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EFFECTS OF 4-HYDROXYPYRAZOLO(3,4-d)PYRIMIDINE UPON THE CATABOLISM OF PURINES BY VARIOUS TISSUES OF THE RAT AND UPON THE RATE OF GROWTH OF MORRIS 5123-C HEPATOMA*

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Abstract—4-Hydroxypyrazolo(3,4-d)pyrimidine (HPP) injected i.p. caused an inhibition of xanthine oxidase activity in the livers and kidneys (but not the spleens) of rats, as measured by catabolism of hypoxanthine-8-^{14}C (*in vivo* and *in vitro*), inosine (8-^{14}C)-5′-monophosphate (*in vitro*), and xanthine-8-^{14}C (*in vitro*). This action of HPP was transient, and there was no evidence of cumulative effects.

Administration of HPP in the diet proved as effective as i.p. injection, but again there was no cumulative effect. It caused a decrease in xanthine oxidase activity and an inhibition of purine biosynthesis in host liver and Morris 5123-C hepatoma. No difference in growth rate of the hepatomas or in body weights of rats maintained on control and HPP-containing diets was observed.

PREVIOUS studies have shown an inverse relationship between the rate of growth of several transplantable hepatomas and the xanthine oxidase activities of the tumors.[1] It was suggested that, although a deficiency of purine catabolism might not be a requisite for neoplasia, the rate of growth of a tumor might be partially determined by the level of xanthine oxidase activity. If this suggestion should be correct, then lowering the xanthine oxidase activity of the tumor *in vivo* should result in an increased rate of growth.

It has been shown that 4-hydroxypyrazolo(3,4-d)pyrimidine (HPP) (Allopurinol) is a powerful inhibitor of xanthine oxidase both *in vivo*[2–4] and *in vitro*[4, 5] in experimental animals and man. This compound causes decreased amounts of uric acid and increased amounts of hypoxanthine and xanthine excreted in the urine.[2–4, 6, 7] In a number of patients the decrease in uric acid excretion exceeded the increase in excretion of hypoxanthine and xanthine.[6] This suggests that one effect of the compound may be a suppression of purine biosynthesis *de novo*.[4, 8] On the other hand, this limited buildup of hypoxanthine and xanthine may be explained by the fact that these purines are utilized for the synthesis of nucleic acids.[9, 10]

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The experiments reported here were performed to determine what dosage, route, and schedule of administration of HPP would cause continuous inhibition of xanthine oxidase of various tissues *in vivo* and whether such inhibition would cause an increase in the rate of growth of the slowly growing Morris 5123-C hepatoma, which has a high xanthine oxidase activity.[1, 11]

MATERIALS AND METHODS

Radioactive substrates. The following radioactive compounds were obtained from the indicated suppliers and used in this study: hypoxanthine-8-^{14}C (specific activity, 30·6 μc/mg), New England Nuclear Corp.; xanthine-8-^{14}C (specific activity, 31·2 μc/mg), Isotope Specialities Co.; inosine(8-^{14}C)-5′-monophosphate, disodium salt heptahydrate (specific activity, 1·04 μc/mg), Schwarz BioResearch, Inc.; and sodium formate-^{14}C (specific activity, 298 μc/mg), New England Nuclear Corp.

Animals and hepatomas. Adult male Sprague-Dawley rats were used in all experiments involving nontumor-bearing animals. Male Buffalo rats served as recipients of bilateral subcutaneous implants of Morris 5123-C hepatoma[12] (generation 49).

Synthetic diet. The standard synthetic diet consisted of the following ingredients in the indicated quantities: sucrose, 654 g; Wesson salt mix, 40 g; oil premix [corn oil, cod liver oil, α-tocophenol (80:20:0·4)], 100 g; Alphacel, 110 g; casein, 200 g; L-cystine, 3 g; choline, 2 g; inositol, 1 g; riboflavin, 6 mg; pyridoxine, 6 mg; thiamine, 6 mg; calcium pantothenate, 30 mg; niacin, 25 mg; menadione, 5 mg; vitamin B_{12}, 0·5 μg; biotin, 125 μg; folic acid, 2 mg. Various quantities of HPP were added to this standard diet in the several experiments.

