Biochemical Pharmacology, Vol. 17, pp. 1213–1216. Pergamon Press. 1968. Printed in Great Britain

THE TERATOGENIC EFFECT OF 1-β-D-ARABINOFURANOSYLCYTOSINE IN THE RAT. PROTECTION BY DEOXYCYTIDINE*

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(*Received* 9 *November* 1967; *accepted* 15 *January* 1968)

Abstract—Single i.p. injections of 1-β-D-arabinofuranosylcytosine (ara-C) at doses ranging from 20–800 mg/kg of body wt. given to the pregnant Wistar rat from day 10–12 of gestation produced malformations which included cleft palate and lip, encephalocele, and deformed appendages and tail in fetuses that survived to day 21 of gestation. No malformations were observed in fetuses at 21 days with any of the doses (5–500 mg/kg) given to the pregnant rat on day 5–9 of gestation. The effectiveness of 1-β-D-2′-deoxyribofuranosylcytosine (CdR) in preventing fetal malformations at 21 days, produced by a single i.p. injection of 150 mg/kg or ara-C given to the 12-day-pregnant rat was dependent on the amount of CdR and of intervals of time between the administration of the two compounds. If the time interval between administration of ara-C (150 mg/kg) and CdR (600 mg/kg) exceeded 10 min, complete protection against ara-C-induced malformations did not occur. 1-β-D-deoxyribofuranosyl-5′-phosphate (dCMP) gave protection in the same range as CdR, but 1-β-D-ribofuranosylcytosine-5′-phosphate (CMP), 1-β-D-ribofuranosylcytosine diphosphate (CDP), 1-β-D-ribofuranosylcytosine (CR) and thymidine (TdR) did not. When tritium-labeled ara-C (282·5 mg/kg) was given i.v. to the 12-day-pregnant rat, about 30 per cent of the injected dose was excreted in the urine within 24 hr, with only minor amounts being deaminated to 1-β-D-ribofuranosyluracil (ara-U); fetuses from these rats extracted 1–2 hr after injection showed about 97·5 per cent of the radioactivity in the trichloracetic acid (TCA), 2·5 per cent in the DNA, and no activity in the RNA and protein fractions.

1-β-D-ARABINOFURANOSYLCYTOSINE (cytosine arabinoside; ara-C) is a potent inhibitor of the growth of bacteria,[1, 2] DNA-containing viruses in cell cultures,[3–5] normal[6] and neoplastic[7–11] mammalian cell lines, regenerating bone marrow,[12] and transplantable mouse tumors and leukemias.[13–17] It inhibits DNA synthesis *in vitro* in human leucocytes[18, 19] and embryonic lung cells[20] and produces megaloblastic changes in bone marrow in man[21, 22] and animals.[23] Ara-C has produced remission in acute leukemia[24] and has been used effectively against herpes keratitis.[25, 26] It suppresses immune reactions in the rabbit[27] and in the mouse,[28] and depresses the SV_{40} antigen in cultures of GMK cells infected with SV_{40}.[29] Ara-C has produced developmental defects in the chick[30] and has induced cerebellar hypoplasia in newborn hamsters.[31]

The inhibitory effect of ara-C can be prevented in bacteria,[1,2] viruses,[3–5] cells in

* This research was supported in part by Grant NCI CA-08748 from the Department of Health, Education and Wefare and by the Berte Abramson Memorial Grant (ACS T-40) for Cancer Research from the American Cancer Society, Inc.

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culture,[7–11] regenerating bone marrow,[12] animal tumors *in vivo*,[32] and can be allayed in man[33] by 1-β-D-2′-deoxyribofuranosylcytosine (CdR).

In view of its wide range of biological activities, ara-C was examined for teratogenic effects in the rat. This report describes the abnormalities it produces after injection into the pregnant rat on various days of gestation and presents evidence of protection against ara-C malformations with CdR and 1-β-D-deoxyribofuranosylcytosine-5′-phosphate (dCMP) but not with 1-β-D-ribofuranosylcytosine-5′-phosphate (CMP), 1-β-D-2′-ribofuranosylcytosine (CR), 1-β-D-ribofuranosylcytosine-5′-diphosphate (CDP) or thymidine (TdR). The metabolism of ara-C in the 12 day-pregnant rat and the fetus is also described. A preliminary report of these findings has been presented previously.[34]

MATERIALS AND METHODS

Chemicals. 1-β-D-arabinofuranosylcytosine (ara-C) was kindly supplied by Dr. C. G. Smith of The Upjohn Company, Kalamazoo, Mich. Tritium-labeled ara-C was obtained from Schwartz Bioresearch, Inc. dCMP, CMP, CDP, CdR, CR and TdR were purchased from Calbiochem, Inc.

Animals. Pregnant CF Wistar female rats of known gestation day, obtained from Carworth Farms and weighing 180–250 g, were used for all experiments. They were maintained in individual cages and fed on Purina chow with water *ad libitum*.

