Antitrypanosomal Effect of Allopurinol: Conversion in vivo to Aminopyrazolopyrimidine Nucleotides by *Trypanosoma cruzi*

Abstract. *The parasite* Trypanosoma cruzi *metabolizes allopurinol by a sequential conversion to allopurinol mononucleotide and aminopurinol mononucleotide. The latter is incorporated into RNA. This transformation of a widely used innocuous agent, allopurinol, into a toxic adenine analog appears to account for the antiprotozoan effect of allopurinol. These unique enzymatic activities appear to occur only in* T. cruzi *and the pathogenic leishmaniae. Allopurinol may serve as a model for the synthesis of similar antiprotozoan agents.*

Investigations from this laboratory of the pathogenic leishmaniae have shown that allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine, HPP], an agent in wide clinical use, is effective in vitro against *Leishmania donovani, L. braziliensis*, and *L. mexicana* (*1, 2*). Subsequent investigations of the metabolism of allopurinol indicated that *L. donovani* converted the drug to ribonucleotide derivatives of 4-aminopyrazolopyrimidine (APP) (*3*), a compound known to be toxic to many cell types. In the experiments reported here we tested allopurinol against another hemoflagellate, *Trypanosoma cruzi*, the causative organism in Chagas' disease, and found that HPP is effective against the culture forms of this organism.

Trypanosoma cruzi, Costa Rica strain, was cultured as described previously (*4*), and the incorporation of isotopes into purines and nucleic acid was studied as described (*2, 5*). As shown in Fig. 1, HPP inhibited the growth of *T. cruzi* by approximately one log at a concentration of 5 to 10 μg/ml; APP was effective against *T. cruzi* to about the same degree as HPP. Oxipurinol, the major metabolic product of allopurinol in man, was without effect, as were allopurinol-1-ribonucleoside (HPPR) and allopurinol ribonucleoside monophosphate (HPPR monophosphate). Purine and pyrimidine bases, and their ribonucleosides and ribonucleotides, were used to reverse the inhibition of growth of *T. cruzi* by HPP and APP. As shown in Fig. 2, only adenine, inosine, and hypoxanthine could compete effectively with HPP. With the exception of inosine, the purine ribonucleosides and ribonucleotides were ineffective, as were the pyrimidine bases, at concentrations ten times that of HPP. These same antagonists of HPP were used in an attempt to reverse the toxicity of APP, and only adenine was effective. None of these natural purines affected growth of the parasites when added to the culture alone.

Table 1. Nucleotides and metabolites of [^{14}C]allopurinol in *Trypanosoma cruzi*. Cell suspensions were incubated for 24 hours with [6-^{14}C]allopurinol (2440 dpm/nmole) or allopurinol [6-^{14}C]-1-ribonucleoside (11,600 dpm/nmole). Perchloric acid extracts of cells were analyzed by high-pressure liquid chromatography on Partisil 10/SAX (Whatman), with a 0.015 to 1.0*M* gradient of KH_2PO_4, *p*H 3.5. Endogenous purines and pyrimidines were quantitated from the ultraviolet peak areas and the allopurinol metabolites were quantitated from the radioactivity in appropriate fractions.

Cellular nucleotides	Control	Concentration (picomoles per 10^6 cells) with ^{14}C-labeled		
		HPP		HPPR (5 μg/ml)
		2.5 μg/ml	25 μg/ml	
Nicotinamide adenine dinucleotide	66	77	19	
Nicotinamide adenine dinucleotide phosphate	19	23	18	
Uridine diphosphate glucose	77	75	28	
Adenosine diphosphate	82	83	48	
Guanosine diphosphate	26	31	13	
Cytidine triphosphate	7	7	5	2
Uridine triphosphate	54	58	56	30
Adenosine triphosphate	136	113	66	58
Guanosine triphosphate	25	28	19	12
HPPR monophosphate		28	106	0.2
APPR monophosphate		2	13	0.04
APPR diphosphate		4	6	0.1
APPR triphosphate		4	6	0.1
Number of cells per milliliter (× 10^6)	52	52	5.8	41
Pyrazolopyrimidine concentration (μg/ml)	None	2.5	25	5