Determination of the effects of treatment with HPP in vivo *upon the catabolism of hypoxanthine, xanthine, and inosine-5′-phosphate* in vitro. After treatment with HPP, the rats were killed by carbon dioxide asphyxiation 24 hr after the last injection, and the tissues of the animals of each group were excised and pooled in ice-cold beakers. After freehand mincing of the tissues with knives, 1-g samples were placed in 10 ml of Krebs-Ringer phosphate buffer containing glucose, ATP, and a labeled substrate (hypoxanthine-^{14}C or xanthine-^{14}C, 2 μc/g tissue) and incubated in a Dubnoff shaking incubator at 37° in an atmosphere of oxygen for 4·5 hr.[13] After incubation the samples were centrifuged, the supernatant fraction was discarded, and the sediment was suspended in 10 ml water and poured into 4 volumes boiling ethanol and boiled for 5 min. The resulting extracts were used for paper chromatography and radioautography,[14] and the radioactive areas of the chromatograms were cut from the paper and assayed by a Tri-Carb liquid scintillation spectrometer.

Other portions of the minced tissues were homogenized by a VirTis model 27 homogenizer and suspended in Krebs-Ringer phosphate buffer containing glucose and ATP at a tissue:buffer ratio of 1:10 (w/v), and the resulting suspension was subjected to sonic vibations by a Raytheon 9 KC Magnetostriction oscillator. To 10-ml portions of the sonically treated homogenate was added 2 μc inosine (8-^{14}C)-5′-monophosphate, and the resulting mixture was incubated in a Dubnoff shaking incubator at 37° in an atmosphere of oxygen. After various intervals of incubation, samples of the mixture were poured into 4 volumes of boiling absolute ethanol, and boiling was continued for 5 min. The resulting extracts were used for chromatography, radioautography, and radioassay.

Determination of the effects of treatment with HPP in vivo *upon the fixation of* ^{14}C

from formate-^{14}C in vitro. Minced tissues were incubated as described above with 20 μc sodium formate-^{14}C/g tissue, and alcoholic extracts were prepared and used for chromatography, radioautography, and radioassay.

Determination of the effects of treatment with HPP in vivo *upon the metabolism of hypoxanthine-*8-^{14}C in vivo. Control rats and rats that had received one or more injections of HPP at specified dosages were given i.p. injections of 40 μc hypoxanthine-8-^{14}C. Forty minutes after the injection of the radioactive hypoxanthine, the rats were killed by carbon dioxide asphyxiation, and the livers, spleens, and kidneys were pooled separately and homogenized in a model 27 VirTis homogenizer. The homogenates were suspended in water (10 ml/g tissue), and extracts were prepared, chromatographed, and assayed as described above.

RESULTS AND DISCUSSION

In the studies of metabolism of labeled substrates, the tissues used were obtained from groups of three, five, or six animals. Although some of the experiments were performed only once, the consistency of the results obtained by the different protocols supports the creditability of the data.

The effects of multiple daily injections of HPP upon the catabolism of hypoxanthine, xanthine, and inosine-5'-monophosphate in vitro. Table 1 contains data that show the

TABLE 1. EFFECT OF TREATMENT WITH HPP *in vivo* ON THE FIXATION *in vitro* OF ^{14}C FROM HYPOXANTHINE-8-^{14}C OR XANTHINE-8-^{14}C BY MINCES OF RAT LIVER

