Biochemical Pharmacology, 1961, Vol. 7, pp. 154–158. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF PHYSIOLOGICAL PURINES ON THE DEVELOPMENT OF THE CHICK EMBRYO*

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(*Received* 14 *December* 1960)

Abstract—The commonly occurring purine bases, ribonucleosides and deoxyribonucleosides were injected into the yolk sac of the 4-day-old chick embryo to determine their toxicity and teratogenic activity. The deoxyribonucleosides were most toxic, with dGuR, dAdR and dHxR having estimated LD_{50}'s of 2, 4 and 4 to 8 μmoles/egg, respectively. The deoxyribosyl derivatives caused marked disturbances in development of the 4-day embryo, with stunting of growth, edema, facial defects, absent bones and shortened extremities. dGuR was most active in producing weight inhibition and severe developmental disturbances; these effects were seen at 1 μmole/egg for dGuR, in contrast to 16 μmoles/egg for dHxR, and at an excess of 8 μmoles/egg for dAdR.

A NUMBER of antimetabolites which interfere with purine and pyrimidine synthesis[1] inhibit the normal growth and development of the chick embryo. Thus, the folic acid antagonists,[2] the glutamine antagonists[3] and the fluorinated pyrimidines[4] are toxic to the chick embryo, and at sublethal doses they induce severe developmental disturbances. Certain physiological purines can prevent the effects of specific antimetabolites by presumably restoring the substance whose production is blocked by the antimetabolite.[5]

In determining the amounts of physiological purines tolerated by the chick embryo during these protection studies, it was noted that the deoxyribosyl derivatives were more toxic than the purine bases or their ribosyl derivatives, and the deoxyribosides also produced developmental disturbances in the surviving embryos.

MATERIALS AND METHODS

Fertile white Leghorn eggs, obtained from a commercial source, were incubated at 38 °C. In most experiments, the drug was injected into the yolk sac through a small hole, produced in the blunt end of the egg, on the fourth day of incubation. The eggs were candled daily, and the dead ones recorded. Those embryos dying between the fifth and tenth days of incubation were discarded, but from the eleventh to eighteenth days the dead embryos were inspected and gross abnormalities recorded. Embryos surviving to the eighteenth day of incubation were sacrificed. These embryos were weighed, inspected for gross abnormalities, and in many instances they were cleared and the skeleton stained with alizarin.[6]

The chemicals were made up in a suspension of 0·5 per cent carboxymethyl cellulose in saline. The volume injected into each egg did not exceed 0·2 ml.

In a few instances drugs were given at 2, 8 or 12 days of incubation. The chemicals given, and the abbreviations used in this paper are: guanine (Gu), guanosine (GuR),

* This research was supported, in part, by grants from the American Cancer Society, Inc (T-40), the Albert and Mary Lasker Foundation, and grants from the National Institutes of Health.

2′-deoxyguanosine (dGuR),* adenine (Ad), adenosine(AdR), 2′-deoxyadenosine (dAdR),* hypoxanthine (Hx), inosine (HxR) and 2′-deoxyinosine (dHxR).*

RESULTS

The toxicity of the physiological purines following yolk sac injection at 4 days of incubation is summarized in Table 1. The number of eggs used, the dosage range and an approximate LD_{50}, estimated from the number of embryos surviving at 10 days following injection, are recorded for each drug. An LD_{50} was estimated from the results of several separate experiments, using the dose ranges shown in Table 1. Toxicity data are variable in separate experiments so that a precise LD_{50} is not useful. The incidence of developmental abnormalities is calculated from the examination of embryos from 11–18 days of incubation, and the surviving embryos sacrificed at 18 days. The most toxic purines in the chick embryo are the 2′-deoxyribosyl derivatives of guanine, adenine and hypoxanthine, in that order, in comparison to the less toxic purine bases and ribosyl derivatives. The deoxyribosyl derivatives also produced developmental abnormalities, and again dGuR proved to be most active.

