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TERATOGENIC EFFECTS OF 6-HYDROXYLAMINOPURINE IN THE RAT—PROTECTION BY INOSINE*

SHAKUNTALA CHAUBE† and M. L. MURPHY

Division of Chemotherapy Research, The Sloan-Kettering Institute for Cancer Research, New York, N.Y., U.S.A.

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Abstract—Single i.p. injections of 6-hydroxylaminopurine (HAP) at doses ranging from 200 to 900 mg/kg of maternal body weight given to the pregnant Wistar rat on day 11 or 12 of gestation produced malformations which included cleft palate, micrognathia 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 (50–600 mg/kg) given on day 9 or 10 of gestation. A single dose of 500 mg/kg of inosine provided complete protection against fetal malformations produced by a single dose of 500 mg/kg of HAP at 21 days when the two compounds were given at zero time and up to 5 min apart to the 12-day pregnant rat. Only partial protection occurred with lower doses of inosine (200–400 mg/kg), with all doses of hypoxanthine (50–1000 mg/kg) and adenine (50–250 mg/kg) as well as with increasing time intervals (10–120 min) between administration of equal amounts (500 mg/kg) of HAP and inosine.

THE ADENINE antagonist, 6-hydroxylaminopurine (HAP), which was synthesized by Giner-Sorolla and Bendich,[1] was found to inhibit the growth of ascitic S 180,[2–4] Ehrlich ascites carcinoma[5] and P 815 leukemic cells[6] in mice. It was active against L5178Y lymphoma cells in culture,[3] mutagenic in phage T4,[7] and caused mitotic inhibition in human epidermoid HEp #2.[8]

In view of its various biological activities, HAP was examined for teratogenic effects in the rat. This report describes the malformations produced in 21-day-old rat fetuses after single injections of HAP into the pregnant rat on days 9–12 of gestation and presents evidence of the protective effects of inosine, hypoxanthine and adenine against HAP-induced malformations in the 12-day fetal rat.

MATERIALS AND METHODS

Chemicals. HAP, inosine, hypoxanthine and adenine were obtained from Nutritional Biochemicals Corp.

Injection solutions. HAP, inosine, hypoxanthine and adenine were suspended in a 0·5% solution of carboxymethylcellulose and prepared fresh on each injection day.

Animals. Pregnant CF Wistar rats of known gestation day, obtained from Carworth

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† Present address: New York University Medical Center, Institute for Rehabilitation Medicine 400 East 34th Street, New York, N.Y. 10016

Farms and weighing 200–250 g, were used in all experiments. They were housed in individual cages and fed on commercial Purina Chow with water *ad libitum*.

Teratogenic and protection studies. A total of 348 rats were treated according to the following schedule: I. On a day selected from day 9–12 of gestation, 106 rats were given single i.p. doses ranging in amounts from 50 to 1000 mg/kg of HAP alone; controls (23 rats) were given the vehicle (carboxymethylcellulose). II. On day 12 of gestation, 118 rats received single i.p. injections of HAP and inosine at zero min (48 rats) or at timed intervals (70 rats). When the compounds were combined, 500 mg/kg of HAP was injected along with single doses of inosine ranging from 50 to 1000 mg/kg at zero min or with 500 mg/kg of inosine at time intervals of 5 to 120 min apart. Controls received 500 mg/kg of HAP (15 rats) or varying amounts of inosine (50–1000 mg/kg) alone (14 rats). III. Also, on day 12 of gestation 55 rats were injected with single i.p. doses of 500 mg/kg of HAP along with single doses of hypoxanthine at 50–1000 mg/kg (31 rats) or adenine at 50–250 mg/kg (24 rats) at zero min; controls were given varying amounts of hypoxanthine (50–1000 mg/kg; 9 rats) or adenine (50–250 mg/kg; 8 rats) alone.

The doses were calculated on a milligram per kilogram basis of the maternal body weight. The day after mating was considered as day 1 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 fetuses and resorbing implantation sites were also recorded. A selected number of specimens from each litter were fixed in 95% ethanol for subsequent clearing and staining in alizarin red for the study of the bony skeleton.[9]

RESULTS

Maternal lethality. Single i.p. injections of 50–300 mg/kg of HAP were not lethal to the pregnant rat on any day of gestation. Fifty percent of the treated rats (estimated maternal LD_{50} dose) died by day 21 when given 400 mg/kg of HAP on day 9 and 900 mg/kg on day 12 of gestation. None of the treated mothers survived a single dose of 1000 mg/kg of HAP given on day 12 of gestation.