Teratogenic and Protection studies

A total of 347 rats were treated as follows: 1) on a day selected for treatment from day 5–12 of gestation, 109 rats were given single i.p. doses ranging in amount from 2·5 to 900 mg/kg of ara-C alone. 2) On day 12 of gestation, 100 rats received single i.p. injections of ara-C and CdR simultaneously (35 rats) or at timed intervals (65 rats). The rats injected simultaneously with the two compounds were given 150 mg/kg of ara-C and varying amounts of CdR ranging from 5–600 mg/kg; the rats which received the two compounds at time intervals were given 150 mg/kg of ara-C and 600 mg/kg of CdR at intervals ranging from 0–360 min apart. Control rats received 150 mg/kg of ara-C (20 rats) or varying amounts of CdR (9 rats) alone. 3) Also on day 12 of gestation, 38 rats received single i.p. injections of 150 mg/kg of ara-C simultaneously with single doses of dCMP, CMP, CDP, CR or TdR at doses ranging from 100–860 (dCMP), 125–750 (CMP), 1460 (CDP), 100–650 (CR) or 150–650 (TdR) mg/kg; controls (28 rats) were given dCMP, CMP, CR or TdR alone. The doses in all experiments were calculated on a mg/kg body weight of each animal; the day after mating was taken as day zero of pregnancy. All animals were sacrificed on day 21 of gestation. Surviving fetuses were removed from the uteri, weighed and examined for gross malformations. Dead and resorbed fetuses were recorded. A selected number of specimens from each litter was fixed in 95% ethanol for subsequent clearing and staining in alizarin red for the study of the bony skeleton.[35]

Ara-C, CdR, CR and TdR were dissolved in an 0·85% solution of physiologic saline; dCMP, CMP and CDP were suspended in a 0·5% solution of carboxymethylcellulose. The solutions were prepared fresh on each injection day.

Metabolic studies

^{3}H-labeled ara-C (sp. act., 0·154 to 1·54 mc/m-mole; the labeled material contained,

besides the main ara-C radioactivity, about 3·5% ara-U and a minor, unidentified contamination) was injected i.v. at a dosage of 282·5 mg/kg into 3 pregnant rats on day 12 of gestation. The rats were sacrificed at 1, 2 and 24 hr, respectively, after the injection of the drug. The embryos of all 3 rats were recovered and the urine of the third rat was collected over 24 hr after the injection. A pregnant control rat received an i.v. injection of saline. The total of 10–12 embryos per rat were pooled, washed in saline, weighed and homogenized with four parts of distilled water and extracted by the Schmidt-Thannhäuser procedure as modified by Schneider.[36] The quantitative analysis of TCA, RNA, DNA and protein fractions were effected by the dry combustion method of Kalberer and Rutschmann.[37] Paper chromatography of the TCA extract and the urine was carried out on Whatman No. 3MM paper (descending technique) in isopropanol: hydrochloric acid: water (680 : 170 : 144, v/v) and *n*-propanol : ethylacetate : water (1 : 4 : 2, v/v) and *n*-propanol: tetrahydrofurfuryl-alcohol: citrate buffer, pH 5·66 (2: 1: 1, v/v) as solvents. The length of the paper chromatogram was then divided into about 45 equal horizontal segments and areas of 1 × 3 cm were cut off and eluted in scintillation vials with 0·4 ml water, incubated for 2 hr at 37° and, after addition of 10 ml Diatol, the radioactivity was determined. A Packard scintillation counter, model 314 EX-2, was used for all evaluations of radioactive samples.

Enzyme studies

Enzyme source. Pregnant rats were sacrificed on day 12 of gestation. The embryos and the maternal liver were removed, washed in 0·85% saline, and homogenized separately in a 0·25 M (1 : 4, v/v) at 0° in a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 100,000 *g* for 60 min at 5° and the supernatant was used for subsequent determinations. The term "tissue homogenate" hereafter refers to this supernatant.

Assay for deamination. In a typical determination, the reaction mixture contained 0·1 ml ara-C (5 μmole), 0·2 ml tissue homogenate and 0·3 ml Krebs–Ringer buffer at pH 6·5. After incubation for 2 hr at 37°, the reaction was stopped by addition of 1·0 ml of 5% cold TCA.[11] After centrifugation for 15 min at 2000 rpm to remove the protein, 0·5 ml of the supernatant was applied to a Conway unit to determine the amount of liberated ammonia.[38] The activity is expressed as the amount of liberated ammonia in μmole/mg protein/hr. The minimum detectable amount of ammonia by this method is 0·05 μmole/mg protein/hr.

Assay for kinase activity. The incubation system consisted of 0·05 ml tissue homogenate, 0·05 ml ara-C and 0·11 ml cofactor mixture. The ara-C amount was 3·3 mμmole and 0·25/μc/0·05 ml. The cofactor mixture contained 3 μmole ATP, 2·5 μmole 3-phosphoglycerate, 3 μmole $MgCl_2$ and 20 μmole Tris-buffered at pH 8·0. After incubation for 1 hr at 37°, 50 μl of the reaction mixture was applied to a DEAE cellulose paper (2 × 2 cm). The paper was immersed in a solution of 0·001 M ammonia formate, water and 95% ethanol to remove the nucleoside. After drying at room temperature in 10 ml of a solution containing 0·4% 2,5-diphenyloxazole and 0·01% 1,4-*bis*-2-(5-phenyloxazolyl)-benzene in toluene, the paper was put into the scintillation counter.[39] The activity is expressed as mμmole/mg protein/hr. Protein concentration in the homogenate was determined by the method of Lowry *et al.*[40] with crystalline bovine albumin as standard.

RESULTS

Maternal lethality. Single i.p. injections of 900 mg/kg of ara-C or 1460, 860, 750, 650, 650 mg/kg of CDP, dCMP, CMP, CR and TdR, respectively, into the 12-day-pregnant rat did not produce any visible sign of toxicity, but a single dose of 3000 mg/kg of ara-C killed 1 of 2 (LD_{50}) and a dose of 5000 mg/kg killed 2 of 2 (LD_{100}) non-pregnant adult rats.

Fetal effects of single doses of ara-C. The effects of single doses of ara-C on the rat fetus when the pregnant rat was treated once from day 5 through 12 is shown in Fig. 1 (A–D). The lowest dose given to the pregnant rat which killed all fetuses by day 21 of gestation was 50 mg/kg and this occurred on day 9 (Fig. 1A). This dose is about 1/6 to 1/10 of that which killed entire litters on the fifth through eleventh day (Fig. 1 A–C) and about 1/18 of that which destroyed litters on the twelfth day (Fig. 1D).

