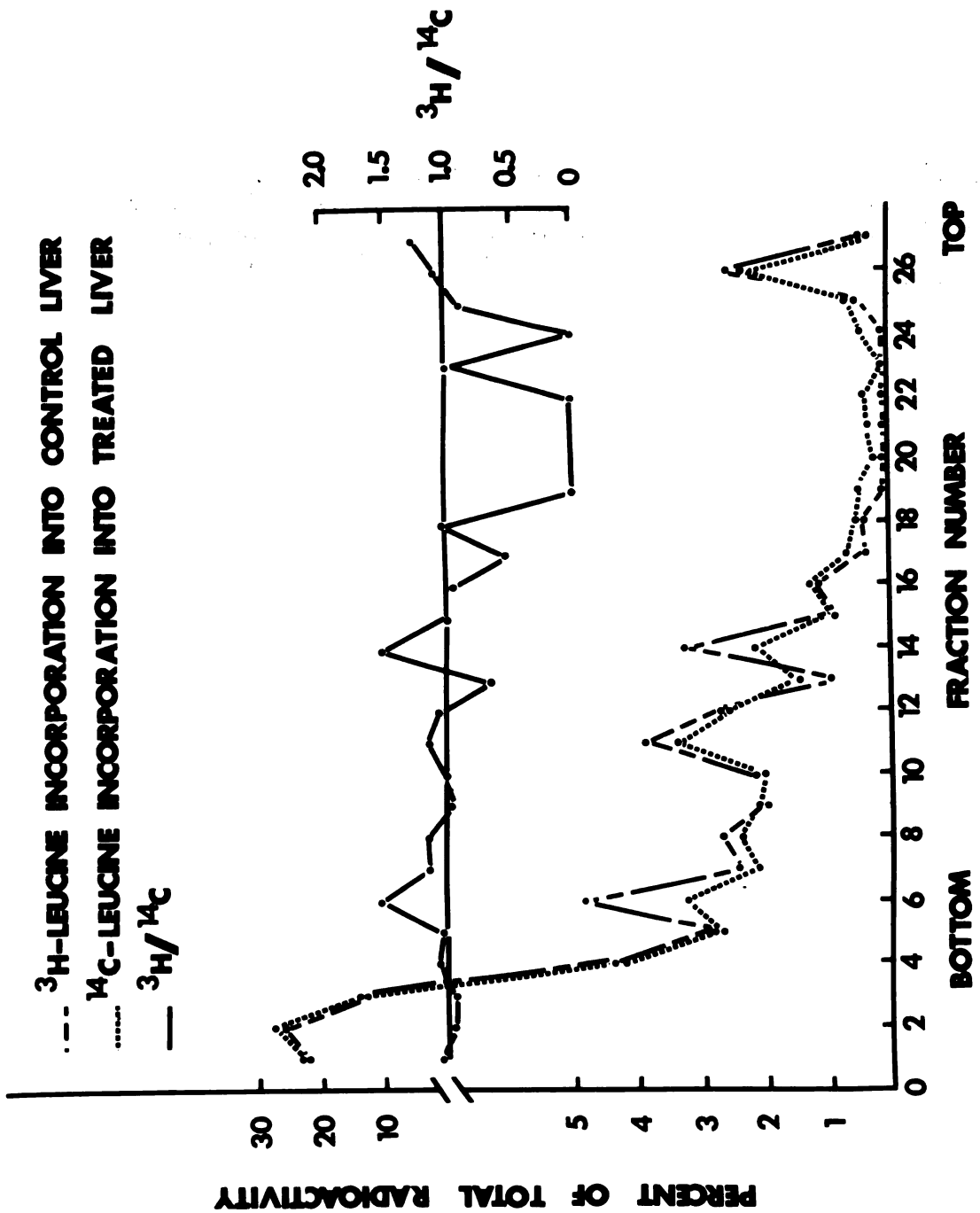
---- ^{3}H-LEUCINE INCORPORATION INTO CONTROL LIVER
..... ^{14}C-LEUCINE INCORPORATION INTO TREATED LIVER
— ^{3}H/^{14}C
PERCENT OF TOTAL RADIOACTIVITY
30
20
10
5
4
3
2
1
0
2.0
1.5
1.0
0.5
0
^{3}H/^{14}C
BOTTOM
2
4
6
8
10
12
14
16
18
20
22
24
26
TOP
FRACTION NUMBER