Single doses of 50–1000 mg/kg of inosine or hypoxanthine or 50–250 mg/kg of adenine were not lethal to the 12-day pregnant rat.

Effects of single doses of HAP in the fetal rat. The effects of single doses of HAP on the rat fetus when the pregnant rat was treated once from day 9 to 12 of gestation are shown in Fig. 1. Although some rats tolerated up to 900 mg/kg (day 12) of HAP, litters were completely destroyed (estimated fetal minimal LD_{100} dose) by day 21 of gestation at doses of 400, 600 and 800 mg/kg when given to the pregnant rat on days 9, 10 and 11 respectively. Single doses of HAP ranging from 50 to 600 mg/kg given on day 9 or 10 were lethal (per cent fetal mortality) to varying degrees but did not produce malformation in fetuses that survived to day 21 of gestation. The lowest dose of HAP which produced malformations was 200 mg/kg (day 12) and the highest, 900 mg/kg (day 12). Within this dose range, single injections of 400 and 500 mg/kg given to the pregnant rat on day 11 and 12, respectively, produced a maximum number of malformed fetuses (100 per cent) with minimum fetal deaths and resorptions (within the control range of 0–5 per cent). Higher doses were accompanied by rise in fetal mortality on both days of gestation.

Twenty-one-day-old fetuses from rats treated on day 11 and 12 of gestation with

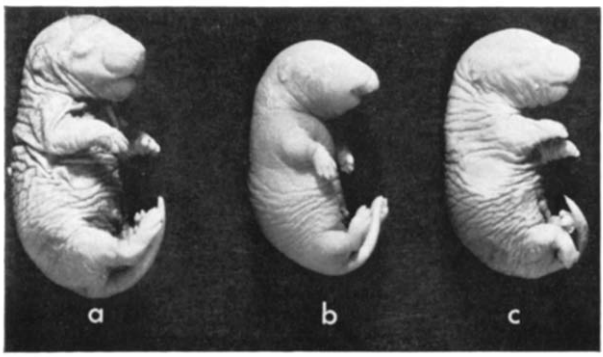

FIG 2. Twenty-one-day-old fetuses from rats treated with HAP. (a) Control; (b and c) fetuses from rats given a single injection of 400 and 800 mg/kg of HAP on day 11 and 12 respectively. Both specimens have malpositioned appendages and short, kinky tails; additionally (c) has syndactylous and brachydactylous fore and rear paws and (b) has cleft lip and micrognathia.

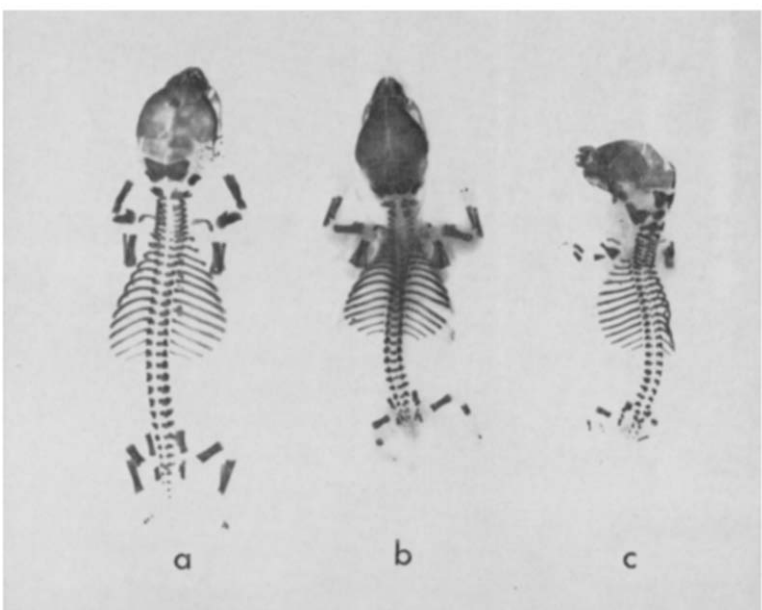

FIG. 3. Skeleton of 21-day-old fetuses stained in alizarin red. (a) Control; (b and c) fetuses from rats given a single dose of 400 and 800 mg/kg of HAP on day 11 and 12 of gestation respectively. Both specimens show retarded femurs and tibiae and the absence of fibulae, metatarsals and caudal vertebrae. Additionally (c) has incompletely ossified skull bones and cervical vertebrae, retarded scapulae, humerii, radii, ulnae and pelvic bones and absent centrae.