Substrate	Compound	Per cent of total radioactivity of the extract		
		Control	After HPP at 20 mg/kg	After HPP at 30 mg/kg
Hypoxanthine-8-^{14}C	Adenosine monophosphate	1	1	1
	Hypoxanthine			9
	Inosine			2
	Xanthine	2	9	30
	Xanthosine	1	2	3
	Allantoin	96	88	57
	(Total ^{14}C on chromatogram, counts/min)	(9560)	(10,000)	(10,700)
Xanthine-8-^{14}C	Xanthine	5	7	11
	Xanthosine	1	1	2
	Allantoin	92	90	86
	Unknowns	2	1	1
	(Total ^{14}C on chromatogram, counts/min)	(15,000)	(15,900)	(11,900)

The animals received 5 daily intraperitoneal injections of HPP at the indicated dosage level and were killed 24 hr after the last injection. The minces were incubated with the labeled substrate for 4·5 hr.

effects of five daily injections of HPP upon the distribution of ^{14}C among soluble components of the minced livers after incubation with hypoxanthine-8-^{14}C or xanthine-8-^{14}C. With either substrate, more than 90% of the radioactivity in the livers of the control animals was present in allantoin. Treatment with HPP at a dosage of 20 mg/kg did not significantly alter the extent of oxidative catabolism by the minced livers, but treatment with 30 mg/kg did result in decreased catabolism of hypoxanthine-

8-^{14}C and possibly a slightly decreased catabolism of xanthine-8-^{14}C. It is likely that a greater decrease in catabolism of xanthine-8-^{14}C would be observed for shorter periods of incubation. Figure 1 shows that treatment at both dosage levels decreased the extent of catabolism of inosine-(8-^{14}C)-5′-monophosphate by homogenates of the livers.

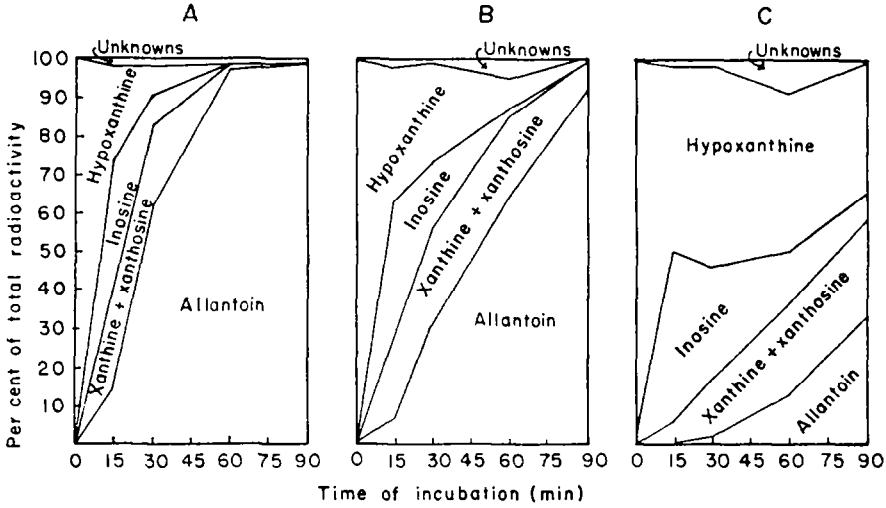

Fig. 1. Effect of treatment with HPP *in vivo* on the fixation *in vitro* of ^{14}C from inosine(8-^{14}C)-5′-monophosphate by homogenates of rat liver. The animals received five daily injections of HPP at a dosage level of 0 (A), 20 (B), or 30 (C) mg/kg and were killed 24 hr after the last injection.

Table 2. Effect of treatment with HPP *in vivo* on the fixation of ^{14}C *in vivo* from hypoxanthine-8-^{14}C by tissues of the rat

Tissue	Dose of HPP (mg/kg)	Per cent of total radioactivity of extract that is present in xanthine + uric acid + allantoin	
		After 1 hr*	After 24 hr†
Liver	0	84	84
	20	14	76
	30	13	73
Spleen	0	93	93
	20	11	87
	30	16	78
Kidney	0	96	96
	20	19	90
	30	22	89

* The animals received 6 daily injections of HPP and received the hypoxanthine-8-^{14}C 1 hr after the last injection; they were killed 40 min after receiving the radioactive compound.

