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METABOLISM OF AMINONUCLEOSIDE-8-^{14}C IN THE RAT AND GUINEA PIG*

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Abstract—The aminonucleoside (PA) of puromycin is nephrotoxic to rats but not to guinea pigs. That guinea pigs are resistant to PA-nephrotoxicity has been confirmed in the Hartley strain.

The metabolic disposition of subcutaneoulsy administered PA in the rat and in the Hartley guinea pig has been determined with the aid of the 8-^{14}C-labeled compound. Unchanged PA is the major urinary excretion product in both the rat and guinea pig 8 hr after PA administration, and allantoin represents the major end-product of metabolism. Guinea pigs, but not rats, excrete part of the PA-8-^{14}C as $^{14}CO_2$. The monodemethylated analog of PA, viz., 6-methylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine, is the major nucleoside metabolite in the urine of rats and guinea pigs. The rat demethylates PA to this monodemethylated analog *in vivo* at a rate approximately 4-fold greater than the guinea pig.

ADMINISTRATION of the aminonucleoside (PA)† of puromycin to rats produces a nephrotic syndrome that is clinically indistinguishable from the neophrotic syndrome of unknown etiology frequently observed in children.[1] Whereas rats,[2, 3] monkeys,[4] and humans[5, 6] are susceptible to the nephrotoxic action of PA, mice, guinea pigs or rabbits are resistant.[4] Species specificity of drugs is often related to differences in the metabolic disposition of the drug in the different species, and it was of interest to compare the metabolism of PA in both a susceptible and a non-susceptible species. In this communication, the metabolic fate of PA in the rat (a susceptible) and in the guinea pig (a non-susceptible species) is compared, using PA labeled with ^{14}C in position 8 of the purine ring.

MATERIALS AND METHODS

Animals and radioactive counting procedures

Animals used in this study were standard laboratory albino rats (SLA) and Hartley strain guinea pigs which were purchased from Simonson Laboratories.‡ For nephrotoxicity studies, PA was administered to 7 guinea pigs at a dose of 12 mg/kg based on initial body weight (averaged for the group), for 11–14 days, with 2 animals serving as

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†Abbreviations used: PA, aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine; MMPA, 6-methylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine; APA, 6-amino-9-(3′amino-3′-deoxy-β-D-ribofuranosyl)purine; IPA, 6-hydroxy-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine.

‡ White Bear Lake, Minn.

controls. Food (Labena Chow),* supplemented with fresh lettuce, and water were allowed *ad libitum* and urine protein was determined daily by the sedimentation method of Shevsky and Stafford as modified by McKay.[7] PA-8^{14}C† was diluted with unlabeled PA‡ as described previously[8] to final sp. act. ranging from 4·60 to 5·57 $\times$ 10^5 dpm/mg. PA-8-^{14}C (15 mg/kg) was administered subcutaneously to guinea pigs weighing between 200 and 250 g and its tissue distribution and excretion were determined at 8 hr. The procedures for the collection of urine, feces, expired CO_2, counting of radioactive tissue samples§ and metabolites, as well as the quantitative determination of unchanged PA in the urine by inverse isotope dilution have been previously described.[8] As an added check, the expiratory $^{14}CO_2$ trapped as $Na_2{}^{14}CO_3$ was also precipitated as $Ba^{14}CO_3$,[9] collected, and counted as $Ba^{14}CO_3$ in a thixotropic gel suspension.[10] In Expt. II, Table 3, the tissues were combusted in a Schöniger apparatus and the $^{14}CO_2$ adsorbed in 1 M methanolic hyamine hydroxide and counted in the system described above. The rat with the bile duct cannula was kept in a restraining cage and bile as well as urine was collected for 8 hr.

Chromatographic separation of metabolites and carrier procedures (*Fig.* 1)

The urine and bile containing radioactive metabolites of PA were diluted with 5–10 vol. of distilled water, adjusted to pH 1 with concentrated hydrochloric acid, and fractioned into 'non-basic,' 'free-base,' and 'nucleoside' fractions by stepwise gradient elution from Bio-Rad AG-50 W $\times$ 4 (H^+) cation-exchange resin, according to a procedure patterned after Wilson *et al.*[11] To remove the buffer salts (NH_4CO_2H), the free-base and nucleoside fractions were acidified to pH 1 with concentrated hydrochloric acid and charged on another column of Bio-Rad AG-50 (H^+). Elution with 0·1 N NH_4OH afforded the desalted fractions which were concentrated *in vacuo* to convenient small volumes for paper chromatography or addition of carriers.