Figure 12

Developmental Changes in Glucose-6-Phosphatase Activity. Enzymic differentiation during postnatal development provides a method for assessing the ability of an animal to respond to developmental cues and increase the level of enzyme activity. Glucose-6-phosphatase activity gradually increased in the rat liver 3 days before birth and then precipitously increased immediately after birth (Greengard, 1969). Enzyme activity ranged from 150 to 200% of the activity found in adult male rats between 1 and 2 days after birth. Neonatal mice had increased levels of glucose-6-phosphatase activity which were about 125% of the activity found in maternal livers (Table 18). There was, in addition, no significant difference between the levels of enzyme activity in maternal or virgin female livers. Glucose-6-phosphatase activity was significantly increased in livers from both control and treated neonatal mice, relative to adults, at 3 and 5 days of age. Cyclophosphamide treatment at 1 or 2 days of age did not affect the level of enzyme activity relative to control at 1 or 3 days after treatment. Cyclophosphamide, therefore, did not affect the increased levels of glucose-6-phosphatase which occurred during enzymic differentiation in the mouse.

Table 18. Glucose-6-phosphatase activity in the livers of treated neonatal mice relative to the enzyme activity in maternal liver

Age at treatment[a] (days)	Age at sacrifice (days)	Relative Enzyme Activity[b]		
		Maternal	Control	Treated
1	2	100 ± 10[c] (4)	122 ± 6 (4)	126 ± 12 (4)
2	3	100 ± 6 (3)	130 ± 7[d] (4)	142 ± 8[d] (4)
2	5	100 ± 2 (4)	126 ± 5[d] (3)	129 ± 6[d] (4)

[a]Cyclophosphamide 80 mg/kg s.c. or normal saline.

[b]Average glucose-6-phosphatase activity in the maternal liver (see Methods) was 1.06 ± 0.18 mmoles Pi/mg protein and was set equal to 100 for the determination of relative activity.

[c]Mean ± SE (number of observations).

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

DISCUSSION

Introduction

The purpose of this study was to examine the molecular basis of drug-induced disruption of development in mice. Cyclophosphamide was the agent selected for these studies because of its potent toxic properties during both embryonic and postnatal development. A single dose of cyclophosphamide disrupted embryonic and postnatal development in mice on days 10 through 15 of gestation (Gibson and Becker, 1968a). A broad spectrum of abnormalities were produced which included gross defects, skeletal anomalies, and soft tissue malformations. Postnatal development in mice was also affected by single doses of cyclophosphamide 24 to 48 hours after birth (Nordlinder, 1969; Short and Gibson, 1971a). Treated mice grew at a reduced rate with increased mortality and were morphologically abnormal at maturity. Cyclophosphamide induced disruption of both embryonic and postnatal development, therefore, was the model system selected to study the molecular basis of drug-induced disruption of development.

A fertilized egg gives rise to an adult organism during the ontogenetic development of an animal. Important developmental processes associated with this transformation include cell division, differential gene expression, metabolic activities, and morphogenic cell movements. Treatments which interfere with these processes may result in abnormal development (see Introduction). Cyclophosphamide was shown in the

present study to interfere with several of the important developmental processes.