B.P.—*facing page 1148*

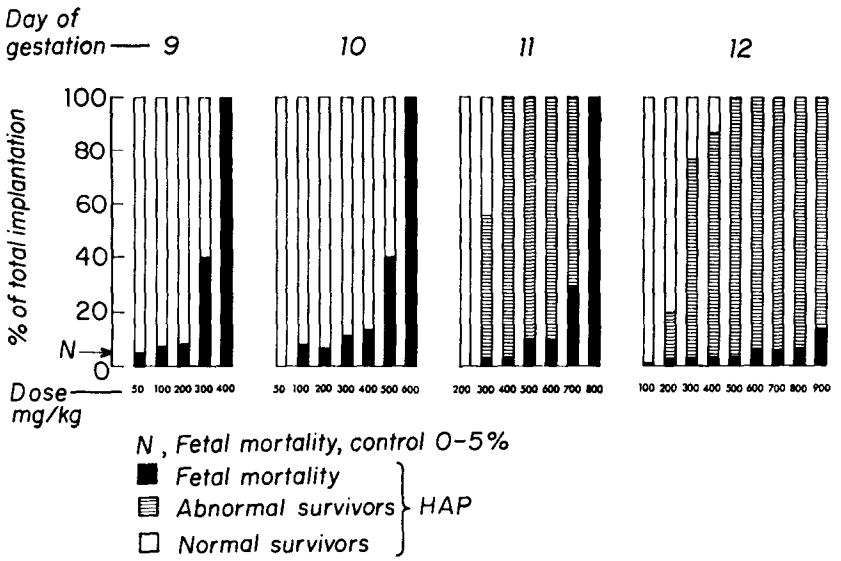

FIG. 1. Lethal and teratogenic effects produced by single i.p. injections of HAP into the pregnant rat from day 9–12 of gestation. Rats were sacrificed on day 21.

HAP are shown in Fig. 2 and the incidence of specific malformations is summarized in Table 1. Malformations of the appendages (malpositioned or clubbed), tail (short, kinky or absent), palate (cleft) and jaws were seen on both days. The incidence of individual malformations increased with the dose of the drug. Skeletal defects associated with gross malformations included short appendicular bones and incomplete ossification of skull bones and the sternebrae (Fig. 3).

TABLE 1. TYPES AND INCIDENCE OF MALFORMATIONS OBSERVED IN 21-DAY RAT FETUSES AFTER SINGLE I.P. INJECTIONS OF HAP INTO 11- AND 12-DAY PREGNANT RATS

	Day of gestation												
	11					12							
Dose (mg/kg)	300	400	500	600	700	200	300	400	500	600	700	800	900
Fetal mortality (%)	N*	N	6	8	29	N	N	N	N	N	6	7	13
Total survivors	35	58	45	20	49	49	60	77	150	55	67	59	26
Number abnormal	19	58	45	20	49	9	46	67	150	55	67	59	26
% with specific malformations:													
Retarded and/or clubbed													
Fore leg	84	78	78	87	100		9	18	40	42	40	39	38
Rear leg	52	77	84	87	100	100	72	92	95	95	95	95	100
Ectro, syn- or brachy-dactylous													
Fore paw	68	79	91	80	82	33	54	69	86	82	82	85	100
Rear paw	63	65	51	64	82	55	61	71	91	89	91	93	100
Cleft palate	5	20	38	42	51			2	3	22	30	29	61
Micrognathia	26	30	49	50	82			2	19	31	34	39	100
Tail: short, kinked or absent	26	30	64	78	82		13	10	53	55	69	69	100

* N, fetal mortality within the control range of 0–5 per cent.

Protective effects of inosine against HAP-induced malformations in the 12-day rat fetus. The teratogenic dose of 500 mg/kg of HAP was selected for these studies because it was the lowest single dose which produced a maximum number of malformed fetuses (100 per cent) and minimum number of fetal resorptions and deaths (fetal mortality was within the control range of 0–5 per cent at 21 days).