† The animals received 5 daily injections of HPP and received the hypoxanthine-8-^{14}C 24 hr after the last injection; they were killed 40 min after receiving the radioactive compound.

The effects of multiple daily injections or single injections of HPP upon the catabolism of hypoxanthine in vivo. The data of Table 2 show that when hypoxanthine-8-^{14}C was injected 1 hr after the sixth daily dose of HPP and the animals were killed 40 min later, there was much less of the total ^{14}C of the livers, spleens, and kidneys present in catabolic products than was present in these products in the corresponding tissues of untreated animals. However, when the hypoxanthine-8-^{14}C was not injected until 24 hr after the last dose of HPP, the results obtained for the tissues of the treated animals were not greatly different from those obtained for the tissues of the control animals. These results, in conjunction with those of Table 1 and Fig. 1, indicate that HPP inhibits the catabolism of hypoxanthine and xanthine but that the effect persists for only a limited time and that a cumulative effect does not result from multiple doses. This hypothesis is substantiated by the data obtained at various intervals after a single injection of HPP (Fig. 2), which show that HPP has a great inhibitory effect

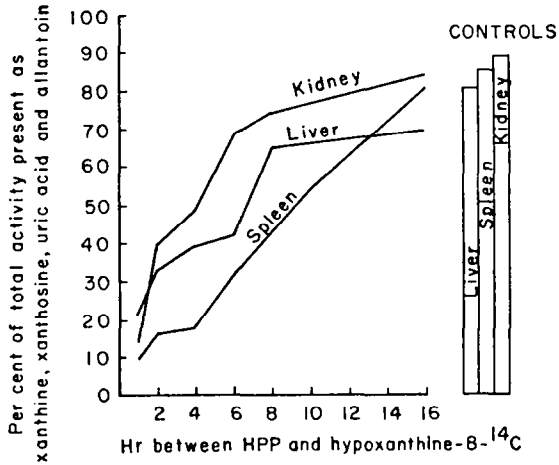

FIG. 2. Effects of a single injection of HPP (20 mg/kg) upon the fixation of ^{14}C *in vivo* from hypoxanthine-8-^{14}C by rat liver, rat spleen, and rat kidney. The animals were killed 40 min after injection of the hypoxanthine-8-^{14}C. The bars show the values for tissues from untreated animals.

only for a short period of time. Therefore, in order to maintain continuous inhibition of catabolism of purines by these tissues it would be necessary to administer the HPP at frequent intervals.

The effects of HPP in the diet upon the catabolism of hypoxanthine in vitro. Since multiple injections per day over an extended period of time seemed impractical, the possibility of maintaining continuous inhibition of the xanthine oxidase by incorporating the HPP in the diet of the animals was investigated. Table 3 contains data for minced tissues of animals on standard synthetic diet and on this diet containing HPP at various concentrations. The data show that inhibition of the oxidation of hypoxanthine-8-^{14}C occurred in liver, kidney, and Morris 5123-C hepatoma, but not in spleen. It also appears that there was no cumulative effect. Concentrations of HPP in the diet (0·04% and 0·06%) calculated to give daily ingestion of approximately 40 mg and 60 mg HPP per kg body weight caused essentially the same inhibitory effect, and this effect was greater than that observed at the 20 mg/kg level.