TABLE 1. TOXICITY OF THE PHYSIOLOGICAL PURINES INJECTED INTO THE YOLK SAC OF THE 4-DAY CHICK EMBRYO
(Survivors sacrificed at 18 days of incubation.)

Compound	Dose range (mg/egg)	No. eggs injected 4 days	No. embryos alive		Abnormal embryos 11–18 days		Approximate LD_{50}	
			10 days	18 days	No.	%	mg/egg	μmoles/egg
Ad	2–8	55	29	23	2	7	4	30
AdR	2–8	55	40	29	0	0	2–4	8–16
dAdR	0·5–4	187	108	85	35	32	1	4
Gu	2–8	65	45	39	0	0	8	53
GuR	2–12	65	49	45	6	12	8	28
dGuR	0·1–4	277	193	153	133	69	0·5	2
Hx	2–8	45	21	18	0	0	2–4	15–30
HxR	2–8	35	22	17	2	9	4–8	15–30
dHxR	0·5–4	127	80	59	29	36	1–2	4–8

The effects of the deoxyribosyl purines were compared simultaneously in one experiment (Table 2). The differences noted were confirmed in a number of other experiments (Table 1). dGuR was highly teratogenic at 0·25 mg/egg, and larger doses produced 100 per cent of abnormal embryos. dAdR was about half as toxic dGuR, but even above the LD_{50} dAdR was weakly teratogenic in embryos surviving to 18 days of incubation. dHxR was least toxic, -and definite teratogenic effects occurred at 4·0 mg/egg. Accordingly, there appeared to be some dissociation between the toxicity and the weight inhibition and teratogenic effects of the deoxyribosides. dAdR at LD_{50} doses at 4 days caused less severe weight inhibition and inconsistent developmental effects in the embryos surviving beyond 10 days, as compared to equivalent toxic doses of dGuR.

The teratogenic effects of dGuR were analyzed in data pooled from several separate experiments (Table 3). Weight inhibition and developmental abnormalities were severe, and were particularly conspicuous in the embryos surviving and sacrificed on the eighteenth day of incubation (Fig. 1). The embryos grossly were stunted and edematous.

* The deoxyribonucleosides were obtained from the California Corporation for Biochemical Research.

TABLE 2. COMPARATIVE TOXICITY AND TERATOGENIC EFFECTS OF THE DEOXYRIBOSYL DERIVATIVES OF THE PHYSIOLOGICAL PURINES
(Injected into the yolk sac at 4 days of incubation; survivors sacrificed at 18 days.)

	2′-Deoxy-guanosine			2′-Deoxy-adenosine			2′-Deoxy-inosine		
Dose (mg/egg)	0·25	0·5	1·0	0·5	1·0	2·0	1·0	2·0	4·0
No. injected, 4 days	20	20	20	20	20	20	20	20	20
No. sacrificed, 18 days	16	9	5	16	7	4	14	5	8
No. of abnormal embryos	12	9	5	0	2	2	2	1	8
Abnormalities:									
Facial coloboma and cleft palate	7	9	5		1				2
Cleft palate	1						1		
Short lower beak	7	8	5		2	2		1	7
Micromelia	12	9	5						
Missing toes	9	9	5						3
Short toes	3								2
Webbed toes	4						1		2
Corneal cysts	1	6	5						
Eyelid defect		1							
Exteriorized viscera			1						
Edema, localized	2	1	2						1
Edema, generalized	3	7	4		1				
Feather inhibition	2	2	3						
Average emb. (wt., g)	12·9	10·4	8·5	15·6	14·1	11·5	15 7	14·5	13·0
18 days s.d.	±1·1	±2·3	±1·7	±2·6	±2·0	±1·1	±1·5	±2·6	±1·6

TABLE 3. TOXICITY AND TERATOGENIC EFFECTS OF 2-DEOXYGUANOSINE
(Injected into the yolk sac at 4 days of incubation; survivors sacrificed at 18 days.)