Inhibition of Cell Division

Cell division is an important aspect of growth in all living systems. Normal growth occurred in three phases which lasted for variable times in rat organs (Winick and Noble, 1965). Organs grew, during the first phase, entirely by cell division. The second phase consisted of growth both by cell division and cell enlargement. During the last phase organs grew only by cell enlargement. These observations concerning growth patterns were supported in the present study. An increased percent of dividing cells (i.e., growth fraction), as measured by an increased ^{14}C-thymidine incorporation into DNA, was observed in embryos and placentae, relative to maternal liver (Table 1). There was, in addition, an indication that the growth fraction of embryonic cells decreased over the observation period. The specific activity of DNA in neonatal tissue suggested that the growth fraction decreased in the order liver, carcass, and brain with liver and carcass equivalent to embryo (Figures 7, 8 and 9). Problems associated with extrapolating growth fractions from DNA specific activities include possible variability in specific activities of the DNA precursor pool and different methodology for measuring DNA specific activities. The data, however, were consistent with the observation that an increased population of dividing cells was associated with early organ growth.

Agents which reduced the population of dividing cells by inhibition of DNA synthesis disrupted normal development. Doses of cytosine arabinoside and hydroxyurea which inhibited DNA synthesis in embryos between 1 and 24 hours after treatment produced anomalies in rats (see

Introduction). DNA synthesis, as measured by ^{14}C-thymidine incorporation into DNA, was reduced in both embryonic and neonatal mice by doses of cyclophosphamide which disrupted normal development. Embryonic DNA synthesis (Figure 1) was reduced only at 12 hours after cyclophosphamide, and DNA synthesis in neonatal liver (Figure 7) and brain (Figure 8) was reduced 1 day after treatment and remained reduced for at least 4 days. The temporal aspects of this biochemical lesion may be related both to the dose and the pharmacokinetic properties of the drug.

The pharmacokinetic properties of cyclophosphamide differed in neonatal and adult mice (Bus *et al.*, 1973). Radioactivity in the plasma of neonatal mice, after ^{14}C-cyclophosphamide, reached peak values 32 minutes after treatment and was eliminated by apparent first-order kinetics. The half-life of plasma radioactivity was 8.8 hours and the time for elimination of 99% of the radioactivity from plasma was calculated to be 2.4 days. Neonatal mice had an ability to metabolize cyclophosphamide since the parent compound represented 76 and 44% of the total plasma radioactivity at 1 and 8.5 hours after treatment, respectively. The half-life for the elimination of radioactivity from the liver and brain was 7 and 11 hours, respectively (unpublished observation, this laboratory). The elimination of plasma radioactivity from adults, on the other hand, was rapid and biphasic with an initial half-life of 1.9 hours.

The pharmacokinetic properties of a teratogenic dose of ^{14}C-cyclophosphamide during embryonic development suggested that the maternal system provided a route for the rapid elimination of cyclophosphamide from the embryo (Gibson and Becker, 1971a). Radioactivity in maternal plasma and embryos reached peak levels between 16 and 32 minutes after ^{14}C-cyclophosphamide and attained equilibrium at very

low levels of radioactivity by 256 minutes after treatment. Cyclophosphamide metabolites were produced more rapidly in the maternal-embryonic system, since the parent compound represented 11, 10, and 27% of the total radioactivity in maternal plasma, urine, and embryo, respectively, 32 minutes after ^{14}C-cyclophosphamide. Approximately 90% of the injected dose was excreted by the mother in urine and feces 24 hours after ^{14}C-cyclophosphamide.

The present studies indicated that a teratogenic dose of ^{14}C-cyclophosphamide, which had a 20-fold higher specific activity than the ^{14}C-cyclophosphamide used by Gibson and Becker (1971a), produced acid soluble radioactivity in the liver, placenta, and embryo (Figure 2). Radioactivity in the acid soluble fraction represented both cyclophosphamide and its metabolites which were not covalently bound to acid precipitable molecules. The initial acid soluble radioactivity, which decreased in the order liver, placenta, and embryo, declined with time and approached equivalent equilibrium values in the three tissues. These observations support a maternal role in cyclophosphamide clearance from the embryo.