Positive identification and quantitation of metabolites were accomplished by inverse isotope dilution with appropriate unlabeled carriers. The carriers were purified by recrystallization, ion-exchange chromatography or by paper chromatography, typically as follows.

An aliquot of the non-basic fraction was diluted with water and unlabeled uric acid (20–40 mg) was added; to another aliquot, similarly diluted, was added carrier allantoin (40–80 mg). The carriers were dissolved by heating the solution and then allowed to crystallize. The recovered uric acid was recrystallized once again from water and allantoin was recrystallized from water or from absolute ethanol–ether. To an aliquot of the desalted free-base fraction (guinea pig urine) was added urea (102 mg) and to the remaining was added a known quantity ($\sim$ 5 μmole in 0·1 N HCl) of solutions of 6-dimethylaminopurine, 6-methylaminopurine, adenine, hypoxanthine (and in the case of rat bile, xanthine in 0·1 N NH_3). The urea was purified by repeated recrystallizations from ethanol–ether and further purified by ion-exchange chromatography. The carrier purine bases were separated by ascending paper chromatography on Whatman 3MM paper in water-saturated *n*-butyl alcohol: concentrated NH_4OH

* Ralston Purina Co., St. Louis, Mo.

† A gift from Dr. Ralph Barclay, Sloan Kettering Institute, New York, N.Y.

‡ We are indebted to Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., for generous samples.

§ In Packard Tri-Carb model 314 liquid scintillation spectrometer or Nuclear Chicargo model no. 703.