Dose (mg/egg)	0·1	0·25	0·50	1·0
No. injected, 4 days	18	107	85	59
No. sacrificed, 18 days	15	79	45	14
No. of abnormal embryos	1	49	44	14
Abnormalities:				
Facial coloboma and cleft palate	1	23	41	14
Cleft palate		6		
Short lower beak		24	40	13
Micromelia		42	43	14
Missing toes		37	42	14
Short toes		20	7	1
Webbed toes		10	4	
Corneal cysts		1	28	11
Eyelid defect		3	4	2
Exteriorized viscera				1
Edema, localized		5	3	5
Edema, generalized		7	18	8
Feather inhibition		4	2	3
Average embryo weight; s.d.	16·0 ±2·2	13·7 ±2·7	10·4 ±2·2	9·7 ±2·3

Many had corneal cysts, with occasional eyelid defects. Feather growth was often inhibited, and the extremities were grossly deformed, with shortened legs and wings and missing toes. The heads were severely distorted with short lower beak and cleft palates.

The gross disturbances corresponded with the bony changes noted in the cleared embryos. Some of the embryos treated at 0·25 mg/egg showed facial coloboma, cleft palate, shortened lower beaks and fusion of the cervical vertebrae. Many had fusion of the humerous and radius and of the femur and tibia. The metatarsals showed fusion and shortening, and the second and fourth toes were often missing.

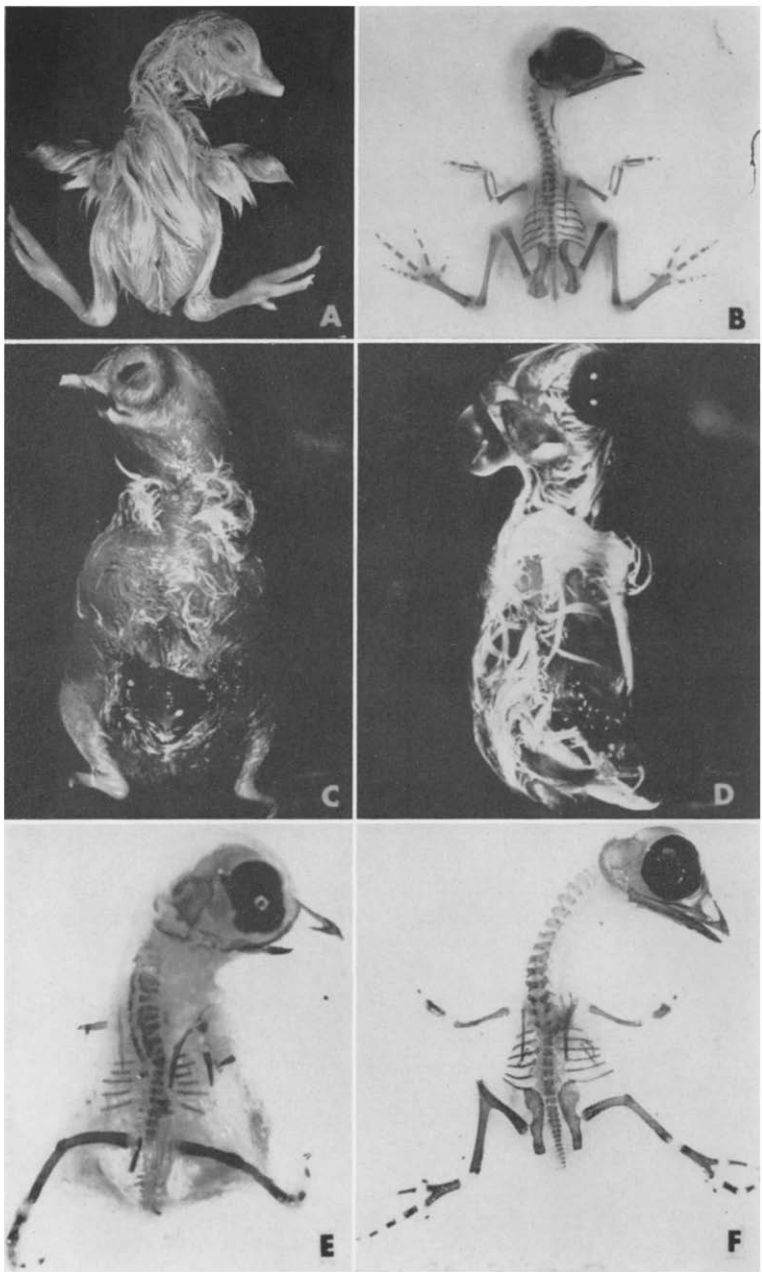

FIG. 1. (Photographs not to scale).