Cyclophosphamide induced disruption of development was correlated with the presence of its metabolites. Drug pretreatment which increased or decreased the cyclophosphamide activating ability of the newborn produced a corresponding increase or decrease in cyclophosphamide toxicity (Bus *et al.*, 1973). The slow elimination of cyclophosphamide in neonatal, as compared to adult, mice may be due, in part, to the poorly developed capacity of these animals to form readily excreted polar metabolites (Short and Gibson, 1971a). Neonatal mice, however, apparently had a sufficient capacity to form toxic metabolites since metabolites were present in the plasma and drug pretreatments which

modified cyclophosphamide metabolism provided a correlation between metabolites and toxicity. The slow release of alkylating metabolites over a prolonged period appeared to be an important aspect of cyclophosphamide neonatal toxicity. A dose of nor-nitrogen mustard, which was equimolar with a toxic cyclophosphamide dose, was cleared from the plasma within one hour and failed to affect the growth and development of mice (Short and Gibson, 1971b).

Cyclophosphamide teratogenicity was correlated with the parent compound in studies which used phenobarbital and SKF 525-A to modify the metabolism of cyclophosphamide (Gibson and Becker, 1968b). Recent evidence, however, has implicated cyclophosphamide metabolites and the alkylation of DNA in the disruption of normal embryonic development. The increased teratogenicity of cyclophosphamide in SKF 525-A pretreated mice was attributed (Bus *et al.*, 1973) to an increased placental transfer and embryonic pool of unmetabolized cyclophosphamide (Gibson and Becker, 1971a) which slowly gave rise to teratogenic alkylating metabolites. Teratogenicity, in addition, was correlated with ^{3}H-cyclophosphamide alkylation of embryonic DNA rather than RNA or protein (Murthy *et al.*, 1971).

The reduction in DNA synthesis observed in embryonic and neonatal mice after cyclophosphamide was consistent with a proposal that development was disrupted by a mechanism dependent on the presence of alkylating metabolites. This proposal was consistent with observations that DNA synthesis, in a variety of systems, was sensitive to both nitrogen mustard alkylating agents as well as cyclophosphamide (see Introduction). Alkylating agents were proposed to inhibit DNA synthesis by covalently interacting with the DNA duplex and preventing strand separation during replication (Roberts *et al.*, 1968). Methodology employed in the present

study did not permit a sufficient yield of radioactive macromolecules to examine the temporal aspects of ^{14}C-cyclophosphamide alkylation of embryonic DNA, RNA, and protein (Table 3).

A teratogenic dose of ^{14}C-cyclophosphamide produced alkylation of embryonic macromolecules as measured by the presence of ^{14}C-cylophosphamide radioactivity in an acid precipitate (Figure 3). The reduced specific activities of embryonic macromolecules (Figure 3 and Tables 4 and 5) and ratio of bound to free drug in the embryo (Figure 4) relative to the adult were indicative of low embryonic levels of alkylating metabolites. Low levels of cyclophosphamide metabolites in the embryo were due either to a placental barrier to the transport of alkylating metabolites (Gibson and Becker, 1971a) or to a poorly developed ability of embryonic mice to metabolically activate the parent compound (Short and Gibson, 1971a). The specific activity of the acid precipitate which decreased in the order liver, placenta and embryo declined with time (Figure 3). The specific activity of the hot acid nucleic acid extract suggested that radioactivity was eliminated from the nucleic acids of the liver and placenta at a more rapid rate than from embryonic nucleic acids (Table 4). The more rapid decline of nucleic acid specific activity (DPM/mg DNA) in the liver relative to the embryo was not due entirely to the synthesis of new DNA since DNA synthesis was greater in embryonic than adult tissue (Table 1). The temporal decline in nucleic acid specific activity suggested that embryos may have a poorly developed ability to repair DNA damaged by alkylating agents. This conclusion was supported by an analysis of dose-embryolethality data which indicated that there was no minimal embryopathic dose for cyclophosphamide and nor-nitrogen mustard (Jusko, 1972). The lack of a

minimally toxic dose suggested that embryos may have a poorly developed capacity to repair damaged DNA.