To determine the fate of HPP in the cell, we added [6-^{14}C]HPP to a midlog culture of *T. cruzi* which we then incubated for 24 hours. The perchloric acid–soluble fraction of the cells was analyzed by high-pressure liquid chromatography, and nucleotide concentrations were calculated. A comparison between concentrations of normal products of purine metabolism and those compounds derived from HPP is given in Table 1. Only HPP, HPPR monophosphate, and APP ribonucleotides were radiolabeled. The HPP was converted with a high efficiency to HPPR monophosphate. Approximately 38 percent of the HPPR monophosphate in the cell was converted to 4-aminopyrazolopyrimidine ribonucleoside monophosphate (APPR monophosphate), and the di- and triphosphates of APPR.

Increasing the HPP from 2.5 μg/ml to 25 μg/ml caused a fourfold increase in the amount of HPPR monophosphate in the cells. The amount of HPPR monophosphate was 106 pmole per 10^6 cells, 1.5 times that of adenosine triphosphate. Only a 1.4-fold increase in the APPR diphosphate and APPR triphosphate was found.

The identity of these compounds was confirmed by hydrolysis with alkaline phosphatase to the respective ribonucleosides and then substantiated by cochromatography with the authentic compounds. No ribonucleotide of oxipurinol or 4-hydroxy-6-aminopyrazolopyrimidine was found, and neither HPPR diphosphate nor HPPR triphosphate was found. The medium in which the cells were incubated was found to contain unchanged [6-^{14}C]HPP (88 percent) and some HPPR (11 percent). There was no oxipurinol in the medium, implying a lack of xanthine oxidase in *T. cruzi*; in addition, there was no HPPR monophosphate, APP, or APPR.

The RNA in the precipitate obtained after perchloric acid extraction of the cells was hydrolyzed sequentially with potassium hydroxide and *Escherichia coli* alkaline phosphatase and the resulting nucleoside mixture was separated by high-pressure liquid chromatography by means of a Partisil-ODS reverse phase column (Whatman) and H_2O:ethanol gradient elution (*3*). A single small component of radioactivity appeared with the retention time of APPR (19 minutes) and was well separated from adenosine (23 minutes) and other components. The ratio of adenosine to APPR in the RNA was 1917:1. Purines and pyrimidines were not labeled.

Nucleotide pools were determined by high-pressure liquid chromatography

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(Table 1). Both nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate were present; cytidine triphosphate was the least abundant of the normal ribonucleoside triphosphates. Although allopurinol inhibited the growth of *T. cruzi* at a concentration of 2.5 μg/ml, there was no significant alteration in purine or pyrimidine ribonucleotide pools. These results are similar to the effects of allopurinol on ribonucleotide pools in *L. donovani* and *L. braziliensis* (*3*), and indicate that depletion of endogenous nucleotide pools is not associated with the toxic effects of allopurinol. The higher concentration of allopurinol decreased the amounts of nucleotides derived from adenine, which is consistent with the data on reversal of the effects of allopurinol (Fig. 2), but probably is not important with regard to the mechanism of action of this compound.

Inasmuch as 11 percent of the original ^{14}C-labeled HPP (2.5 μg/ml) was converted to HPPR and, since HPPR is found in man after administration of allopurinol (*6*), the ^{14}C-labeled ribonucleoside was tested (Table 1). An equimolar concentration of HPPR was converted only to insignificant amounts of HPPR monophosphate and APP ribonucleotides, in accord with its absence of toxicity to these organisms (Fig. 1). This could be due to cleavage of a small amount of HPPR to HPP with subsequent conversion to nucleotides of HPP and APP, or direct phosphorylation of HPPR. This is a very minor transformation and there are insufficient data to permit meaningful conclusions on this point.

