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^{14}C-Cyclophosphamide Alkylation of Mouse Embryo Macromolecules (37862)

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Cyclophosphamide is an alkylating agent that requires metabolic activation to produce cytotoxicity (1). The metabolic activation of cyclophosphamide occurs primarily in the liver (2) and requires an NADPH oxygen-dependent system located in the microsomal fraction (3). Mouse embryos and placentas have a poorly developed ability to metabolically activate the parent compound (4).

Cyclophosphamide was teratogenic in Swiss–Webster mice (5), and the degree of teratogenicity was modified by the extent of maternal metabolic activation (6.) The placental transfer of ^{14}C-cyclophosphamide was demonstrated in mice (7), and the binding of ^{3}H-cyclophosphamide to embryonic DNA, but not RNA or protein, was correlated with teratogenicity (8). The correlation between DNA alkylation and cyclophosphamide teratogenicity (8) was obtained by measuring drug binding to macromolecules after various doses of cyclophosphamide or modification of metabolic activation at only one time after treatment. The present studies were undertaken to evaluate the temporal aspects of the alkylation of embryonic macromolecules by cyclophosphamide.

Methods. Radioactive ring-labeled 5-^{14}C-cyclophosphamide (7.6–8.2 mCi/mmole, New England Nuclear, Boston, MA) was diluted with nonradioactive cyclophosphamide to give 250 μCi/10 mg cyclophosphamide (6.5 mCi/mmole). 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). A teratogenic dose (20 mg/kg) of cyclophosphamide (5), containing 500 μCi/kg of ^{14}C-cyclophosphamide, was administered intraperitoneally to pregnant Swiss–Webster mice on Day 11 of gestation.

Groups of 3–5 mice were sacrificed by cervical dislocation at various times after treatment. Approximately 100–300 mg of maternal liver, placental (pooled from each maternal animal), and embryonic tissue (pooled from each maternal animal) was homogenized in 5 ml of 0.2 *N* perchloric acid (PCA) that contained 1 m*M* nonradioactive cyclophosphamide to minimize reversible binding. The precipitate was washed with 3 successive 5-ml portions of 0.2 *N* PCA. Lipids were extracted from the precipitate with washes of absolute methanol which contained 1 *M* sodium acetate, methanol:chloroform (2:1), absolute ethanol, and two ether washes. Nucleic acids were extracted by heating the precipitate in 5 ml of 0.6 *N* PCA for 30 min at 70°. The precipitate was washed with 0.2 *N* PCA and dissolved in 0.1 *N* sodium hydroxide overnight at 40°.

Radioactivity in the initial acid-soluble hot-acid extract and protein fraction was 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). Radioactivity was measured after the addition of a toluene-base counting solution containing 5 g 2,5-diphenyloxazole (PPO),

200 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP), and 1 liter toluene. DNA was measured (9) in the hot-PCA extract and protein was measured (10) in the alkaline-solubilized precipitate. A nomograph (California Corp. for Biochemical Research) of the optical density at 260 and 280 nm was used to quantitate 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. Separate determinations were made for each maternal animal, and means and standard errors (SE) were calculated.

Results. Initial attempts to measure radioactivity in maternal and embryonic DNA and RNA isolated by a phenol technique (11) were unsuccessful due to the small amount of available tissue and an inability to obtain nucleic acids with sufficient specific activity for accurate determinations. The acid-precipitation procedure, however, provided readily detectable levels of radioactivity when small amounts of tissues were used.

Acid-soluble radioactivity was present in maternal liver, placenta, and embryo for at least 10 hr after ^{14}C-cyclophosphamide administration and was eliminated from all of the tissues studied in an apparent biphasic fashion (Fig. 1). Acid-precipitable radioactivity was present in all of the tissues studied during the 10-hr observation period (Fig. 2). The specific activity of the acid precipitate reached maximal values within 1.5 hr after treatment and declined with time. The decline in specific activity appeared to plateau toward the end of the observation period.