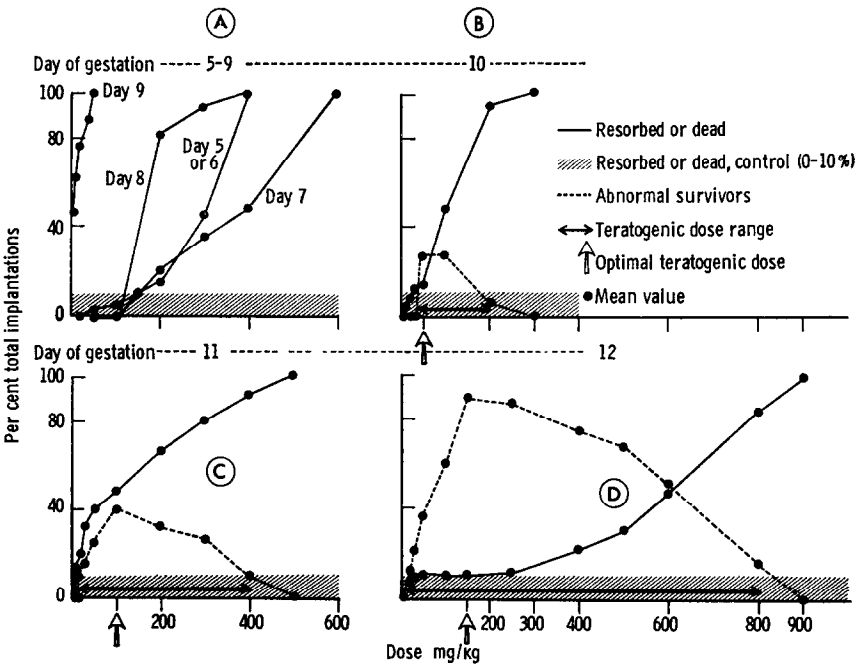

FIG. 1. Lethal and teratogenic effects produced by single i.p. injections of ara-C into the pregnant rat from day 5–12 of gestation; sacrificed on day 21.

Single doses of ara-C ranging from 5–400 mg/kg injected into pregnant rats from day 5 through 9 of gestation did not produce any malformations in fetuses that survived to day 21 (Fig. 1A), nor were malformations observed in fetuses from rats treated with 5 or 10 mg/kg on day 10, 11 or 12. The lowest dose of ara-C which produced malformations was 20 mg/kg (day 11 or 12) (Fig. 1C, D) and the highest was 800 mg/kg (day 12) (Fig. 1D). Within this dose range, single injections of 50, 100 and 150 mg/kg given to the pregnant rat on day 10, 11 and 12 of gestation, respectively, produced a maximum number of malformed fetuses and a minimum number of resorptions or deaths. This dose is referred to as the *optimal teratogenic dose* in Fig. 1

TABLE 1. TERATOGENIC EFFECTS OF ARA-C IN THE RAT FETUS (SINGLE I.P, INJECTIONS INTO THE PREGNANT RAT FROM DAY 10–12 OF GESTATION; SACRIFICED ON DAY 21)

Fetal effects	Day of gestation															
	10			11						12						
Teratogenic dose (mg/kg)	50	100	200	20	50	100	200	300	400	20	50	100	150	250	400–600	800
Fetal mortality (%)	13	48	94	25	39	47	70	76	91	N*	N	N	N	12	22–52	83
Total survivors	45	27	3	39	22	29	8	14	3	66	58	69	35	30	39	5
No. abnormal	14	15	3	5	9	22	8	14	3	8	24	47	35	30	39	5
Numbers with specific abnormalities																
Encephalocele†			1						2							
Cleft palate	6	14	3			11	8	13	3		2	24	25	26	39	5
Cleft lip	4	10	2			4	6	13	3							
Retarded‡ and/or clubbed																
Foreleg			1			1	8	13	3		1	20	20	26	39	5
Rear leg			1			13	8	13	3	3	10	25	29	26	39	5
Ectro-, syn-, poly- or brachydactylous																
Forepaw	1							1	3	4	11	22	29	26	39	5
Rear Paw			1	5	9	16	4	14	3		16	32	26	30	39	5
Tail, short, kinky or absent	6	6	3				4	14	3	3	5	18	15	26	39	5
Micrognathia or agnathia			1			9	8	13	3			7	6	26	39	5

* N = 1–10% (Control value).
† Hernial protrusion of the brain substance through a cleft in the skull.
‡ Microgenesis and incomplete ossification.

(arrow). Below the optimal teratogenic dose, the rise in malformations was accompanied by a corresponding rise in resorptions and deaths on day 10 and 11, but not on day 12; above this dose, malformations decreased as resorptions increased, presumably because the majority of the embryos were affected severely enough to cause death. At the optimal teratogenic dose, there were from 2–3 times as many malformed embryos at 21 days from rats treated on day 12 than on day 10 or 11 of gestation.

Representative specimens from pregnant rats treated with ara-C on different days of gestation are shown in Fig. 2, and the incidence of specific malformations is summarized in Table 1. Malformations which occurred in fetuses from pregnant rats treated on all 3 days included cleft palate, micrognathia, and deformed rear appendages, paws and tail. Cleft lip (uni or bilateral) and encephalocele occurred on day 10 and 11 and deformed forelegs on day 11 and 12. The incidence of malformations increased with the dose on all 3 days. Cleft lip occurred independently or concomittantly with cleft palate. Deformation of the appendages included malpositioning (clubbed) and retardation of growth resulting in short appendages and, in extreme cases, in hemimelia or phocomelia and a variety of digital defects in the paws (poly-, hepta-, hexa-, syn,- brachy-, dactyly and diplopodia) (Fig. 3). Skeletal defects associated with gross malformations included incomplete ossification, distortion and fusion of the bones of the skull and appendages. Other defects included fused ribs and vertebrae and incompletely ossified sternebrae (Figs. 4 and 5).