Adenine and its congeners can reverse the toxic effect of allopurinol in *T. cruzi* (Fig. 2) and in the pathogenic leishmaniae (*2*). Only adenine, however, can act as an antagonist to APP. The inhibitory forms of HPP and APP are probably their ribonucleotides, whose formation would require the action of a phosphoribosyltransferase. This activation would be expected to be competitively inhibited by the natural purine substrates of these phosphoribosyltransferases. The alternative pathway for formation of HPP nucleotides would be by way of a two-step activation, first through nucleoside phosphorylase to form a ribonucleoside and then a nucleoside kinase. Direct activation by a phosphoribosyltransferase is more probable since allopurinol ribonucleoside was found not to be active against *T. cruzi*

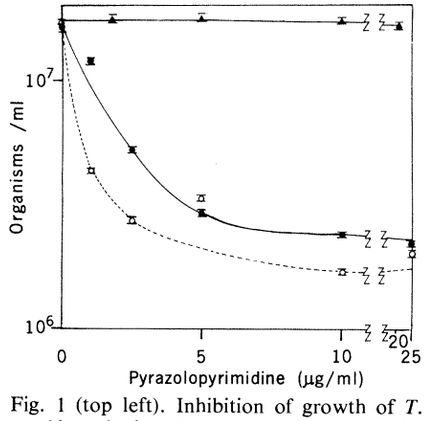

Fig. 1 (top left). Inhibition of growth of *T. cruzi* by substituted pyrazolopyrimidines. The organisms were incubated with increasing concentrations of allopurinol (●), aminopyrazolo(3,4-*d*)pyrimidine (○), or allopurinol-1-riboside and allopurinol monophosphate (▲), for 5 to 7 days until the control cultures had reached stationary phase. The bars indicate the ranges of three replicate counts on each flask, usually less than ± 2 percent.

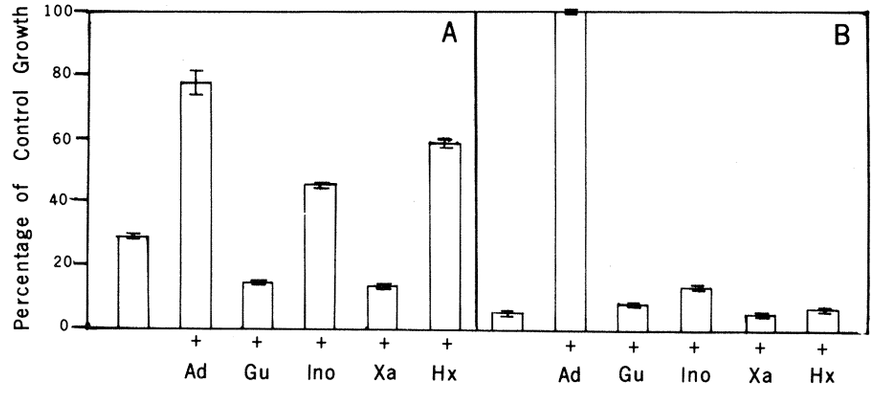

Fig. 2 (top right). Reversal of effect of substituted pyrazolopyrimidine by purines in *T. cruzi*. Allopurinol (A) and aminopyrazolo(3,4-*d*)-pyrimidine (B) were present at a concentration of 5 μg/ml. The antagonists were present at a concentration of 50 μg/ml. The bars indicate the ranges of three replicate counts on each flask, usually less than ± 2 percent.