The results of experiments in which 500 mg/kg of HAP and varying amounts of inosine were injected at zero min into the 12-day pregnant rat are summarized in Fig. 4. Inosine alone injected at single doses of 100–1000 mg/kg into the 12-day pregnant rat was not teratogenic and produced fetal mortality in the same range as the controls at 21 days. A minimal dose of 500 mg/kg of inosine was required to

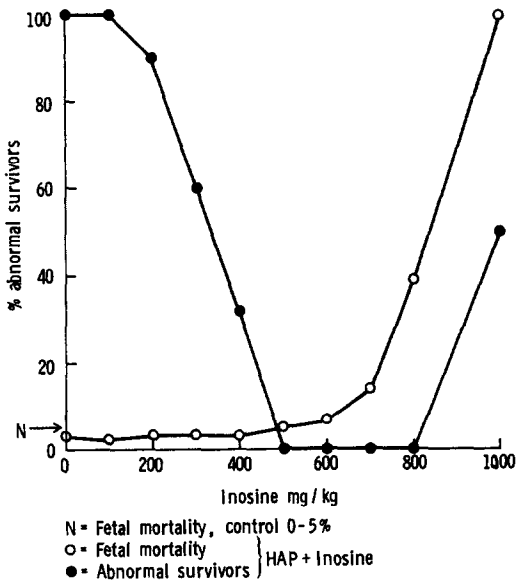

FIG. 4. Fetal effects produced by interaction of various doses of inosine (50–1000 mg/kg) and a single dose of HAP (500 mg/kg) injected at zero min into the 12-day pregnant rat. Rats were sacrificed on day 21 of gestation.

provide complete protection (100 per cent normal survivors) to the 12-day rat fetus against malformations produced by a single dose of 500 mg/kg of HAP at 21 days. This interaction was not toxic to the fetus, i.e. there was no increase in fetal mortality over that of controls (0–5 per cent). Inosine was also effective at higher doses, i.e. at 600–800 mg/kg, in providing complete protection against HAP-induced malformations, but these reactions were accompanied by increased fetal mortality. A dose of 1000 mg/kg of inosine gave 91 per cent fetal mortality and produced 100 per cent abnormal survivors at 21 days. At lower doses (200–400 mg/kg), inosine provided partial protection, reducing the number of abnormal survivors (by 10–73 per cent) and decreasing the incidence of all selected malformations (Table 2) without increasing the range of fetal deaths and resorptions above those of controls. The lowest dose of 100 mg/kg of inosine was not effective against HAP-induced malformations (100 per cent abnormal survivors obtained at day 21).

Results of experiments in which the protective role of inosine was tested over a period of time are shown in Fig. 5. In these studies a single dose of 500 mg/kg of

TABLE 2. PROTECTIVE EFFECTS OF VARYING AMOUNTS OF INOSINE AGAINST A SINGLE DOSE OF 500 mg/kg OF HAP WHEN INJECTED AT ZERO min INTO THE 12-DAY PREGNANT RAT*

	HAP† (500 mg/kg; 3·3 m-moles/kg)										Control
Inosine (mg/kg)	0	100	200	300	400	500	600	700	800	1000‡	100–1000
		0·36	0·75	1·1	1·4	1·8	2·2	2·5	2·8	3·6	0·36–3·6
Fetal mortality (%)	N§	N	N	N	N	N	8	14	39	56	N
Total survivors	150	52	49	62	47	83	37	42	30	18	147
Number abnormal	150	52	44	36	15	0	0	0	0	9	0
% With specific malformations:											
Retarded and/or clubbed											
Fore leg	40	15	5	6	0	0	0	0	0	0	0
Rear leg	86	79	66	36	26	0	0	0	0	79	0
Syn-, ectro-, brachy-dactylous											
Fore paw	60	57	55	44	40	0	0	0	0	33	0
Rear paw	90	85	75	50	33	0	0	0	0	44	0
Cleft palate	3	3	0	0	0	0	0	0	0	55	0
Tail: short, kinked or absent	38	27	18	6	0	0	0	0	0	0	0

* Rats were sacrificed on day 21 of gestation.
†HAP, 6-hydroxylaminopurine.
‡ 20 per cent of the treated mothers died at this dosage combination.
§ N, fetal mortality within the control range of 0–5 per cent.

