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THE EFFECTS OF 1-β-D-ARABINOFURANOSYLCYTOSINE ON THE DEVELOPING CHICK EMBYRO*

DAVID A. KARNOFSKY and CORINNE R. LACON

Division of Clinical Chemotherapy, Sloan-Kettering Institute for Cancer Research and Cornell University Medical College, New York, N.Y., U.S.A.

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Abstract—1-β D-Arabinofuranosylcytosine (CA) inhibits the development of the chick embryo when it is injected into the yolk sac on day 4 of incubation. The LD_{50} is about 0·025 mg/egg; those embryos surviving to 18 days are stunted, and the developmental abnormalities consist of facial coloboma, absence of the pelvic skeleton and other bone deletions, corneal cysts, and feather inhibition. Embryos injected at later stages show progressively less severe abnormalities. In those treated on day 8 and later, weight inhibition, feather disturbances, and cerebellar atrophy were the major detectable defects by gross examination. The effects of CA resemble those caused by 2′-deoxyguanosine and are presumed to be due to a dCDP deficiency caused by a block in the reduction of CDP to dCDP, thus interfering with DNA synthesis. Qualitatively these effects are similar to those caused by other antimetabolites inhibiting, in various ways, the availability of precursors necessary for the synthesis of DNA.

1-β-D-ARABINOFURANOSYLCYTOSINE (cytosine arabinoside, CA) is a potent antimetabolite, which is reported to interfere with the proliferation of DNA viruses[1–4] and bacterial growth;[5,6] block cell division and inhibit DNA synthesis in tissue culture;[7–10] prevent the growth of various types of transplanted tumors and leukemia in mice,[11–13] suppress immunologic reactions *in vivo* and *in vitro*;[12,14] induce chromosomal abnormalities in human leukocytes and lung cells *in vitro*;[15,16] produce megaloblastosis in the bone marrow with leukopenia, thrombocytopenia, and anemia;[17] cause developmental defects in the fetal rat[18] and cerebellar hypoplasia in neonatal hamsters;[19] induce temporary remissions in acute leukemia in children and adults;[20] and to be effective against herpetic keratitis.[21] The wide range of activity of CA as a growth-inhibiting agent suggests that it may also produce significant effects on the chick embryo. This report describes developmental abnormalities following the administration of CA to the chick embryo at various stages of development.

MATERIALS AND METHODS

Fertile white Leghorn eggs, obtained from a commercial source, were incubated at 38°. In most experiments, the drug was injected on the fourth day of incubation, but in some instances it was given as a single dose, on day 3, 5, 6, 7, 8, or 12 of incubation. The drug was injected into the yolk sac through a small hole made in the blunt end of the egg; in one experiment, injections were made through a window onto the chorioallantoic membrane of the 8-day embryo. The eggs were candled

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daily, and the dead ones recorded. The embryos dying between days 5 and 10 of incubation were discarded, but from the days 11 to 18 the dead embryos were inspected and gross abnormalities recorded. Embryos surviving to day 18 of incubation were killed, weighed, inspected for gross abnormalities, and, in many instances, cleared and the skeleton stained with alizarin.[22, 23]

CA* was dissolved in isotonic saline before use. The volume injected into each egg did not exceed 0·2 ml.

RESULTS

Toxicity of CA *for the chick embryo*

The toxicity of single doses of CA, injected into the yolk sac of chick embryos at stages of incubation from days 3 to 12, ranged from an approximate LD_{50} of 0·02 mg/egg at 3 days to 0·5–1·0 mg/egg at 12 days (Table 1). The LD_{50} doses were

TABLE 1. APPROXIMATE LD_{50} DOSE OF CA INJECTED INTO THE YOLK SAC OF THE CHICK EMBRYO AT VARIOUS STAGES OF DEVELOPMENT AND BASED ON SURVIVAL TO 18 DAYS

Age at injection (days)	Number of eggs injected	Approximate LD_{50} (mg/egg)
3	45	0·02
4	214	0·025
5	45	0·04–0·06
6	30	0·08
7	45	0·08–0·12
8	30	0·1 –0·2*
12	25	0·5 –1·0