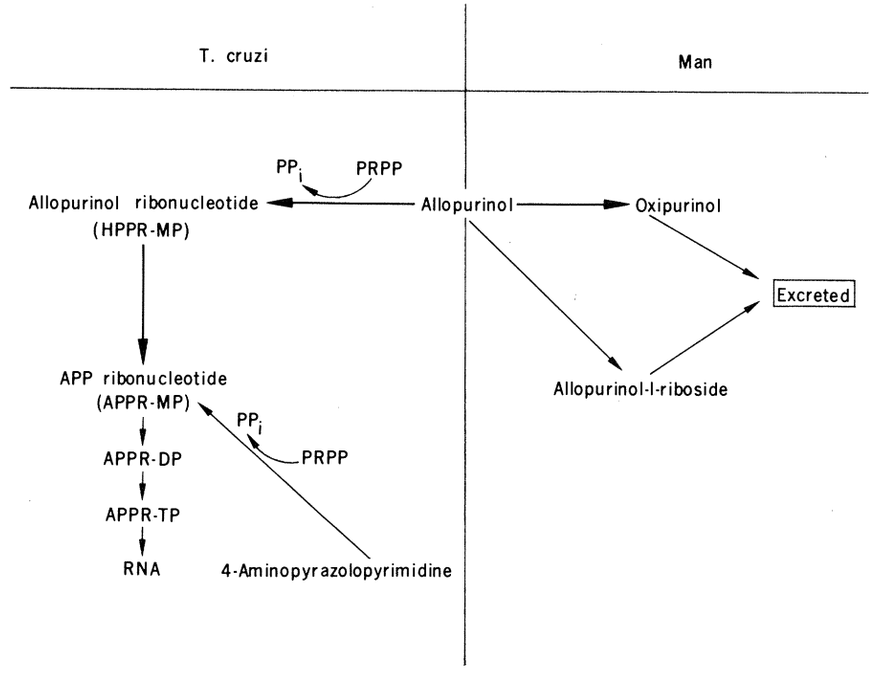

Fig. 3 (bottom right). Metabolic transformation of allopurinol in *T. cruzi* and man. Approximately 90 percent of orally administered allopurinol is converted in man to oxipurinol by xanthine oxidase; the remainder is converted by nucleoside phosphorylase to allopurinol riboside. Human erythrocytes, when incubated with allopurinol, will synthesize trace amounts of 1-allopurinol-5′-phosphate; in addition, small amounts of oxipurinol-7-riboside have been recovered from human urine. The major metabolic pathway of allopurinol in *T. cruzi* is to allopurinol ribonucleotide (*HPPR-MP*). This probably is mediated through a phosphoribosyltransferase since there is no evidence for an intermediate of allopurinol riboside. The HPPR monophosphate is subsequently converted by the adenylosuccinate synthetase to APP ribonucleotide (*APPR-MP*). This compound is converted then to the diphosphate (*DP*) and triphosphate (*TP*) and incorporated into RNA. Aminopyrazolopyrimidine can be converted directly to APPR monophosphate by a ribosyltransferase, probably the adenine phosphoribosyltransferase; PP_i, inorganic pyrophosphate; *PRPP*, phosphoribosylpyrophosphate.

and was not converted to nucleotides efficiently. Also, the data indicate that HPPR monophosphate may act as an analog of inosinic acid which can be converted by adenylosuccinate synthetase to APPR monophosphate in the parasite. The amination of HPPR monophosphate in *T. cruzi* is a reaction which does not occur in mammalian cells (*7*).

Since HPP must be converted to a ribonucleotide to be active, the reversal of the inhibition by hypoxanthine may be explained as a result of competition of the two bases for the hypoxanthine-guanine phosphoribosyltransferase. Alternatively, the inosinic acid formed from hypoxanthine may compete with HPPR monophosphate for adenylosuccinate synthetase. The reversal of allopurinol's activity by adenine could be due to a preliminary deamination of adenine to hypoxanthine with an action as described, or its activation by an adenine phosphoribosyltransferase to adenosine monophosphate which could compete with APPR monophosphate for conversion to the triphosphate and incorporation into RNA. The obverse, activation of APP by an adenine phosphoribosyltransferase, would not be antagonized by hypoxanthine or inosine and the product, APPR monophosphate, would be directly available for conversion to the di- and triphosphate forms and introduction into cellular RNA. Adenine would compete with APP for the phosphoribosyltransferase or as a nucleotide into cellular RNA. This proposed scheme is summarized in Fig. 3.

Thus, *T. cruzi*, like the pathogenic leishmaniae, appears to possess a phosphoribosyltransferase capable of converting HPP efficiently to HPPR monophosphate and an adenylosuccinate synthetase which can aminate HPPR monophosphate to APPR monophosphate. *Trypanosoma cruzi*, because of enzymatic differences between itself and its host, transforms a compound which is innocuous for mammalian cells into one which is toxic for the parasite. It will be of considerable interest to attempt to exploit these enzymatic differences in the chemotherapy of Chagas' disease.