Doses of cyclophosphamide which disrupted embryonic and neonatal development, in summary, reduced the population of dividing cells as measured by ^{14}C-thymidine incorporation into DNA. The evidence indicated that development was affected by a mechanism which was dependent on the presence of alkylating metabolites. The more prolonged inhibition of DNA synthesis in neonatal, relative to embryonic, mice was probably due to the more prolonged exposure of these animals to alkylating metabolites which were formed from the slowly eliminated parent compound.

Interference with Differential Gene Expression

Differential gene expression refers to the utilization of genetic information in the production of unique tissues with characteristic properties. This process includes both the transcription of specific messenger RNA molecules and the translation of these molecules into proteins characteristic of a particular tissue. Agents that interfered with the transcription of genetic information disrupted development in a variety of systems (see Introduction). These agents presumably acted to prevent an ordered sequence of gene transcription which was essential for normal development. As a result of insufficient genetic information, therefore, the tissues were unable to synthesize proteins essential for normal developmental functions.

A teratogenic dose of cyclophosphamide did not affect maternal liver, placental, or embryonic RNA synthesis as measured by ^{14}C-precursor incorporation into RNA, at 1 or 3 days after treatment (Short, 1971). There was a trend towards reduced RNA synthesis in

the embryo; however, the differences were not statistically significant. Cyclophosphamide treatment in neonatal mice, on the other hand, inhibited RNA synthesis in both the liver (Figure 7) and brain (Figure 8). The data in neonatal mice were consistent with the observation that cyclophosphamide inhibited the *in vivo* synthesis of RNA by hamster plasmacytomas at 24 and 48 hours after treatment (Wheeler and Alexander, 1969). The more dramatic inhibition of RNA synthesis in neonatal, relative to embryonic, mice may be attributed either to the larger dose of cyclophosphamide used in these studies or to the more prolonged exposure of these animals to alkylating metabolites as discussed above. A more prolonged exposure to alkylating metabolites, for example, may have inactivated a greater portion of the genome for RNA synthesis.

Protein synthesis, as measured by ^{14}C-leucine incorporation into protein, was studied to determine if drug treatment affected the ability of mice to synthesize proteins essential for normal development. Embryonic protein synthesis was not reduced until 3 and 4 days after treatment when the embryos were visibly deformed (Short, 1971). An inhibition of protein synthesis, therefore, was concluded to be a result rather than a cause of abnormal development. Protein synthesis in neonatal mice after cyclophosphamide did not reveal a consistent pattern which would indicate that drug treatment altered protein synthesis (Figures 7, 8 and 9), even though growth was reduced. The presence of messenger RNA molecules, which were long-lived, represented a small portion of total RNA synthesis, or whose synthesis was insensitive to alkylating agents may have maintained protein synthesis at normal values. Since these studies did not measure the specific activity of the intracellular amino acid pool variations in this parameter, on the other hand, may have masked drug induced changes in protein synthesis.

The possibility that cyclophosphamide affected the synthesis of specific proteins rather than total protein synthesis was tested by examining the electrophoretic pattern of newly synthesized proteins. The data indicated that within the resolving power of the methodology employed in this study, drug treatment did not affect the qualitative aspects of liver protein synthesis in neonatal mice (Figures 11 and 12). The levels of glucose-6-phosphatase activity were measured in the livers from cyclophosphamide treated neonatal mice to determine if drug treatment affected the synthesis of a specific enzyme protein. The levels of enzyme activity were significantly elevated in both control and treated neonatal mice relative to adult females and there was no indication of a drug effect (Table 18). Cyclophosphamide treatment, therefore, was concluded not to affect the qualitative aspects of protein synthesis in neonatal mouse livers.