* The LD_{50} dose was similar when CA was injected onto the chorioallantioic membrane.

estimated from a large number of separate trials (the LD_{50} is the dose that allows about 50 per cent of the treated embryos to survive until day 18 of incubation). Twice the estimated LD_{50} dose is usually fatal to the entire group before day 18. One half the LD_{50} generally permits the majority of embryos to survive to 18 days but, for unexplained reasons, there is a variable mortality, up to 20 per cent, among saline-injected control embryos. These embryos generally die shortly after injection, and the injected survivors do not exhibit a significant number of developmental abnormalities; the figure is less than 1 per cent. While the volume of the egg is about 50 ml, the LD_{50} of CA increased approximately 25–50 times, from 0·025 mg/egg on day 4 to 0·5–1·0 mg/egg on day 12. During the same period, the embryo increases in weight about 50 times, from about 0·10 g at 4 days to 5·0 g at 12 days.[24]

Effects of CA *on the* 4*-day chick embryo*

The most extensive data with CA were obtained from embryos injected on day 4 of incubation. At this stage, organogenesis is largely complete,[25] and drug-induced gross developmental defects are often compatible with the survival of the embryos to 18 days of incubation. Table 2 summarizes the toxicity and the developmental defects

* Cytosine arabinoside was generously provided by The Upjohn Co., Kalamazoo, Mich.

observed at 18 days when CA was injected at various doses into the yolk sac of the 4-day embryos. Embryos dying before 18 days at near LD_{50} or supralethal doses are severely deformed; these embryos were examined grossly, but they were not studied in detail.

TABLE 2. EFFECTS OF VARIOUS DOSES OF CA INJECTED INTO THE YOLK SAC OF THE 4-DAY CHICK EMBRYO; SURVIVORS SACRIFICED AT 18 DAYS

Dose (mg/egg)	0·01	0·025	0·05	0·1
Eggs injected	20	64	150	45
Embryos sacrificed, 18 days	10	30	17	2
Survivors, abnormal	4	19	17	2
Gross abnormalities				
Facial coloboma + cleft palate	1	9	9	2
Cleft palate	1	2	2	
Short lower beak	3	16	16	2
Corneal cysts		3	1	
Other eye defects (eyelid defect + small eyes)		3	7	2
Micromelia	3	10	12	2
Missing toes	3	13	16	2
Short toes	3	3	11	
Edema, generalized	2	10	17	2
Feather inhibition		7	10	1
Average embryo wt. (g ± S.D.)	10·8 ± 3.3	11·5 ± 2·7	8·4 ± 2·7	3·4

The gross effects in the embryos killed at 18 days are listed in Table 2. They consist of weight inhibition (normal 18-day embryos average 16–18 g), defects in the development of the beak and face ranging from short lower beaks to facial coloboma and cleft palate, micromelia with a high incidence of short and missing toes, generalized edema, feather inhibition, and occasional corneal cysts and eye and eyelid defects.

The dose causing the most severe effects, while still permitting many of the embryos to survive to 18 days, range between 0·025 and 0·050 mg/egg. Figure 1 (1B-D) shows examples of the gross abnormalities found in the 18-day embryo, ranging from a short lower beak and missing toes (1B), to an embryo with a short beak, missing toes, and feather inhibition (1C), to the most severe form of the syndrome with facial coloboma, cleft palate, short upper beak, eye defects, micromelia, missing toes, edema, and almost complete absence of feathers (1D).

The osseous defects observed grossly were studied in more detail in the cleared specimens (Fig. 2). The skeletal abnormalities ranged from a defective pelvic girdle (2b) to its complete absence (2D), shortened to complete absence of toes, beak defects and facial coloboma due to missing facial bones, and vertebral and rib defects. Figure 3 shows in more detail the range of skeletal defects caused by CA, and compares the upper beak, lower beak, left lower leg, and pelvic girdle with an untreated 18-day control. Figure 3 includes an intermediate and a severely affected embryo; it is noted that the lower beak and pelvic girdle fail to develop in severely affected embryos.