(caption on reverse)

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Caption FIG. 1.

A. Normal 18-day chick embryo.

B. Alizarin-stained skeleton of normal 18-day chick embryo.

C. Eighteen-day chick embryo, treated at 4 days with 0·5 mg of 2′-deoxyguanosine injected into the yolk sac. The embryo is moderately stunted and markedly edematous. There is a facial coloboma, a cleft palate and a short lower beak. Feather development is abnormal. There is marked shortening of the metatarsals and metacarpals, with only a single toe on each leg.

D. Eighteen-day chick embryo treated at 4 days with 0·5 mg of 2′-deoxyguanosine. The egg also received 4 mg of guanosine which does not affect the action of dGuR. The embryo is stunted. There are large corneal cysts. A facial coloboma and cleft palate are present. The long bones are shortened. There is incomplete closure of the lower abdominal wall.

E. Alizarin-stained, cleared skeleton of an 18-day chick embryo, treated at 4 days with 0·5 mg of 2′-deoxyguanosine injected into the yolk sac. The embryo is severely stunted and edematous. There is absence of calcified facial bones except the premaxilla (incisive); there is a short lower beak, the vertebrae are fused in the cervical and thoracic region, and poorly formed, the ribs are short and of irregular length, the femur and tibia are fused and the fibula missing; wings are poorly formed and the metacarpals and phalanges are incomplete.

F. Alizarin-stained, cleared skeleton of 18-day chick embryo treated at 4 days with 0·25 mg of 2′-deoxyguanosine injected into the yolk sac. The head is normal. The vertebrae appear normal. The humerus–radius and femur–tibia are fused, the ulna and fibula are missing. The terminal digits are poorly formed and toes 1 and 4 are shortened.

At 0·5 mg/egg the severe changes were more consistent, with face and rib defects, fusion of long bones, and absence of the ulnae. In some embryos the metacarpals and phalanges were missing. Embryos surviving 1·0 mg/egg until the eighteenth day were affected most severely. Facial bones were almost entirely absent, vertebrae were fused in a random manner, ribs were short and uneven, the long bones were fused, and metacarpals, metatarsals and phalanges missing. The ilium was missing in some cases, and the ischium and pubis were absent in all animals examined at this dose.

The LD_{50} of dGuR injected into the yolk sac at 2 days of incubation was approximately 0·05–0·10 mg/egg. Teratogenic effects were observed in embryos dying within 10 days after injection, but those surviving to 18 days were grossly normal. The toxicity and teratogenic action of dGuR thus appear to be increased in the 2-day as compared to the 4-day embryo, in that those affected did not survive to 18 days. At from 8 to 12 days of incubation, the toxic effects of dGuR and dAdR injected into the yolk sac were markedly reduced, and doses up to 10 mg/egg were without effect.

DISCUSSION

Of the physiological purines studied, the deoxynucleosides, particularly dGuR, were uniquely active in producing growth inhibition and developmental abnormalities. Distinct differences between toxicity and teratogenic activity were observed when the embryos were treated at 4 days (Table 4).