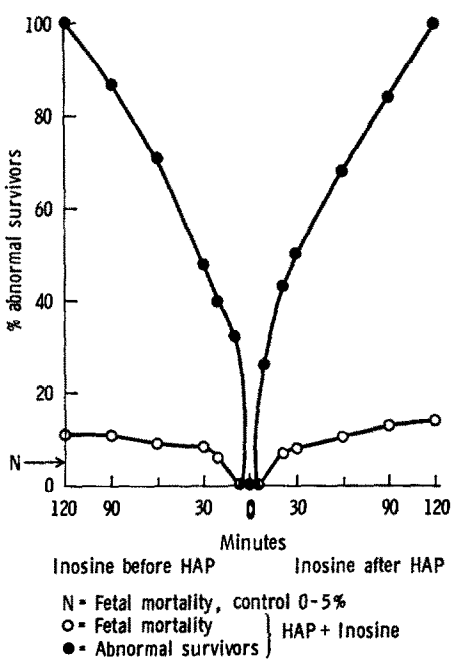

FIG. 5. Fetal effects produced by timed interaction of a single dose of 500 mg/kg of HAP and 500 mg/kg of inosine in the 12-day pregnant rat. Rats were sacrificed on day 21 of gestation.

inosine was injected into the 12-day pregnant rat before or after a single injection of 500 mg/kg of HAP at time intervals ranging from 5 to 120 min. There were no significant differences in the results of the two sets of experiments. In both, complete protection against HAP-induced malformations was observed up to 5 min, partial protection from 10 to 90 min (74–16 per cent) when inosine was given after and (8–1 4per cent when inosine was given before HAP) no protection at 120 min. The type and incidence of malformations produced are shown in Table 3. The feta

TABLE 3. PROTECTIVE EFFECTS OF A SINGLE DOSE OF 500 mg/kg OF INOSINE AGAINST SPECIFIC ABNORMALITIES PRODUCED BY A SINGLE INJECTION OF 500 mg/kg OF HAP, WHEN THE TWO COMPOUNDS ARE ADMINISTERED AT DIFFERENT TIME INTERVALS TO THE 12-DAY PREGNANT RAT*

Fetal effects	Inosine (500 mg/kg)									HAP† (500 mg/kg)	Inosine (500 mg/kg)
	Minutes before HAP					Minutes after HAP					
	120	60–90	10–30	5	0	0	10–30	30–90	120		
Fetal mortality (%)	11	7–10	N‡–8	N	N	N	N–7	10–12	12	N	N
Abnormal fetuses (%)	100	71–85	32–48	0	0	0	26–50	68–84	100	100	0
% With specific malformations:											
Retarded and/or clubbed											
Fore leg	2	0–2	0	0	0	0	0	0–4	2	40	0
Rear leg	84	84–90	44–91	0	0	0	46–62	82–86	100	95	0
Syn-, ectra-, brachy-dactylous											
Fore paw	70	67–70	44–70	0	0	0	38–75	78–91	100	86	0
Rear paw	97	67–82	40–67	0	0	0	38–40	57–69	92	91	0
Cleft palate	0	0	0	0	0	0	0	0	0	3	0
Tail: short, kinked or absent	63	27–29	16–22	0	0	0	0	34–45	40	53	0

* Rats were sacrificed on day 21 of gestation.
† HAP, 6-hydroxylaminopurine.
‡ N, fetal mortality within the control range of 0–5%.

palate and forelegs (excluding paws) received maximum protection over the entire period of time (10–120 min) in both sets of experiments while the rest of the abnormalities, viz. rear legs, fore and rear paws, and tail, were protected to various degrees at different intervals of time.

Comparison of the protective effects of inosine, hypoxanthine and adenine against HAP-induced malformations in the 12-day rat fetus. The protective effects of hypoxanthine and adenine against HAP-induced malformations are shown in Table 4. Single doses of hypoxanthine at 50–1000 mg/kg or adenine at 50–250 mg/kg injected into the 12-day pregnant rat were not teratogenic to the fetus at 21 days of gestation. At biologically equivalent doses, hypoxanthine and adenine were only partially, but about equally, effective in protecting the 12-day embryo against malformations produced by 500 mg/kg of HAP at 21 days. As a result of this protection, the number of abnormal fetuses and the incidence (100–500 and 100–200 mg/kg of hypoxanthine and adenine respectively) or only the incidence (50 and 700 mg/kg of hypoxanthine