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References and Notes

1. M. A. Pfaller and J. J. Marr, *Antimicrob. Agents Chemother.* **5**, 469 (1974).
2. J. J. Marr and R. L. Berens, *J. Infect. Dis.* **136**, 724 (1977).
3. D. J. Nelson, C. J. L. Bugge, G. B. Elion, R. L. Berens, J. J. Marr, in preparation.
4. R. L. Berens, R. Brun, S. M. Krassner, *J. Parasitol.* **62**, 360 (1976).
5. J. J. Marr, R. L. Berens, D. J. Nelson, *Biochim. Biophys. Acta*, in press.
6. T. A. Krenitsky, G. B. Elion, R. A. Strelitz, G. H. Hitchings, *J. Biol. Chem.* **242**, 2675 (1967).
7. D. J. Nelson, C. J. L. Bugge, W. C. Krasny, G. B. Elion, *Biochem. Pharmacol.* **22**, 2003 (1973).
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Children Absorb Tris-BP Flame Retardant from Sleepwear: Urine Contains the Mutagenic Metabolite, 2,3-Dibromopropanol

Abstract. *The flame retardant, tris(2,3-dibromopropyl)phosphate (tris-BP), which is a mutagen and causes cancer and sterility in animals is absorbed from fabric by people. 2,3-Dibromopropanol, a metabolite of tris-BP and a mutagen itself, has been found in the urine samples of ten children who were wearing or who had worn tris-BP–treated sleepwear. Eight of these children were wearing well-washed sleepwear and the possibility of absorption of tris-BP from well-washed sleepwear is discussed. 2,3-Dibromopropanol was not found in the urines of one child and one adult who had never worn tris-BP–treated garments.*

To evaluate the hazard to human health posed by a chemical, both its toxic effects and human exposure levels should be defined. We now report human absorption of the flame retardant tris-BP, the major chemical used in sleepwear between 1973 and 1977 for the purpose of complying with federal regulations designed to reduce burn injuries in children. About 50 million children were exposed to this chemical before it was banned from use in children's clothing in April 1977. Amounts of about 5 percent of fabric weight had been padded onto children's sleepwear fabric.

Tris-BP produces mutations in bacteria (*1*, *2*), cancer when fed to rats and mice (*3*) or when painted on the skin of mice (*4*), and testicular atrophy and sterility in rabbits after application to the skin (*5*). Similar chemicals have long been known to penetrate human skin (*6*), and fabric treated with tris-BP causes an allergic reaction in previously sensitized individuals (*7*). Radioactivity (^{14}C) from labeled tris-BP has been found in urine, feces, and body organs of rabbits exposed to fabric treated with tris-BP (*8*), and the mutagenic tris-BP metabolite, 2,3-dibromopropanol, has been found in urine of animals dermally exposed to tris-BP (*9*, *10*).

Table 1. Morning urine samples were obtained from a 7-year-old child. On days 1 and 2 and 8 to 12 the child was wearing repeatedly washed sleepwear that may have been tris-BP–treated; on days 3 to 7 she was wearing new tris-BP–treated pajamas. Urine samples were hydrolyzed with glusulase, an enzyme mixture containing glucuronidase and sulfatase, extracted with ethyl acetate, and analyzed by negative ion atmospheric pressure ionization mass spectrometry.

Day	New treated pajamas	Dibromopropanol (mg/ml)
1	No	0.4
2	No	0.4
3	Yes	11
4	Yes	29
5	Yes	*
6	Yes	21
7	Yes	18
8	No	9
9	No	14
10	No	6
11	No	6
12	No	8

*The urine was lost.

Human skin absorption of tris-BP was not found in a previous study in which 2,3-dibromopropanol could be detected at a level of 200 ng/ml (*10*). However, a rough estimate, based on human absorption studies with structurally similar compounds, suggested that a child wearing sleepwear treated with tris-BP might absorb a considerable daily dose and that doses sufficient to give levels of less than 200 ng/ml in the urine may pose a considerable risk (*11*). Using a very sensitive analytical method for brominated compounds—negative ion mass spectrometry (*12–15*)—we have now found 2,3-dibromopropanol (up to 29 ng/ml) in the urine of children wearing tris-BP–treated sleepwear.

Morning urine samples were collected from a 7-year-old female child who had been wearing repeatedly washed sleepwear, some of which had originally been treated with tris-BP. The child then wore new tris-BP–treated pajamas (100 percent polyester) for 5 nights (*16*). Morning urine samples were collected during the corresponding 5 days and then for the

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