Table 3. Catabolism *in vitro* of hypoxanthine-8-^{14}C by minced tissues of rats fed synthetic diet and synthetic diet containing HPP

	Radioactivity of the compound as per cent of the total radioactivity of extract									
	Experiment 1*				Experiment 2*				Experiment 3*	
Concentration of HPP in diet (%)†	0	0·02	0	0·02	0	0·02	0·04	0·06	0	0·06
Time on diet (days):	16	16	30	30	7	7	7	7	48	48
Liver										
Xanthine + xanthosine	13	39	6	36	0	14	34	31	9	27
Uric acid	5	1	1	0	0	1	0	1	0	1
Allantoin	80	15	93	32	98	73	31	32	87	47
Sum	98	55	100	68	98	88	65	64	96	75
Kidney										
Xanthine + xanthosine	52	31	52	22	46	35	16	13	45	4
Uric acid	12	3	11	1	9	5	0	1	4	0
Allantoin	4	2	11	1	3	2	0	1	5	1
Sum	68	36	74	24	58	42	16	15	54	5
Spleen										
Xanthine + xanthosine	24	14	14	17	28	20	15	11	29	18
Uric acid	61	74	73	65	57	64	72	79	35	43
Allantoin	7	8	12	13	3	4	4	5	17	20
Sum	92	96	99	95	88	88	91	95	81	81
5123-C Hepatoma										
Xanthine + xanthosine									19	33
Uric acid									1	0
Allantoin									69	27
Sum									89	60

* In experiments 1 and 2 normal Sprague-Dawley rats were used. In experiment 3 Buffalo rats bearing Morris 5123-C hepatoma were used.

† For the indicated concentrations of HPP in the diet, it is estimated that the animals would ingest approximately 0, 20, 40, and 60 mg HPP/kg body weight per day.

No evidence of toxicity to HPP was observed at these dosage levels, and animals of the treated group and control group gained weight similarly.

The effects of HPP in the diet upon the fixation of ^{14}C from formate-^{14}C in vitro. Table 4 contains data for the fixation of ^{14}C from formate-^{14}C *in vitro* by minced tissues of animals maintained on the synthetic diet and on this diet containing HPP (calculated to give a daily ingestion of approximately 60 mg HPP/kg body weight) for 48 days. The data show that while HPP did not substantially decrease the radioactivity fixed in the amino acids and carboxylic acids, it did decrease the amount of activity in the purines and lower the total amount of ^{14}C incorporated by both the host liver and Morris 5123-C hepatoma. These results indicate that HPP not only inhibits xanthine oxidase activity but also suppresses purine biosynthesis. This fact is consistent with the observed inhibition of the activity of avian glutamine ribosylpyrophosphate-5-phosphate amidotransferase by the ribonucleotide of HPP and the suggestion that this ribonucleotide might inhibit purine biosynthesis by a pseudo-feedback mechanism.[8]

The effect of HPP in the diet upon the rate of growth of Morris 5123-C hepatoma. Rats that had received implants of Morris 5123-C hepatoma bilaterally and subcutaneously 7 days earlier were divided into two groups of 20 animals each. The control group was maintained on the complete synthetic diet, and the treated group received the synthetic diet containing 0·06% HPP. The rats were kept on these diets

TABLE 4. FIXATION OF ^{14}C *in vitro* FROM FORMATE-^{14}C BY MINCED HOST LIVER AND HEPATOMA 5123-C OF RATS FED SYNTHETIC DIET AND SYNTHETIC DIET CONTAINING HPP*

	Radioactivity (counts/min)					
	Host liver			Hep. 5123-C		
	Control	HPP-treated	HPP tr. as % of cont.	Control	HPP-treated	HPP tr. as % of cont.
Lactic acid	379	341	90	893	570	64
Malic acid	55	115	209	79	152	192
Citric acid	82	124	151	114	201	176
Serine	3428	3018	88	2920	2922	100
Glucose	2402	2132	89	1210	938	78
Alanine	966	895	93	786	627	80
Glutamine	425	365	86	85		
Aspartic acid	152	135	89	111	86	77
Glutamic acid	159	177	111	184	95	52
Formylglycinamide riboside	649	480	74	502	280	56
Formylglycinamide ribotide	88			144		
Hypoxanthine		508		452	267	59
Adenine	991	918	93			
Xanthine		300		558	160	29
Inosine	1290	526	41	745	151	20
Adenosine	5350	465	9	2002		
IMP	688	1110	161	249	126	51
A5P	13,910	2440	18	1756	386	22
ADP	3080	515	17	208	117	56
ATP	616	232	38	297	124	42
NAD	1590	358	22	221		
GMP	284	94	33	129		
Unknowns	5104	3580	70	3373	4329	128
Allantoin	1940	652	34	801	138	17
Urea	10,200	5080	50	1990	1532	77
Total ^{14}C fixed	53,828	24,560	46	19,809	13,201	67