TABLE 4. COMPARISON OF THE PROTECTIVE EFFECTS OF VARYING AMOUNTS OF INOSINE, HYPOXANTHINE AND ADENINE AGAINST MALFORMATIONS PRODUCED BY A SINGLE DOSE OF 500 mg/kg OF HAP WHEN INJECTED AT ZERO MIN INTO THE 12-DAY PREGNANT RAT*

	HAP† (500 mg/kg; 3·3 m-moles/kg)																		
Protective agent	Inosine					Hypoxanthine						Adenine				HAP	Inosine	Hypoxanthine	adenine
(mg/kg)	100	200	400	500–800	1000‡	50	100	200	250	500	700	50	100	200§	250§	500	100–1000	50–700	50–250
(m-moles/kg)	0·36	0·75	1·4	1·8–2·9	3·6	0·36	0·73	1·4	1·8	3·6	5·1	0·37	0·74	1·4	1·8	3·3	0·36–3·6	0·36–5·1	0·37–1·8
Fetal mortality (%)	N‖	N	N	N–39	56	N	N	N	7	9	10	N	8	57	69	N	N	N	N
Abnormal fetuses (%)	100	90	32	0	50	100	80	82	80	76	100	100	87	87	100	100	0	0	0
% With specific malformations:																			
Retarded and/or clubbed																			
Fore leg	15	5	0	0	0	5	15	6	9	9	18	25	22	37	57	40	0	0	0
Rear leg	79	66	26	0	79	92	79	47	48	33	58	80	74	100	85	95	0	0	0
Syn-, ectro-, brachy-dactylous																			
Fore paw	57	55	40	0	33	86	81	65	70	48	89	71	79	62	85	86	0	0	0
Rear paw	85	75	33	0	44	67	66	47	53	36	63	77	80	50	57	91	0	0	0
Cleft palate	3	0	0	0	55	0	2	3	3	3	26	17	34	50	57	3	0	0	0
Tail: short, kinked or absent	27	18	0	0	0	35	38	28	27	9	5	54	63	50	57	53	0	0	0

* Rats were sacrificed on day 21 of gestation.
† HAP, 6-hydroxylaminopurine.
‡ 20 per cent of the treated mothers died at this dosage combination.
§ 66 per cent of the treated mothers died at this dosage combination.
‖ N, fetal mortality within the control range of 0–5 per cent.

and adenine respectively) of some malformations were lowered in fetuses that survived to day 21 of gestation. While no increase in fetal mortality over that of controls resulted from interaction of 500 mg/kg of HAP and 50–200 mg/kg of hypoxanthine or 50 mg/kg of adenine, combinations of this amount of HAP with higher doses of the two compounds, i.e. 250–1000 and 100–250 mg/kg of hypoxanthine and adenine, respectively, not only increased the fetal mortality significantly over that of controls but also in some cases killed from 66 per cent (200, 250 mg/kg of adenine) to 80 per cent (1000 mg/kg of hypoxanthine) of the treated mothers by day 21 of gestation.

A comparison between the protective effects of biologically equivalent dose of inosine, hypoxanthine and adenine showed that 200 mg/kg of inosine and 100 mg/kg of hypoxanthine or adenine were about equally (90, 80 and 87 per cent abnormal survivors respectively) effective in protecting the 12-day fetus against HAP-induced malformations at 21 days. But whereas the effectiveness of inosine increased from 58 per cent (32 per cent abnormal fetuses) to 90 per cent (100 per cent normal fetuses) between the doses of 400 and 800 mg/kg, the effectiveness of hypoxanthine (200–500 mg/kg) and adenine (200 mg/kg) remained about the same; i.e. between 82–76 per cent abnormal fetuses with hypoxanthine and 87 per cent with adenine, for most of the doses except 250 mg/kg of adenine (100 per cent abnormal fetuses).

DISCUSSION

These experiments have demonstrated that single i.p. injections of HAP into the 11- or 12-day pregnant rat at approximately $\frac{2}{9}$ (200 mg/kg on day 12) to $\frac{8}{9}$ (800 mg/kg on day 12) of the dose which was lethal to the adult (estimated maternal LD_{50} dose was 900 mg/kg) on day 12 of gestation produced malformations which included deformed appendages, paws, jaws and tail and cleft palate. No malformations were produced by single doses of inosine (50–1000 mg/kg), hypoxanthine (50–1000 mg/kg) or adenine (50–250 mg/kg).