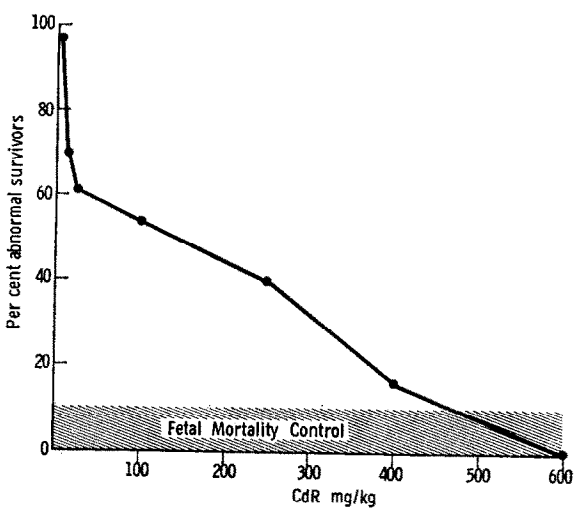

FIG. 6. Fetal effects produced by interaction of varying doses of CdR (5–600 mg/kg) and a single dose of ara-C (150 mg/kg) injected simultaneously into the 12-day-pregnant rat; sacrificed on day 21 of gestation.

Protection against ara-C-induced malformations in the 12-day fetal rat. The optimal teratogenic dose of ara-C, *viz*, 150 mg/kg, was selected for the study of the protective effects of CdR, dCMP, CMP, CDP, CR and TdR.

The results of experiments in which 150 mg/kg of ara-C was injected simultaneously with varying amounts of CdR into the 12-day-pregnant rat are summarized in Fig. 6. A minimal dose of 600 mg/kg of CdR was required to provide complete protection of

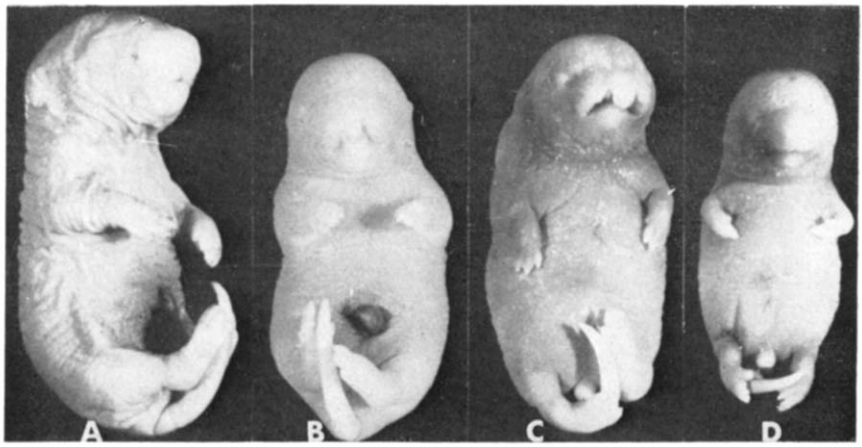

FIG. 2. Representative fetuses from pregnant rats treated with ara-C on different days of gestation. A, control; B, C and D, fetuses from pregnant rats which received a single injection of 100 mg/kg of ara-C on day 10, 11 and 12 of gestation respectively. Abnormalities include cleft lip, cleft palate (not visible), retarded and clubbed appendages, deformed paws, short mandibles and short tail.

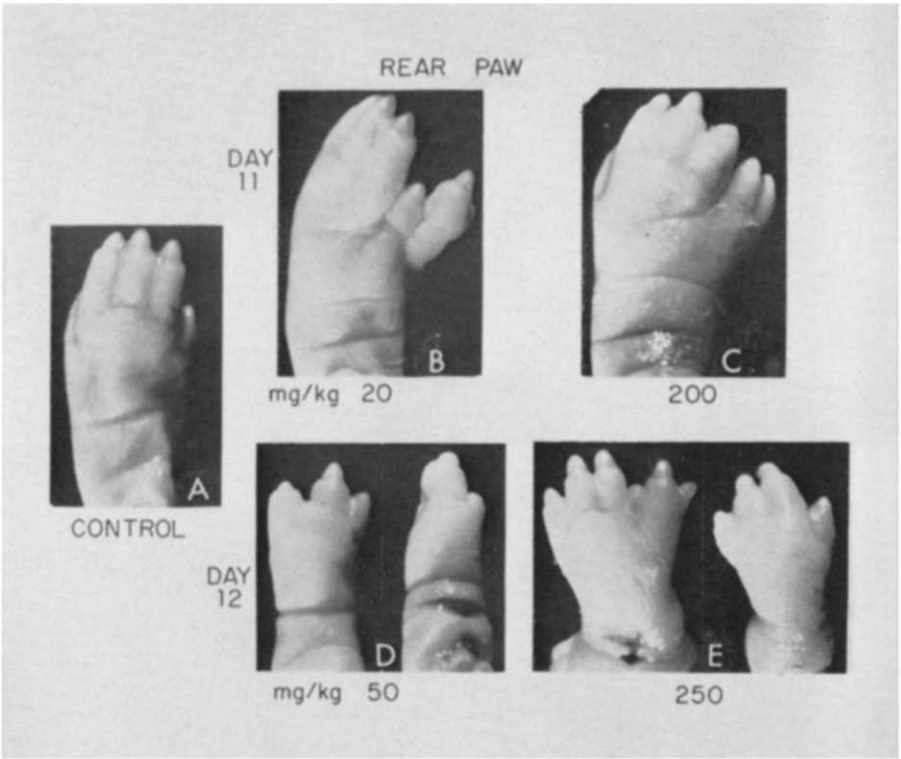

FIG. 3. Rear paws of fetuses showing different types of digital malformations produced by ara-C (single i.p. injections of different doses of ara-C into the 11- or 12-day-pregnant rat; sacrificed on day 21). A, control; B, polydactylous paw with a hyperphalangeous, distally duplicated extra digit; C, dichirus paw with partial duplication approximating a mirror image; D, brachydactyly accompanied by ectrodactyly; E, example on the left is a paw with polydactylous first digit and brachydactylous second digit, and example on the right is a paw which is ectro- and brachydactylous.