Effects of CA *administered at various stages of development from* 3 *to* 12 *days*

The observed alterations in embryonic development produced by CA depend largely on the stage of development when the drug is given (Table 3). On day 3, doses

TABLE 3. EFFECTS OF CA INJECTED INTO THE YOLK SAC OF THE CHICK EMBRYO AT VARIOUS STAGES OF DEVELOPMENT IN THE RANGE OF THE LD_{50} DOSE; EMBRYOS SACRIFICED AT 18 DAYS

Age of injection (days) Dose (mg/egg)	3 0·02–0·04	4 0·025–0·05	5 0·04–0·06	6 0·06–0·08	7 0·06–0·12	8 0·1–0·2	12 0·5–1·0
Eggs injected	30	214	30	30	72	50	15
Embryos sacrificed, 18 days	7	47	14	21	42	22	7
Survivors, abnormal	5	36	14	21	40	22	6
Gross abnormalities							
Facial coloboma + cleft palate	3	18					
Cleft palate	1	4	11				
Twisted upper beak				17	33	15	
Parrot upper beak					9	8	
Short upper beak						6	4
Short lower beak	4	32	14	21	24		
Corneal cysts	1	4	1	7			
Other eye defects (eyelid defect and small eyes)	2	10	1	12	6		
Micromelia		22	8	20	15		
Missing toes	1	29	11	20	7		
Short toes	1	14	11	18	28	4	
Edema, generalized	2	23	10	18	10	1	2
Feather inhibition		17	11	20	25	22	6
Feather clubbing			5	3	10	13	
Average embryo weight (g ± S.D.)	12·2 ± 3·6	10·2 ± 3·2	9·6 ± 3·8	7·5 ± 2·6	7·2 ± 1·4	8·4 ± 2·1	14·5 ± 2·3

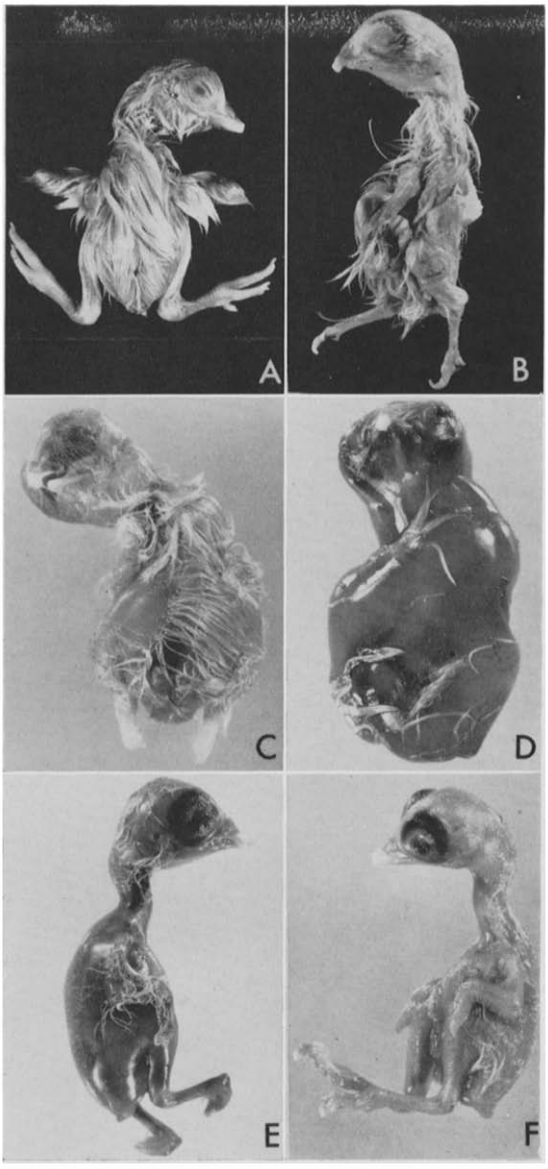

FIG. 1. Effects of CA on the gross appearance of the chick embryo.

A. Normal embryo, sacrificed at 18 days.

B. CA 0·05 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. Note short lower beak, curved upper beak, and missing toes.