We did not include in this publication embryonic or fetal specimens which showed a variety of malformations associated with the different visceral organs. The histopathology of the visceral anomalies are described in a separate publication.[10]

In vivo in sarcoma 180 ascites cell,[4, 5] in cultured L5178Y lymphoma cells[4] and *in vitro* and in cell-free extracts of Ehrlich ascites cells,[11] HAP has been shown to markedly inhibit the conversion of hypoxanthine to adenine and guanine nucleotides and, to a somewhat lesser extent, the conversion of adenine to adenylic and of hypoxanthine to inosinic acid. This HAP-induced reduction in the utilization of inosinic acid (IMP) in turn resulted in inhibition of DNA, RNA and protein synthesis.[11] In mice bearing Ehrlich ascites carcinoma[5] and sarcoma 180 ascites cells,[5] the growth inhibitory effect of HAP was partially prevented by addition of adenine in the diet; similarly in cultured L5178Y lymphoma cells HAP-induced growth inhibition was prevented by addition of either adenine or hypoxanthine[5] to the media. Adenine appeared to be the more effective of the two agents in both systems.[4, 5] These findings suggest that HAP functions as an antimetabolite of both adenine and hypoxanthine, being a competitive inhibitor of the utilization of adenine and playing a somewhat complex, noncompetitive role as an analog of hypoxanthine.

Although the exact mechanism by which HAP produces developmental defects in the rat fetus cannot be adduced at this time, its selective action on the embryo when

the pregnant rat is treated is probably the result of its effect on the DNA of embryonic tissues for different periods of time and different concentrations of the drug. The teratogenic effects of HAP in the 12-day fetal rat could be prevented to varying degrees by inosine, hypoxanthine and adenine. Inosine, however, was the most effective of the three compounds, providing complete protection against drug-induced malformations at a dose which was approx. $\frac{4}{7}$ that of HAP (1·86 m-moles/kg of inosine vs. 3·3 m-moles/kg of HAP) when the two agents were given at zero min or up to 5 min apart to the 12-day pregnant rat. Only partial protection against HAP-induced malformations was observed at 21 days with lower doses of inosine (200–400 mg/kg) and with all doses of hypoxanthine (100–700 mg/kg) and adenine (100–200 mg/kg) when these compounds were given at zero min or from 10 to 90 min apart (inosine only) to the 12-day pregnant rat. It was interesting to note that the degree of protection when HAP and inosine were given at various time intervals apart was the same whether inosine was injected before or after HAP. The reason for this is not clear. The protective role of inosine, however, against the teratogenic action of HAP in the rat embryo may be attributed to the antimetabolic action of the analog.

It has been shown that in the rat HAP is rapidly reduced in the kidney to form adenine.[2, 3] Administration of large amounts of adenine to rats and mice was found to be nephrotoxic as a result of its oxidation *in vivo* to the sparingly soluble 2,8-dioxyadenine and deposition of the latter as a crystalline occlusion in the renal tubules.[12, 13] The high percentage of maternal deaths (66 per cent) observed in these studies when 500 mg/kg of HAP and 200 or 250 mg/kg of adenine were injected at zero min into the 12-day pregnant rat was probably the result of the additive effects of the two compounds in the maternal kidney tissue.

The adenosine analog, 6-hydroxylamino-9-β-D-ribofuranosylpurine (HAPR),[6, 14, 15] which showed antitumor activity against several experimental mouse neoplasms,[6, 16, 17] and the analog of 2,6-diaminopurine, 2,6-dihydroxylaminopurine (DHAP),[18] which was active against L1210 and P815 mouse leukemias,[18] were not teratogenic in the 12-day pregnant rat (authors' unpublished data). In the adult rat, HAPR is presumably hydrolyzed by adenosine deaminase to give inosine and hydroxylamine.* Perhaps the lack of teratogenic activity of HAPR is due to its rapid conversion and excretion by the maternal organism.

Substitution of the sulfhydryl group in the 6-position of xanthine, hypoxanthine or inosine to form mercaptopurine (MP), 6-thioguanine (TG) and their nucleosides, 6-mercaptopurine riboside (MPR) and 6-thioguanosine (TGR), as well as the 9-ethyl and 9-butyl analogs of 6-mercaptopurine (9 EMP, 9 BMP) were teratogenic in the 11 or 12-day pregnant rats, whereas 6-chloropurine (CP), 6-chloropurine riboside (CPR), 2,6-diaminopurine (DAP) and azaguanine were not.[19]

* A. Giner-Sorolla, personal communication.

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