B.P.—*facing page 1218*

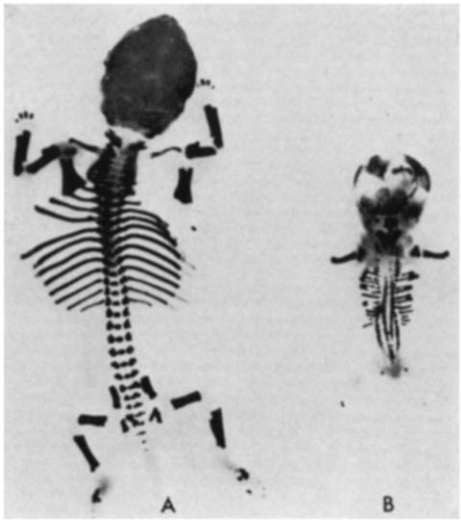

Fig. 4. Fetal skeleton at 21 days, stained in alizarin red. A, control; B, a fetus from a pregnant rat which received 400 mg/kg of ara-C on day 12 of gestation. Note severe retardation (general), incomplete ossification of skull bones, fused and fragmented ribs, split vertebral arches, bifid vertebral centra and partial or complete absence of the limb bones.

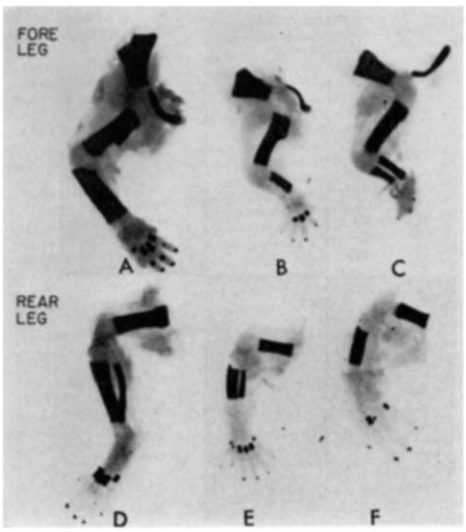

FIG. 5. Skeleton of the forelegs and rear legs of fetuses at 21 days, stained in alizarin red. A and D, controls; B and E, fetuses from pregnant rats which were treated with a single injection of 100 mg/kg, and C and F, with 150 mg/kg of ara-C on day 12 of gestation. Besides general retardation of the limb bones, note polydactyly in E, malpositioning (clubbed) in E and F, ectrodactyly in C and F, and absence of fibula in F.

the fetus against abnormalities produced by a single dose of 150 mg/kg of ara-C, whereas lower doses, i.e. 12–400 mg/kg, were only partially effective in reducing the number of abnormal embryos and lowering the incidence of all selected abnormalities except that of the rear paw (Table 2) at 21 days. Interaction of the two compounds did not enhance the percentage of fetal mortality over that of the control.

TABLE 2. PROTECTIVE EFFECTS OF VARYING AMOUNTS OF CdR AGAINST SPECIFIC ABNORMALITIES PRODUCED BY A SINGLE INJECTION OF 150 MG/KG ARA-C WHEN THE TWO COMPOUNDS ARE ADMINISTERED SIMULTANEOUSLY TO THE 12-DAY-PREGNANT RAT; SACRIFICED ON DAY 21 OF GESTATION

Ara-C (mg/kg)*	150								0
CdR (mg/kg)†	0	5	12	25	100	250	400	600	62–1000
Fetal mortality (%)	N‡	N	N	N	N	N	N	N	N
Total survivors	35	58	51	49	46	52	60	46	75
No. abnormal	35	56	36	30	25	21	11	0	0
Per cent with specific abnormalities									
Cleft palate	74	60	47	46	36	19	0	0	0
Retarded§ and/or clubbed									
Rear leg	83	68	55	33	28	9·5	0	0	0
Foreleg	60	1·7	2·7	6	8	0	0	0	0
Poly- or ectrodactylous rear paw	80	82	80	73	80	90	73	0	0
Ectro- or syndactylous forepaw	77	69	58	43	44	47	27	0	0
Short, kinky tail	51	17	11	3	8	0	0	0	0

* ara-C: 1-β-D-arabinofuranosylcytosine.
† CdR: 1-β-D-2′-deoxyribofuranosylcytosine.
‡ N = 0–10% (control value).
§ Microgenesis and incomplete ossification.

Further evidence of the protective role of CdR was obtained from experiments in which a single dose of 600 mg/kg of CdR was injected into the 12-day-pregnant rat *before* or *after* a single dose of 150 mg/kg of ara-C at time intervals of 10, 20, 40, 60, 120, 240 and 360 min. The results of these experiments are shown in Fig. 7. A dose of 600 mg/kg of CdR given *before* a dose of 150 mg/kg of ara-C provided complete protection against the teratogenic effects of the latter up to 20 min, partial protection up to 240 min (80 per cent at 40 min; 64% at 120 min) and none at 360 min. When 600 mg/kg of CdR was given *after* a dose of 150 mg/kg of ara-C, complete protection occurred up to 10 min and partial protection thereafter (88 per cent at 20 min; 80 per cent at 90 min; 7 per cent at 360 min). The type and incidence of abnormalities which were observed in all partially protected fetuses are shown in Table 3. Two abnormalities which received complete protection over the entire period of time (40–360 min, in experiments in which CdR was given *before* ara-C) were those of the tail and the foreleg. The abnormality which received least protection was the forepaw. All others (rear leg, palate, rear paw) were protected to a varying degree over different periods of time (Table 3).