C. CA 0·05 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. The beak is shortened; there are missing toes, edema, and feather inhibition.

D. CA 0·05 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. The upper beak is shortened, with facial coloboma and cleft palate, short lower beak, eyelid defect, micromelia, missing toes, edema, and very severe feather inhibition.

E. CA 0·1 mg/egg, 8-day yolk sac injection, sacrificed at 18 days. There is a crossed beak and feather inhibition.

F. CA 0·8 mg/egg, 12-day yolk sac injection, sacrificed at 18 days. There is slight shortening of the upper beak and feathers are moderately inhibited.

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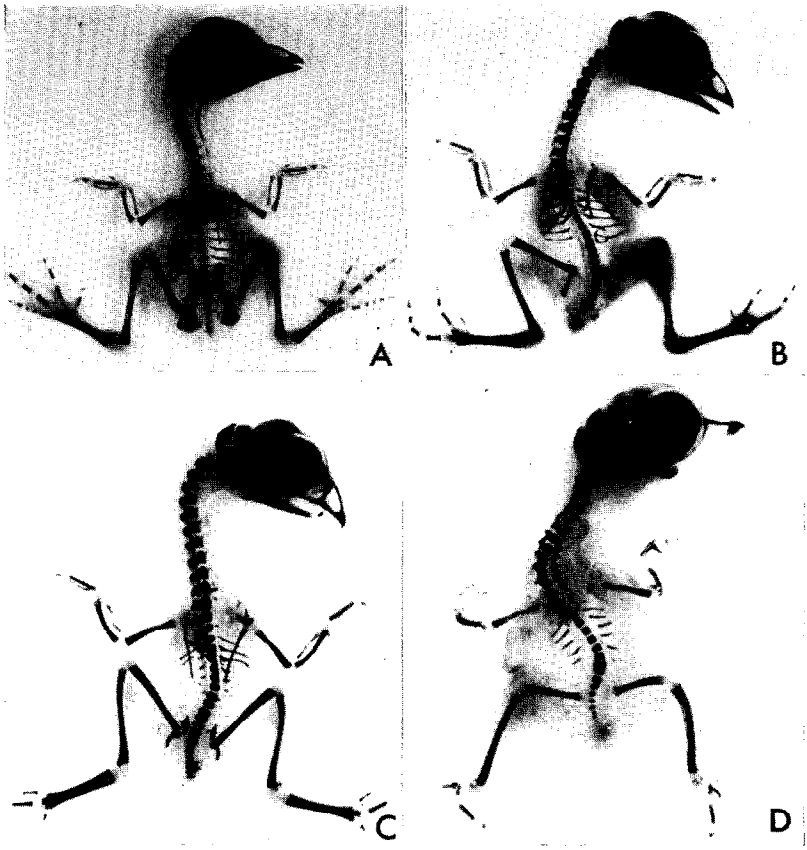

FIG. 2. Effects of CA on the skeleton of the chick embryo (cleared and stained with alizarin S).
A. Normal embryo sacrificed at 18 days.
B. CA 0·025 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. The pelvic girdle is almost absent and the fourth toe missing on one foot and shortened on the other foot.
C. CA 0·05 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. The lower beak is shortened, the first toe is missing and distal toe digits are missing; the first and second phalanges of the second and third toes are fused, and the pelvic girdle is almost absent.
D. CA 0·05 mg/egg, 4-day yolk sac injection, sacrificed at 18 days. There is a facial coloboma and cleft palate and the lower beak is almost absent; the femur and humerus are angulated; in addition there is spinal curvature, ribs are of uneven length, the metacarpals and metatarsals are missing, only two toes are present with missing distal phalanges, and the pelvic girdle is absent.