Radioactive molecules were solubilized from the acid precipitates of tissue by hot acid (Table I). The acid extract contained DNA, as measured by the diphenylamine reagent, and protein and nucleic acids, as measured by the optical density at 280 and 260 nm. The expression of radioactivity in terms of the amount of extracted DNA indi-

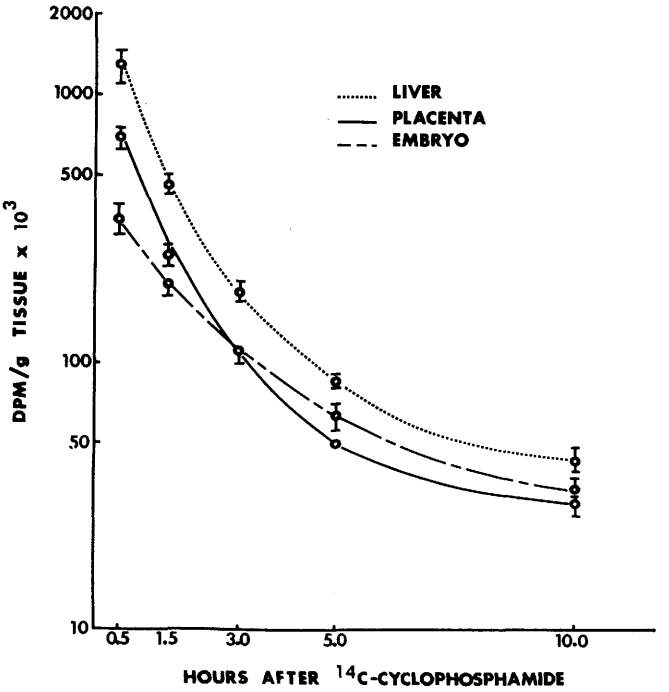

FIG. 1. Acid-soluble radioactivity in the maternal liver, placenta, and embryo as a function of time after 500 μCi/kg (20 mg/kg) ^{14}C-cyclophosphamide. The values plotted are the mean $\pm$ SE for observations on at least 4 maternal animals.

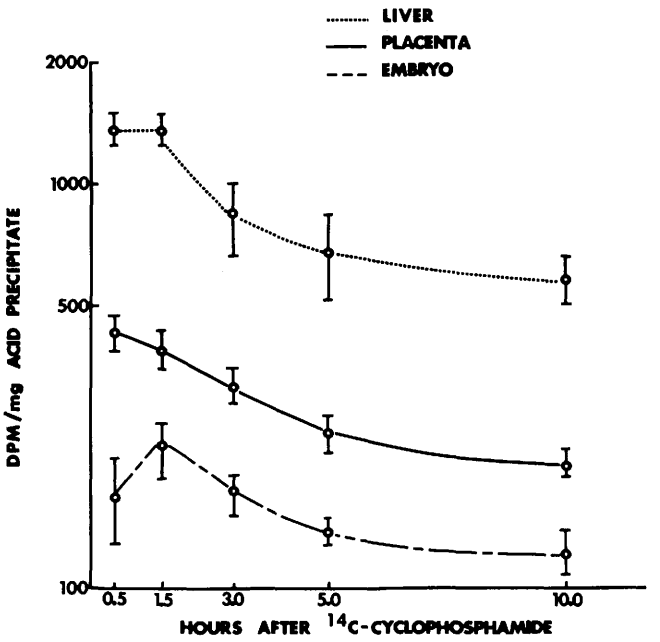

FIG. 2. Radioactivity in the acid-washed lipid-extracted acid precipitate from maternal liver, placenta, and embryo as a function of time after 500 μCi/kg (20 mg/kg) ^{14}C-cyclophosphamide. The values plotted are the mean ± SE for observations on 3–5 maternal animals.

cated that the specific activity of this fraction decreased in the order liver, placenta, and embryo at all of the times studied. Radioactivity expressed in terms of total nucleic acids was lower than the specific activity expressed in terms of 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 hr 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 also associated with the protein fraction (Table II). The specific activity of this fraction decreased in the order liver, placenta, and embryo. Radioactivity in both the protein and nucleic-acid fractions, expressed in terms of protein, indicated that the specific

TABLE I. ^{14}C-Cyclophosphamide Radioactivity in the Nucleic-Acid Extract from Maternal Liver, Placenta, and Embryo.[a]

Hours after ^{14}C-cyclophosphamide[b]	DPM/mg DNA[c] × 10^{-2}			DPM/mg NA[d] × 10^{-2}		
	Liver	Placenta	Embryo	Liver	Placenta	Embryo
0.5	126.8 ± 19.1[e]	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	6.8 ± 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 0.6 *N* perchloric acid for 30 min at 70°.