TABLE 3. PROTECTIVE EFFECTS OF A SINGLE DOSE OF 600 MG/KG OF CDR AGAINST SPECIFIC ABNORMALITIES PRODUCED BY A SINGLE INJECTION OF 150 MG/KG OF ARA-C WHEN THE TWO COMPOUNDS ARE ADMINISTERED AT DIFFERENT TIME INTERVALS TO THE 12-DAY-PREGNANT RAT; SACRIFICED ON DAY 21 OF GESTATION

Fetal effects	CdR* (600 mg/kg) Minutes before ara-C†							CdR (600 mg/kg) Minutes after ara-C							Ara-C (150 mg/kg)	CdR (62–1000 mg/kg)
	360	240	920	90	40	20–10	0	10	20	40	90	120	240	360		
Fetal mortality (%)	N‡	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Total survivors	31	22	58	67	48	96	46	38	49	38	25	40	56	40	154	30
No. abnormal	31	20	21	22	10	0	0	0	6	6	5	9	18	37	154	0
Per cent with selected abnormalities																
Short, kinky tail	0	0	0	0	0	0	0	0	0	0	20	22	22	57	51	0
Retarded§ and/or clubbed																
Foreleg	0	0	0	0	0	0	0	0	0	16	21	11	16	13	59	0
Rear leg	32	35	28	18	0	0	0	0	16	33	26	33	33	38	84	0
Cleft palate	51	50	43	4	0	0	0	0	0	0	0	0	0	73	77	0
Poly- or ectrodactylous rear paw	64	65	71	62	70	0	0	0	66	66	60	89	94	97	82	0
Ectrodactylous forepaw	100	100	100	41	39	0	0	0	16	33	40	33	33	38	82	0

* CdR: 1-β-D-2′-deoxyribofuranosylcytosine.
† ara-C: 1-β-D-arabinofuranosylcytosine.
‡ N = 0–10% (Control Value).
§ Microgenesis and incomplete ossification.

TABLE 4. PROTECTIVE EFFECTS OF SINGLE DOSES OF dCMP, CMP, CDP, CR AND TdR AGAINST ABNORMALITIES PRODUCED BY A SINGLE INJECTION OF 150 MG/KG OF ARA-C WHEN INJECTED SIMULTANEOUSLY INTO THE 12-DAY-PREGNANT RAT; SACRIFICED ON DAY 21 OF GESTATION

	With ara-C* (150 mg/kg)														
Agent†	dCMP				CMP			CDP		CR		TdR			Ara-C
(mg/kg)	860	550	400	100	600	400	125	1460	650	300	100	650	425	150	150
(m-mole/kg)	2·6	1·7	1·2	0·3	1·7	1·2	0·3	2·6	2·6	1·2	0·4	2·6	1·7	0·6	0·6
Fetal mortality (%)	N‡	N	N	N	68	16	15	31	78	80	47	100	N	N	N
Abnormal survivors (%)	0	21	30	47	100	100	100	100	100	100	100	§	100	100	100

	Without ara-C													
Agent	dCMP				CMP			CDP		CR		TdR		
(mg/kg)	860	500	400	125	600	400	125	1460	700	250	125	1000	500	150
(m-mole/kg)	2·6	1·5	1·2	0·3	1·7	1·2	0·3	2·6	2·8	1·0	0·5	4·1	2·0	0·6
Fetal mortality (%)	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Abnormal survivors (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Ara-C: 1-β-D-arabinofuranosylcytosine.

† dCMP: 1-β-D-deoxyribofuranosylcytosine-5′-phosphate; CMP, 1-β-D-ribofuranosylcytosine-5′-phosphate; CDP, 1-β-D-ribofuranosylcytosine-5′-diphosphate; CR, 1-β-D-2′-ribofuranosylcytosine; TdR, thymidine.

‡ N = control value (0–10%).

§ Complete litter resorption in 2 rats.

The protective effects of dCMP, CMP CDP, CR and TdR against ara-C-induced malformation in the 12-day fetal rat are shown in Table 4. A single dose of 860 mg/kg of dCMP injected into the 12-day-pregnant rat simultaneously with 150 mg/kg of ara-C gave complete protection against ara-C-induced malformations at 21 days, while decreasing amounts, i.e. 550, 400 and 100 mg/kg, gave 79, 70 and 53 per cent protection respectively. The protective effects of dCMP are comparable on a millimole per kilogram basis to those seen with equivalent amounts of CdR. Single doses of 150, 425 and 650 mg/kg of TdR, 125, 400 and 600 mg/kg of CMP, 100, 300 and

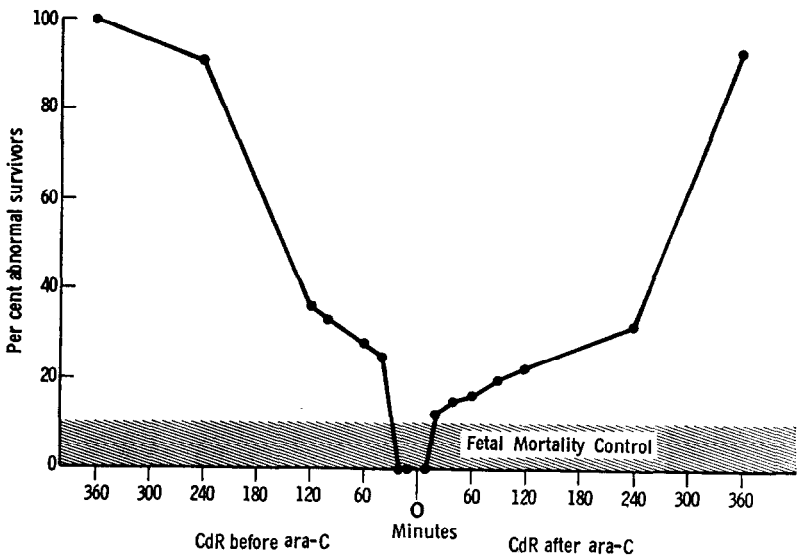

FIG. 7. Fetal effects produced by timed interaction of a single dose of 150 mg/kg of ara-C and 600 mg/kg of CdR in the 12-day-pregnant rat; sacrificed on day 21 of gestation.