* The animals were maintained on the standard synthetic diet or the standard synthetic diet containing 0·06% HPP for a period of 48 days. The minced tissues were incubated with formate-^{14}C for 4·5 hr.

for 48 days and were housed in a room with alternating 6-hr periods of light and dark. The tumors were palpated and measured three times a week with calipers.

From the caliper measurements, the approximate weights of the tumors were calculated, with the assumption that the tumors were prolate spheroids with a density of 1. It was considered that a tumor having a calculated weight of 200 mg was large enough to permit a reliable, reproducible measurement; and the day that a particular tumor reached approximately that weight was designated as Day 0 for that tumor. Subsequent calculated weights of that tumor were expressed as ratios to the weight on Day 0. Average values of the ratios based upon the measurements of five or more tumors were plotted versus time on semilog paper, and the straight lines of best fit were determined by the method of least squares (Fig. 3). (It was assumed that the tumor was increasing in weight logarithmically during this period of growth.) By Student's *t*-test it was determined that there was no significant difference in the slopes of the two curves; i.e. there was no discernible difference in the rates of growth of the Morris 5123-C hepatomas of the control and HPP-treated groups.

The length of time necessary for the tumor to reach a size of 200 mg was also determined for each group. Twenty-two tumors in the control group gave a mean of 34·5 ± 6·0 (S.D.) days; 19 tumors in the treated group gave a mean of 39·7 ± 6·5 days. Thus there was no significant difference between the control group and the HPP-treated group in the length of time after implantation necessary for the tumors to reach a size of 200 mg.

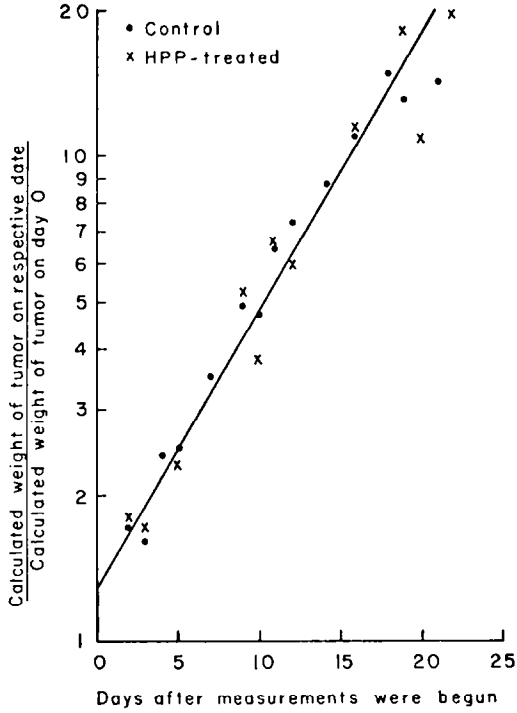

Fig. 3. Failure of HPP in the diet to alter the rate of growth of Morris 5123-C hepatoma. Day 0 represents the day on which the calculated weight of the tumor was approximately 200 mg, and this weight was used as the reference value for subsequent weights. Only one line is drawn through the data because the lines of best fit almost coincide.

Although HPP in the diet apparently accomplished sustained inhibition of purine catabolism in the Morris 5123-C hepatoma *in vivo*, and also caused a decrease in purine biosynthesis, possibly by a pseudo-feedback mechanism,[8] it did not alter the rate of growth of the tumor as measured. This is consistent with the results obtained by Hitchings,[15] which showed that the rate of growth of adenocarcinoma 755 was not changed upon the administration of HPP.

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