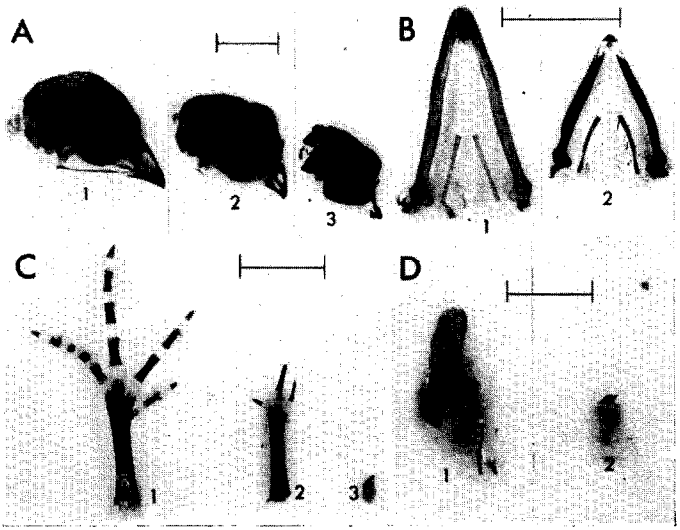

FIG. 3. Effect of CA on skeletal components of the chick embryo sacrificed at 18 days (cleared and stained with alizarin S; the bar equals 1 cm).

A. Upper beak and skull. B. Lower beak. C. Left foot. D. Pelvic girdle.

1. Normal embryo, sacrificed at 18 days. 2. CA 0·05 mg/egg on day 4, sacrificed at 18 days 3. CA 0·1 mg/egg on day 4, sacrificed at 18 days; lower beak and pelvic girdle were not found.

of CA producing developmental abnormalities are usually lethal before day 18; the survivors resemble embryos treated on day 4.

Facial colobomas are produced on days 4 and 5, but embryos injected beyond this period, on days 6, 7, or 8, develop a short lower beak or twisted beak. There is thus an abrupt change in the severity of the facial abnormalities in embryos treated on day 4 or 5 when compared to those treated later (Fig. 1, D and E; Fig. 4). Cleft

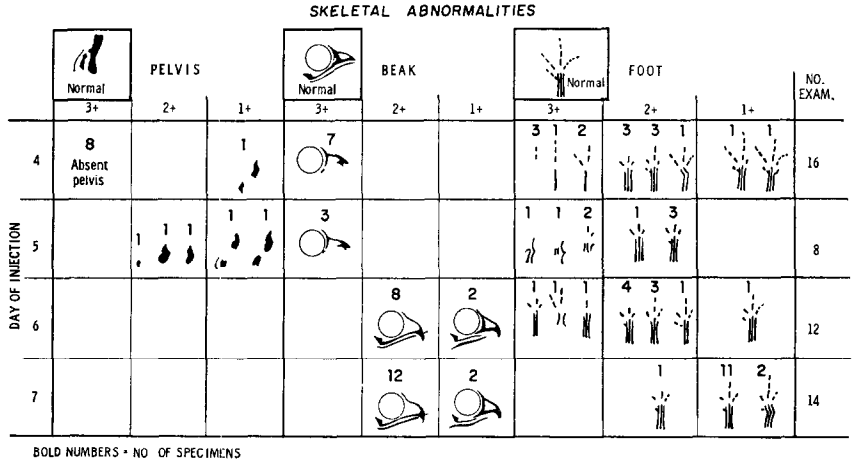

FIG. 4. Skeletal abnormalities observed when teratogenic doses of cytosine arabinoside are given during days 4 to 7 of development. Of the embryos treated at 4, 5, 6, or 7 days of incubation, 16, 8, 12, and 14 of the embryos respectively which survived to 18 days were cleared and studied for skeletal defects. The 3+ refers to maximal, 2+ to moderate, 1+ to definite or minimal defects. When CA was injected at 4 days, the pelvis failed to develop (3+); when injected at 5 days, the pelvis was retarded but present; on day 6 and beyond, the pelvis was normal. Severe facial coloboma occurred if CA was injected on the day 4 or 5, but embryos injected on day 6 or 7 showed parrot or twisted upper beaks, and curved upper beaks. Severe defects of the feet occurred if eggs were injected on day 4, 5, or 6, but were largely absent if injected on day 7. The data indicate the importance of the developmental stage of the embryo in determining its response to a teratogenic agent. These embryos studied all survived to 18 days, whereas many embryos at this dose range succumbed earlier in development, suggesting that more severe defects, incompatible with survival, had been produced. These could be followed by sacrificing the embryos at earlier stages.