650 mg/kg of CR or a dose of 1460 mg/kg of CDP injected at zero min along with a single dose of 150 mg/kg of ara-C into the 12-day-pregnant rat did not give any protection against ara-C-induced malformations at 21 days. All the doses of CdR and dCMP and the lower doses (150 and 425 mg/kg) of TdR did not potentiate the lethal effect of ara-C in the fetus, but all doses of CMP (125, 400 and 600 mg/kg), CR (650, 300 and 100 mg/kg) and 650 mg/kg of TdR and 1460 mg/kg of CDP did.

Metabolic studies. Table 5 shows the results of the experiments in which three 12-day-pregnant rats were injected with ^{3}H-labeled ara-C. It is obvious that only a very small part (about 0·03 per cent) of the total amount of injected radioactivity reached the fetuses. Only the TCA and the DNA extracts showed radioactivity, whereas the RNA and protein fractions showed no radioactivity at all. Furthermore, only 1 and 2 hr after injection of ^{3}H-ara-C could radioactivity be found in the TCA and DNA extracts, whereas 24 hr after the injection none of the extracts showed any radioactivity. The ratios of radioactivities in TCA and DNA extracts are much in favor of TCA-soluble material (about 50:1). The paper chromatograms of the TCA extracts revealed only trace amounts of activity in the regions where 3′ or 5′ ara-C-phosphates would be localized. As compared to the injection solution, which

TABLE 5. DISTRIBUTION OF RADIOACTIVITY AFTER I.V. INJECTION (282·5 MG/KG) OF ^{3}H-ARA-C INTO THE 12-DAY-PREGNANT RAT

Rat No.	Injected amount (cpm × 10°)	Time of Sacrifice (hr after injection)	Radioactivity found in fractions: TCA extract (cpm)	TCA extract (% of all extracts)	RNA extract (cpm)	DNA extract (cpm)	DNA extract (% of all extracts)	Protein (cpm)
67–316	27·2	1	7736	97·8	0	168	2·2	0
67–317	27·3	2	6844	97·7	0	160	2·3	0
67–269	1·5	24	0	0	0	0	0	0

is obviously contaminated with ara-U and a not yet identified second substance, the TCA extract shows an increase in the deaminated product ara-U (from 3·5 per cent in the injection solution to 40 per cent in the TCA total counts). The shift may be due to a real deamination of ara-C or to accumulation or slower excretion of the contaminating ara-U of the injection solution.

The urine collection revealed that about 30 per cent of the injected radioactivity is excreted within 24 hr in the urine. A paper chromatogram of the injection solution and the urine revealed a slight relative increase (about 5 per cent of total) of the deaminated product ara-U in the urine as compared to the injection solution (Fig. 8). Here again the question arises whether this is a consequence of enzymatic deamination or a problem of distribution and excretion of the injected labeled material.

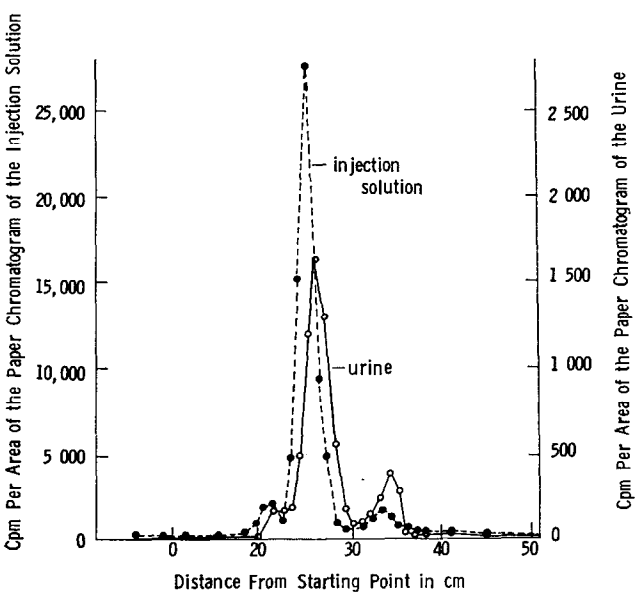

FIG. 8. Paper chromatogram of aliquots of injection solution and 24-hr urine of the 12-day-pregnant rat after a single i.v. injection of ^{3}H-ara-C (282·5 mg/kg); system, isopropanol:hydrochloric acid: water (680:170:144, v/v). Large peak represents ara-C; small peak to the left of ara-C is unidentified; small peak to the right of ara-C represents ara-U.

TABLE 6. DEAMINASE AND KINASE ACTIVITY IN THE 12-DAY-OLD RAT EMBRYO AND IN THE LIVER OF THE 12-DAY-PREGNANT RAT

Tissue homogenate	Deaminase activity (μmole/mg protein/hr)	Kinase activity (mμmole/mg protein/hr)
Whole embryo	> 0·05	2·35
Maternal liver	> 0·05	0·11

The results of enzymatic assays for kinase and deaminase activities in the tissue homogenates of whole embryo and maternal liver are shown in Table 6. The kinase necessary for the phosphorylation of ara-C is available in large amounts in the 12-day-old embryo and its activity is 21 times as high in the embryo as in the maternal liver homogenate. By comparison, the amount of deaminase in both the embryonic and maternal tissues is much less.