TABLE 4.

	dGuR	dAdR (μmoles/egg)	dHxR
Approximate LD_{50}	2	4	4–8
Effective teratogenic dose	1	8	16

While abnormal purine deoxynucleosides have not been available for study thus far, the toxicity of the physiological deoxyribonucleosides is consistent with observations on the enhanced activity of certain abnormal pyrimidine deoxyribonucleosides. When injected into the yolk sac of the 4-day embryo, 5-fluorodeoxyuridine may be lethal or teratogenic at 0·05 μg/egg, whereas comparable effects occur with 3·0 μg of 5-fluorouridine per egg and 200 μg of 5-fluorouracil per egg.[4] 5-Bromo- and 5-iodo-deoxyuridine cause growth inhibition when injected into the yolk sac of the 4-day embryo at doses of 50 and 75 μg/egg, respectively, whereas 5-bromo- and 5-iodo-uracil and their ribosyl derivatives are non-toxic up to 5000 μg/egg.[7] These data suggest that the embryo, *in vivo*, at 4 days may be unusually susceptible to deoxyribonucleosides, possibly because it cannot readily catabolize these compounds. Furthermore, the lower toxicity of the purine and pyrimidine bases and nucleosides suggest that the 4-day embryo cannot readily convert them to the more toxic deoxyribonucleosides. If this generalization can be supported by direct biochemical evidence, the 4-day chick embryo represents a favorable environment in which to study the inherent biological activity of preformed deoxyribonucleosides. Reichard, however, found that soluble enzymes extracted from 5-day old chick embryos reduced ribonucleotides of uracil, cytosine[8] and guanine[9] to their corresponding deoxyribonucleotides. This is not necessarily consistent with our *in ovo* observation that guanosine is neither highly toxic nor teratogenic, and it should be noted that 5′-guanylic acid is also comparatively nontoxic (approximate LD_{50}, 5 mg/egg) and non-teratogenic.

Inhibitory effects of dAdR on nucleic acid metabolism *in vitro* have been reported. Klenow and Langer[10] found that dAdR inhibited the incorporation of ^{14}C-formate into the DNA-thymine of Ehrlich cells *in vitro*; in contrast, dAdR had little effect on Yoshida ascites cells. The amount of acid-soluble thymine compounds was increased in the Ehrlich ascites cells treated with dAdR, indicating that dAdR did not interfere with thymine formation but with the synthesis of DNA.[11]

The effect of dAdR was reversed by dGuR, but not dHxR,[12] suggesting that dAdR inhibited the formation of dGuR from a closely related precursor. Prusoff[13] also found that dAdR blocked the utilization of ^{14}C-formate for the biosynthesis of DNA-thymine. In the most recent report, Maley and Maley[14] demonstrated that by incubating 4-day chick embryo suspensions for 2 hr *in vitro* with dAdR, cytidine-2-^{14}C and uridine-2-^{14}C incorporation was blocked, and the incorporation of thymidine-^{3}H into DNA was partially inhibited. While dGuR was less active than dAdR, it also inhibited the incorporation of precursors into DNA.

Our *in vivo* results indicate that dGuR is more active than dAdR and dHxR, particularly in regard to teratogenic activity. Our results thus differ quantitatively from those obtained with the chick embryo,[14] since dAdR is more active than dGuR *in vitro*, and the reverse of the *in vitro* results on Ehrlich ascites cells, as reported by Klenow and Langer[10], and Prusoff,[13] since they found dGuR ineffective.

dGuR causes profound developmental disturbances in the 4-day chick embryo consisting of growth inhibition, edema, beak defects and skeletal disturbances, particularly of the extremities. Similar effects have been seen in 4-day embryos treated with the glutamine antagonist, O-diazoacetyl-l-serine (azaserine)[3] and the fluorinated pyrimidines.[4] The teratogenic effects of azaserine can be prevented by adenine and the purine nucleosides,[5] although guanine is conspicuously ineffective, and thymidine will protect against the teratogenic and toxic effects of 5-fluorodeoxyuridine.[7] Azaserine, 5-fluorouracil and dGuR cause their most striking effects in embryos treated at 4 days of incubation; the high level of teratogenic activity at 4 days is in contrast to drugs which exhibit maximal effects at 8 days; these include cortisone,[6] insulin,[15] nicotanimide antagonists and thallium.[16] These data suggest the chick embryo at 4 days is unusually susceptible to drugs which interfere with nucleic acid metabolism.

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