The protein content of tissue is dependent both on the synthesis of new protein and the degradation of existing protein. The decline in specific activity of radioactive rat liver protein was used to evaluate drug effects on protein degradation which were observed to follow first order kinetics (Arias *et al.*, 1969). The decline in protein radioactivity after cyclophosphamide treatment was measured by two parameters in neonatal mice whose proteins were pre-labeled with ^{14}C-guanidoarginine. The decline in protein specific activity (DPM/mg protein) was attributed to both the synthesis and degradation of protein while the decline in organ radioactivity (incorporated DPM/organ) was due primarily to protein degradation. Protein half-life, as measured by the time required for the protein specific activity to decline to 50% of the initial value, was significantly reduced in the soluble and particulate fraction of neonatal mouse liver and brain as well as the soluble fraction from the carcass after cyclophosphamide treatment

(Table 16). The reduced half-life of proteins, as measured by the decline in protein specific activity, could be attributed to increased synthesis of new proteins, increased degradation of radioactive proteins, or a combination of both factors. Protein half-life, as measured by the decline in organ protein radioactivity, was reduced in the soluble and particulate fraction of the liver; however, no effect was observed in the brain after cyclophosphamide (Table 16). This drug induced reduction in half-life was attributed to an increased protein degradation assuming that the radioactive isotope was not re-utilized to a significant extent.

Protein half-life, as measured by the decline in both protein specific activity and organ radioactivity, revealed that cyclophosphamide affected both the synthesis and degradation of neonatal proteins. A comparison of the protein half-lives, as measured by these two parameters, indicated that cyclophosphamide primarily increased brain protein synthesis without affecting protein degradation. The drug-induced reduction of half-life of liver protein, on the other hand, could not be resolved into separate components of synthesis and degradation but rather appeared to be a combination of both increased protein synthesis and degradation. Drug treatment appeared, however, to have a greater effect on protein degradation in the liver as estimated by the greater reduction in half-life for organ protein radioactivity relative to the half-life for protein specific activity.

The changes in protein half-life and content in neonatal liver and brain may be explained in terms of the cytotoxicity of cyclophosphamide. Cyclophosphamide produced cell death, as measured by a reduced DNA concentration, in both the liver (Table 7) and brain (Table 8). The extent of cell death was more evident in the liver than in the brain. The DNA

concentration of the liver, for example, was more severely reduced than in the brain and there was about a 50% reduction in the liver DNA content. The brain DNA content, on the other hand, was not significantly affected by drug treatment. Cyclophosphamide was proposed to damage liver cells and produce cell death. Proteins, therefore, were eliminated more rapidly from the liver as a result of catabolic processes associated with cell death. The liver, in the present study, was not able to maintain tissue protein content by increased synthetic activities, with the result that organ protein was significantly reduced 5 days after treatment (Table 17). The reduction in organ protein was not due to a failure of the liver to grow since there was no significant drug effect on liver weight at this time (Table 6). The cytotoxicity of cyclophosphamide was not as pronounced in the brain and cell death did not appear to contribute to an increased degradation of proteins. The protein half-life studies indicated that there was an increased protein synthesis in the brains of drug treated neonates; however, there was no change in the amount of brain protein (Table 17). An increased rate of protein synthesis may have occurred in brain cells damaged but not destroyed by alkylating agents.

Conclusions

These studies demonstrate that pharmacokinetic studies, a consideration of metabolites, and an examination of biochemical lesions can contribute to a better understanding of drug disrupted development. Cyclophosphamide induced disruption of embryonic and postnatal development in mice was attributed to or correlated with the presence of alkylating metabolites (Murthy *et al.*, 1971; Bus *et al.*, 1973). The present study provided additional support for an alkylating mechanism

of developmental toxicity. A comparison of the pharmacokinetic properties of cyclophosphamide in both adult and neonatal mice provided an explanation for the increased toxicity of cytophosphamide in newborn mice (Bus *et al.*, 1973). The biochemical lesions observed in this study form the basis for conclusions concerning the mechanism by which cyclophosphamide, and drugs in general, may disrupt development.

Hydroxyurea and cytosine arabinoside, agents which reduced DNA synthesis in rat embryos, were proposed to produce their teratogenic effects by reducing the proliferating cell populations of specific tissues as a result of cell death (Scott *et al.*, 1971). As the tissues, which were sensitive to the action of these agents, reached more advanced stages of development they lacked a sufficient population or type of cells to conduct the genetically prescribed morphogenetic role of the tissue.