palates did not occur in embryos treated on day 6 or later. Eyelid defects and micromelia were not found after day 7, but feather defects occur in embryos treated at any stage through day 12. The pelvic girdle was frequently absent on day 4; embryos treated on day 5 showed a defective pelvis, and beyond this period CA did not cause abnormalities in the pelvic bones (Fig. 4). Toe defects of decreasing severity were found in embryos receiving a single injection of CA at any time from day 4 to 7 (Fig. 4). The embryos injected on day 12 showed weight and feather inhibition, but were otherwise apparently normal (Fig. 1F). Embryos surviving a single CA injection between days 6 and 8 of incubation weighed less at 18 days than those treated once between days 4 and 5 (Table 3). Presumably the embryos treated earlier were more severely damaged, and only the less severely affected ones survived to 18 days, whereas lethal defects were less conspicuous in the embryos treated at a later stage, and there were more survivors at 18 days.

DISCUSSION

Cytosine arabinoside inhibits the development of the chick embryo *in vivo* and causes a variety of developmental defects, which are related to the stage of development at the time of treatment. The dose of CA required to cause growth inhibition or death of the chick embryo increases progressively from 0·025 mg/embryo at 4 days to 0·5–1·0 mg/embryo at 12 days, which parallels the increase in mass of the embryo from about 0·1 g at 4 days to 5·0 g at 12 days. Thus there is a direct correlation between the increasing weight of the embryo and the LD_{50} dose of CA. The amount of CA injected into the egg at 4 days is about 1×10^{-4} m-mole; this is calculated to be 2×10^{-3} m-mole/l. egg, or 1 m-mole/kg chick embryo. While CA was injected into the yolk sac in most studies, and the rate and efficiency of its absorption from the yolk is not known, the toxicity of CA was not increased when it was placed on the chorioallantoic membrane. This suggests that CA is rapidly absorbed from the yolk sac.

The chick embryo at 4 days (Hamburger-Hamilton, Stages 23–24)[25] is differentiated to a considerable degree with a well-defined external form, limb buds, eyes, central nervous system, and visceral arches. The skeletal structures are not yet evident, and these subsequently develop in their separate sequences. Goff[26] has diagrammed the developmental progression of various parts of the appendicular skeleton, and related its temporal status to the developmental defects produced by X-rays administered at different stages of development.

Qualitatively similar phenomena occur with CA; embryos treated on day 4 or 5 and surviving to 18 days show profound skeletal defects, such as facial coloboma, cleft palate, absence of the pelvic bones, and deletions of bones of the extremity. By day 6, however, the bones of the face and pelvis have differentiated beyond the susceptible stage, and CA does not cause major deletions of the skeleton except for bones of the feet. Beak deformities, general evidence of growth inhibition, and defective feather development occur in embryos treated at the later stages. These results suggest that CA produces embryonic defects by acting on specific susceptible processes evolving in the embryo; if injury to a specific differentiating organ prevents its growth or disturbs its structure, and its loss or malfunction is incompatible with survival, the embryo may die within a few hours or days. If nonvital structures are affected, as far as survival *in vivo* is concerned, the abnormal embryo can survive to hatching. Embryos at later stages are more differentiated, and drugs are less likely to produce disturbances in differentiation and structure of vital organs. The older embryos, in other words, respond to growth-inhibiting drugs as do the more mature members of the species.

The duration of the action of a single injection of CA *in ovo* on the chick embryo is uncertain, but an effective dose acts for a sufficient period to produce its effect on susceptible cells and organ systems. CA then may be inactivated by the embryo tissues, or bound in some compartment of the egg in an unavailable form. While there are no *in ovo* data available, the embryo may be presumed to have a cytidine deaminase, which will convert CA to uracil arabinoside, a relatively inactive compound.[27, 28] The activity of cytidine deaminase may be expected to vary at different stages of development. An embryo, while displaying stigmata of the CA-induced defects, may continue to grow and survive as long as the abnormalities are not immediately lethal. While skeletal abnormalities can be readily observed, and the

skeleton is conspicuously affected because of its complex interrelationships, less structured organ systems such as the lungs, liver, and kidneys are not so obviously altered. The cerebellum is severely inhibited in chick embryos treated on day 12, however, and this has also been observed in newborn hamsters injected with CA.[19] Further studies will undoubtedly reveal a number of other developmental defects caused by CA.