DISCUSSION

These experiments demonstrated that single i.p. injections of ara-C given to the pregnant rat at approximately 1/60 (50 mg/kg, day 10) to 1/150 (20 mg/kg, day 12) of the dose which was lethal to the adult (LD_{50} = 3000 mg/kg) from day 10–12 of gestation produced malformations in the fetuses that survived to day 21. No malformations occurred with any of the doses of ara-C given from day 5–9 or with CdR, dCMP, CMP, CDP, CR or TdR given on day 12 of gestation.

In several biological systems the inhibitory effects of ara-C on DNA synthesis can be prevented by CdR,[1–3,5,7–10,18,20,32,33,41] but not by TdR, UdR, CR, CMP or cytosine.[7,9,10,41] The data in Table 2 indicate that 600 mg/kg of CdR counteracted the teratogenic effects of at least 140 mg/kg of ara-C (i.e. 10 mg/kg of ara-C is non-teratogenic) and its effectiveness declined with the increase in time intervals between the administration of the two compounds (Fig. 7). Furthermore, the protective effects seen at 240 (68 per cent) and 360 (7 per cent) minutes in experiments in which CdR was given *after* ara-C (Fig. 7) were equivalent to the effects produced when ara-C, between 30–50 (Fig. 1D) and 100–150 (Fig. 1D) mg/kg was given alone. This suggests that at 240 min, from 20–33 per cent of the teratogenic effect of ara-C had already been completed, and at 360 min (6 hr) the effect at these doses is irreversible. It appears therefore that the degree of protection by CdR and the time over which it occurred are related to the amount of the deoxyriboside derivative entering the embryonic cells and to its rate of catabolism and excretion by the pregnant rat. The protective effect of equivalent amounts of dCMP was in the same range as CdR.

Thymidine, CMP and CDP did not give any protection against ara-C-induced malformations in the rat and potentiated to varying degrees its lethal effects in the fetus.

The mechanism by which ara-C inhibits cell proliferation has been proposed: ara-C is phosphorylated to the diphosphonucleotide (ara-CDP), which inhibits the reduction of cytidylic acid diphosphate (CDP) to deoxycytidylic acid diphosphate (dCDP),[7,42] thereby causing deoxycytidylic acid triphosphate (dCTP) deficiency; this dCTP-induced deficiency prevents DNA synthesis and cell division.[7] With isolated enzymes derived from *Escherichia coli*, ara-C nucleotides were not incorpor-

ated into the newly formed RNA or DNA, nor were they markedly inhibitory in these systems.[43] In the chick embryo homogenates, the reduction of cytidylic acid is strongly inhibited by the triphosphates of the deoxynucleotides of thymine, guanine and adenine.[44] Reports appearing recently show that small amounts of ara-C are incorporated into DNA of cultured animal cells.[6–10,45] This incorporation prevents subsequent DNA-synthesis and replication, but it does not affect RNA or protein synthesis.[6–10,45] Chromosomal breaks in human leucocytes[18,19] and embryonic lung cells[20] in culture on exposure to ara-C during DNA synthesis have been described.

Metabolic studies in man have demonstrated that ara-C is readily deaminated to ara-U[33] by a pyrimidine nucleoside deaminase which is present in several human tissues.[46] A similar deaminase activity has been reported in bacteria,[1] in 11 different species of animals,[46] and in PPLO-infected KB cells.[47] No evidence of deamination of ara-C, CR or CdR was observed *in vitro* and in homogenized Burkitt's tumor cells,[11] in mouse L 1210 leukemia cells[11] *in vitro* in four tissues of the adult rat,[46] and in embryonic tissues of the rat[48,49] and the chick.[50] The deamination data for ara-C, e.g. Burkitt's tumor cells,[11] mouse L 1210 leukemia cells,[11] and the fetal rat, indicate that ara-U is not the basis of ara-C cytotoxicity.

The studies with radioactive ara-C revealed that most of the radioactivity found in the embryos was concentrated in the TCA extract and only a minor amount (2·5 per cent) was being incorporated into the DNA. The form of radioactivity incorporated in the latter was not identified. The paper chromatograms of the TCA extracts revealed radioactive ara-C and ara-U, but showed no relevant radioactivity in the regions of 3′ and 5′-ara-C phosphates. This observation was unexpected, especially since the kinase necessary for the phosphorylation of ara-C is available in large amounts in the 12-day-old rat embryo. Perhaps the fact that no detectable amounts of the nucleotide of ara-C were found might be explained by the short-lived, transient stage of the nucleotide. The increase in the product of deamination, *viz.* ara-U, is considerable only in the TCA extracts (about 30 per cent), whereas the 24-hr urine sample of the pregnant rat showed in comparison to the injection solution an increase of only about 5 per cent. The discrepancy between the results *in vitro* of kinase and deaminase determinations and the findings *in vivo* of phosphorylated and deaminated products of ara-C, cannot be explained at this time. The mechanism by which ara-C produces developmental defects in the rat is not clear. Since ara-C is incorporated into the DNA of the rat embryo cells, it would presumably interfere with the DNA synthesis of these rapidly proliferating cells. But whether this small amount of incorporation of ara-C is alone sufficient to produce the extensive developmental defects observed in these studies is not clear.

Acknowledgement—The authors are grateful to Misses Jane Sodergren and Dionne Bolling of the Pharmacology Division, the Sloan-Kettering Institute, New York, N.Y., for their skilled technical assistance in the metabolic and radioactive studies reported here.

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