Cyclophosphamide reduced the population of proliferating cells, as measured by ^{14}C-thymidine incorporation into DNA, in both embryonic and neonatal mice. Drug treatment, in addition, was associated with cell death, as measured by a reduced DNA concentration and content. Cell death, as a normal developmental process, is an essential feature of the normal morphogenesis of many organs (Markert and Ursprung, 1971). The death and disintegration of strategically placed cells is important in the separation of digits, opening of the eyelids, and to the formation of the central canals of many ducts and organs. A more generalized drug induced cell death may cause a premature abnormal differentiation of certain organs. A teratogenic dose of cyclophosphamide, for example, produced an increased incidence of digital defects, embryos with open eyes, and hydrocephalus in mouse embryos (Gibson and Becker, 1968a). The drug effect on digital formation showed a high degree of

day-dependency and followed an orderly progression with time: polydactyly, syndactyly, ectrodactyly, and adactyly. Drug induced cell death, in addition, may have contributed to a premature opening of the eyelids. Damage to the proliferating layers of the brain produced disturbances in brain morphogenesis (Menkes *et al.*, 1970). Cell death, in addition to producing abnormal morphogenesis, also reduces the cell population. As a result of reduced cell populations tissue may be unable to conduct normal morphogenetic functions. A reduced population of proliferating cells, for example, may provide an explanation for the failure of palate shelves to move and fuse in cyclophosphamide treated mice (Gibson and Becker, 1968a).

The role of reduced DNA synthesis in cyclophosphamide neonatal toxicity was complicated by the observation that DNA synthesis was reduced by a dose of hydroxyurea which did not affect postnatal development. Since the reduction in DNA synthesis induced by hydroxyurea lasted only 24 hours in rat embryos, the difference between the effects of cyclophosphamide and hydroxyurea in neonatal mice may be due to the more prolonged inhibition of DNA synthesis produced by cyclophosphamide. Cyclophosphamide alternatively may have produced toxicity by a mechanism unrelated to the inhibition of DNA synthesis. This proposal was supported by the observation that teratogenic doses of cyclophosphamide (Figure 1) and hydroxyurea (see Introduction) inhibited DNA synthesis for different periods of time.

Agents which covalently interact with nucleic acids may disrupt differential gene expression during development. Alkylation of DNA may block the snythesis of a messenger RNA molecule which codes for a developmentally essential protein. DNA damaged by an alkylating agent may be repaired by a mechanism with low fidelity and consequently code

for faulty proteins. Corticosteroids produced cleft palates in embryonic mice. A three day latent period between the time of optimal drug sensitivity and palate closure was attributed to a reduced transcription of messenger RNA molecules which directed the synthesis of proteins essential for palate closure (Zimmerman, 1970). Since cyclophosphamide produced cleft palate with a similar latent period (Gibson and Becker, 1968a), drug treatment may have affected the transcription process. This proposal was supported by a reduced RNA synthesis in cyclophosphamide treated neonates. There was, however, no change in either RNA synthesis by embryonic mice or protein synthesis by embryonic and neonatal mice which would suggest that cyclophosphamide affected the transcription process. A cyclophosphamide induced disruption of the transcription process, as a general mechanism of drug action during development, therefore remains an unanswered question. The qualitative aspects of protein synthesis, in addition, indicated that drug treatment did not affect the synthesis of specific neonatal liver proteins. The resolving power of the methodology employed in this study, however, did not exclude the possibility that drug treatment affected the qualitative aspects of protein synthesis.

The acquisition of tissue specific proteins is an important aspect of differentiation. The protein content of a tissue is dependent both on the synthesis of new proteins and the degradation of existing proteins. Agents which interfere with either of these processes may disrupt the differentiation process and produce abnormal development. Cyclophosphamide increased neonatal protein degradation, as measured by a reduced protein half-life, and disrupted normal postnatal development. These observations were consistent with a proposal that drug treatment either produced cell death or labilized protein molecules for

degradation. The protein specificity of this effect, however, was not studied. Agents which affect protein turnover may change the level of essential proteins and disrupt regulated processes essential for development.

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