The mechanism of action of CA on cellular proliferation has been widely studied. Chu and Fischer[10] have shown that CA is phosphorylated to the diphosphonucleotide level, and CADP blocks the reduction of cytidylic acid disphosphate (CDP) to deoxycytidylic acid disphosphate (dCDP); dCTP is an essential component of DNA, and the induced dCTP deficiency prevents DNA synthesis and cell division.[7, 29] An additional mechanism of action may involve the competitive inhibition of the conversion of dCR to dCMP and dCDP.[29] The effects of CA on a number of experimental systems can be prevented by the use of dCR; these include viruses,[1] bacteria,[5] cells in tissue culture,[8–10] tumor-bearing mice,[30] and rat fetuses.[18] While the protective action of dCR is complete in some cases, in other systems an excess of dCR has not given complete protection against CA.[9, 30] Evidence has been presented that CA is incorporated into DNA,[9, 30] and Silagi[9] has suggested that the inability of dCR to protect completely against L cells in tissue culture is due to inability of CA-containing DNA to replicate normally. Cardeilhac and Cohen,[29] however, in a bacterial system, were unable to obtain any evidence for the incorporation of CA into DNA or RNA. CA produces chromosome breaks in cultures of human leukocytes[15] and human lung cells,[16] which may be explained by the inhibition of DNA synthesis as well as by the incorporation of CA into DNA. Nichols and Heneen[16] have made the interesting observation that CA produces chromosome changes in human embryo lung cells in tissue culture which enter mitosis during a 3-hr exposure. These cells presumably were not in the S period at the time of exposure, and they suggest that the S period may be longer than that calculated from demonstrable ^{3}H-TdR incorporation, or that CA somehow acts on the replicated chromosome. This observation further broadens the inquiry on the mechanism of action of CA. It is postulated, however, in the chick embryo that CA inhibits the reduction of CDP to dCDP, and DNA synthesis is thereby inhibited. This has important consequences on regions of the embryo engaged in critical steps in differentiation at the time of treatment. The possibility that the teratogenic effects of CA may be related to its incorporation into DNA, or to some other mechanism, has not been excluded.

A number of different groups of chemicals produce defects in the chick embryo characteristic for each group.[23] The developmental abnormalities caused by CA resemble those of a series of antimetabolites and related substances which interfere with purine and pyrimidine metabolism. Perhaps the most interesting agent in this group is the physiological purine nucleoside, deoxyguanoside (dGUR).[31] The injection of 0·25–0·50 mg of dGUR per egg into the yolk sac at 4 days, about 10 times the teratogenic dose of CA, produces developmental abnormalities similar to those seen with CA. One difference, observed both with dGUR[31] and X-rays,[26] is the fusion of the femur and tibia, which is rarely seen with CA. dAdR is less active by weight and is less teratogenic than dGUR.[31] The effects of dGUR can be prevented by dCR, even if given 9–12 hr after dGUR,[32] and dGUR appears to have a mechanism of action in the chick embryo similar to CA. Other antimetabolites, known to interfere

with purine and pyrimidine anabolism, have produced a qualitatively similar spectrum of teratogenic activity. The drugs include the folic acid antagonists,[33] azaserine,[34] and certain fluorinated pyrimidines.[35] Although these drugs act through different biochemical pathways, they produce similar defects which contrast with the distinctive abnormalities seen following the injection of other classes of teratogens in the chick embryo, such as the adrenal steroids, nicotinamide antagonists, cholinesterase inhibitors, lead, and thallium.[23] These data suggest that drugs which interfere with DNA synthesis and cell division by limiting the nucleotides available for DNA synthesis produce a similar pattern of toxicity and developmental abnormalities in the chick embryo.

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