[b] 500 μCi/kg of ^{14}C-cyclophosphamide (20 mg/kg) was administered intraperitoneally 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 animals.

TABLE II. ^{14}C-Cyclophosphamide Radioactivity in the Protein and Nucleic-Acid Fractions from Maternal Liver, Placenta, and Embryo.

Hours after ^{14}C-cyclophosphamide[a]	Protein fraction[b] DPM/mg protein[c] × 10^{-2}			Nucleic-acid fraction[d] DPM/mg protein[e] × 10^{-2}		
	Liver	Placenta	Embryo	Liver	Placenta	Embryo
0.5	14.9 ± 0.8[f]	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 μCi/kg of ^{14}C-cyclophosphamide (20 mg/kg) was administered intraperitoneally on Day 11 of gestation.

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

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

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

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

[f] Mean ± SE for at least 3 animals.

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 hr after treatment.

Discussion. Cyclophosphamide was teratogenic in mice (5). There was conflicting evidence, however, as to whether toxicity was produced by the parent compound (6, 7, 4, 12) or alkylating metabolites (8). The observations made in the present study support the role of alkylating metabolites in the disruption of normal embryonic development.

The distribution of ^{14}C-cyclophosphamide in pregnant mice pretreated with either phenobarbital or SKF 525-A suggested that there was a placental barrier to the transport of cyclophosphamide metabolites (7). The observed differences in specific activity of the various tissue fractions from maternal liver, placenta, and embryo after ^{14}C-cyclophosphamide supported the existence of such a barrier. Metabolites of ^{14}C-cyclophosphamide were present in the embryo since there was alkylation of the embryonic acid precipitate, nucleic acids, and protein as measured by the presence of radioactivity in these fractions. The biochemical lesions observed in embryonic mice after a teratogenic dose of cyclophosphamide, in addition, were characteristic of alkylating agents (13). Alkylating cyclophosphamide metabolites may have crossed the placental barrier or have been generated within the embryo.

The decline in specific activity of the nucleic acid and protein fractions after ^{14}C-cyclophosphamide was used as an indication of repair processes which were reported for DNA damaged by alkylating agents (14). The embryo, relative to the maternal liver and placenta, had a poor ability to remove ^{14}C-cyclophosphamide radioactivity from the nucleic-acid fraction. These differences were not due entirely to the synthesis of new DNA since DNA synthesis, as measured by ^{14}C-thymidine incorporation, was greater in the embryo than the maternal liver (13). The specific activity of the protein fraction declined with time but there was no indication of a difference between maternal and embryonic tissue to remove ^{14}C-cyclophosphamide radioactivity.

A teratogenic dose produced an alkylation of embryonic macromolecules. Embryonic mice, in addition, were not able to remove ^{14}C-cyclophosphamide radioactivity from the nucleic-acid fraction as effectively as tissues from their mothers. Abnormal embryonic development, therefore, may be due in part either to an inability of the embryo to repair DNA damaged by alkylating agents or to an error-prone repair process which does not depend on the excision of regions of DNA damaged by alkylating agents. Abnormal development, in either case, may result from the transcription of faulty genetic informa-

tion and the disruption of a developmentally directed program of gene expression.

Summary. A teratogenic dose of ^{14}C-cyclophosphamide, an alkylating agent, was administered to pregnant mice to measure the alkylation of embryonic macromolecules as a function of time after treatment. Acid-soluble and acid-insoluble radioactivity was present in the maternal liver, placenta, and embryo during a 10-hr observation period after ^{14}C-cyclophosphamide. The alkylation of macromolecules was demonstrated by the presence of radioactivity in the acid precipitate and nucleic acid and protein fractions of the tissues. The decline in specific activity of the nucleic-acid fraction after ^{14}C-cyclophosphamide suggested that embryos had a poor ability, relative to the adult, to remove alkylated metabolite of ^{14}C-cyclophosphamide from this fraction. These studies indicated that a teratogenic dose of ^{14}C-cyclophosphamide produced alkylation of embryonic macromolecules and suggested that embryos differed from adults in their ability to repair macromolecules damaged by alkylating agents.

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