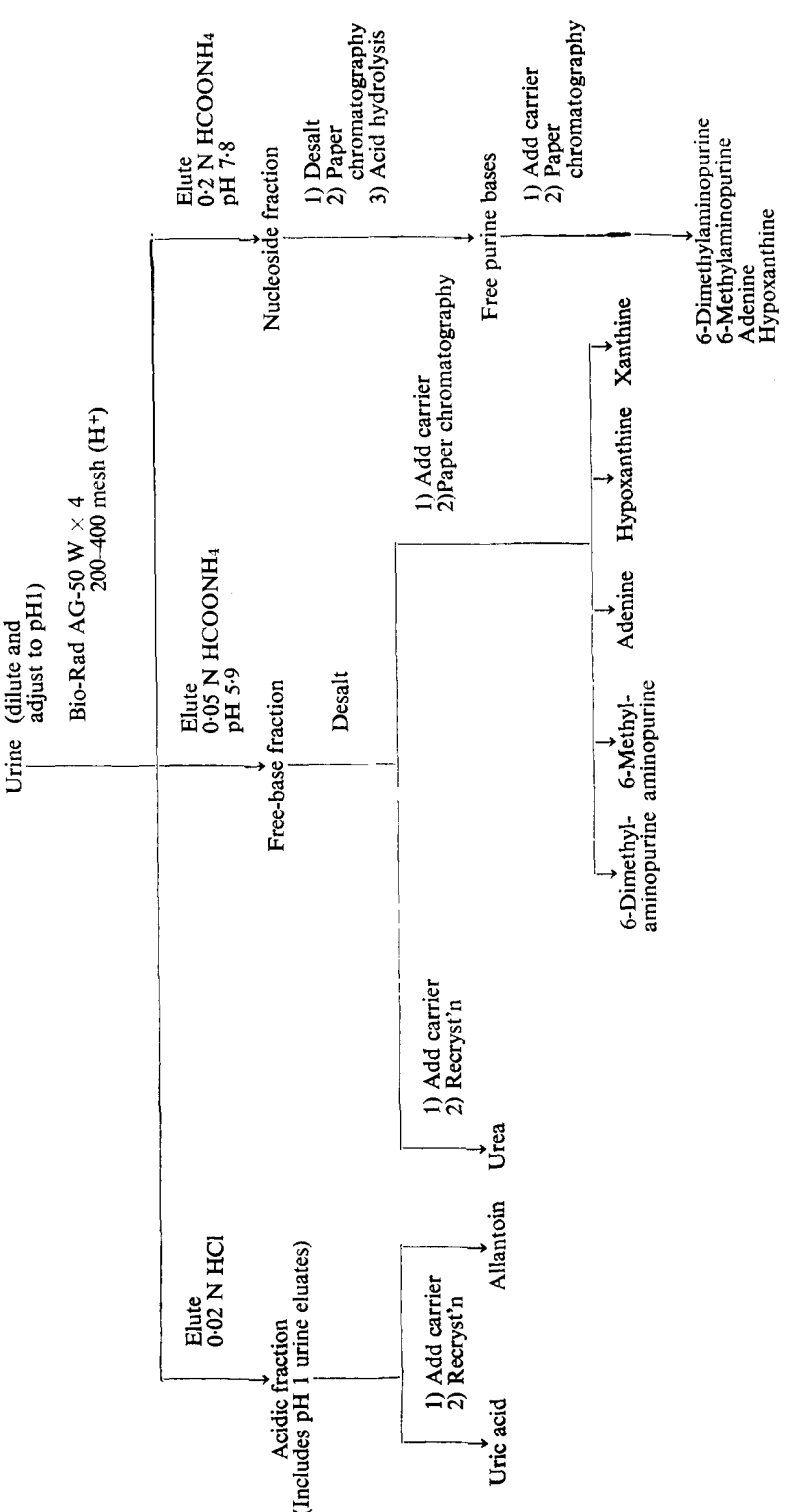

FIG. 1. Separation and identification of urinary metabolites of PA.

(100:1) (solvent D of Wilson *et al.*[11]). The individual areas corresponding in R_f to the purine bases were visualized under u.v. light, cut out, and extracted 3 times with 10 ml of 0·1 N HCl at room temperature (paper pulverized and fibers sedimented by centrifugation). The identity of the bases was verified by the position of their u.v. absorption maxima at acid and neutral pH's, and their concentrations were determined on the basis of their extinction coefficients. Aliquots of the extracts were assayed for radioactivity and the specific radioactivities of the carriers were then calculated.

The desalted nucleoside fraction was subjected initially to paper chromatography on Whatman 3MM paper (ascending) with *n*-butyl alchol: formic acid: water (77:33:10) (solvent A of Wilson *et al.*[11]). The areas corresponding in R_f to the various aminonucleosides[11] were cut out, eluted with water, then with 0·1 N HCl, and the extracts hydrolyzed with 3–4 N HCl for 1 hr. Addition of carrier purine bases and further work-up proceeded as in the free-base fraction described above. This degradative (hydrolytic) procedure was necessary since carrier compounds corresponding to the aminonucleoside metabolites were not available.

In several instances, the procedures deviated somewhat from that described above, and this will be noted, when appropriate, in the text or tables.

RESULTS

PA administration to guinea pigs

Since previous investigators[3] did not specify the strain of guinea pigs used in their study of the nephrotoxicity of PA in different species, it was necessary to determine whether PA was nephrotoxic to the Hartley strain of guinea pigs used in this study. Daily s.c. administration of PA to these guinea pigs did not induce proteinuria, although evidence of systemic toxicity was noted in that weight gain was arrested throughout the injection course and some deaths resulted. Three of the 7 animals treated with PA died by day 9 (on days 3, 6, and 9) and 1 control animal developed diarrhea on day 6 and died on day 11. Two of the 4 surviving PA animals and the remaining control were sacrificed on day 11. All organs appeared grossly normal and histologically the kidneys of both PA-treated guinea pigs were likewise normal. PA administration to the 2 remaining animals was continued through day 14 and urine proteins were determined through day 22; however, urine protein excretion levels remained considerably below 5 mg/day/pig except on day 21 when it was 8 mg/day. Thus, although PA appeared to be somewhat toxic to guinea pigs of this strain in the dosage administered, nephrotoxicity was not manifest.

Distribution and excretion of PA-8-^{14}C in the guinea pig

The organ distribution and excretion of radioactivity 8 hr after s.c. administration of ^{14}C-labeled PA to guinea pigs are shown in Table 1. As in the rat,[8] a large percentage of the administered radioactivity appeared in the urine (41–52 per cent) and feces (24–31 per cent), although the urinary excretion rate was significantly less for the guinea pig than for the rat. (The rat excreted 65 per cent of the administered radioactivity in the urine over 8 hr.[8]) Approximately 2 per cent of the dose appeared as $^{14}CO_2$ (Expt. I). Since $^{14}CO_2$ excretion after PA-8-^{14}C administration was not observed previously in the rat, and the recovery of radioactivity was not optimal in Expt. I, the study was repeated in another guinea pig (Expt. II). That radioactive CO_2 was actually expired was not confirmed in this experiment; however, in a third experiment

(Expt. III), where the recovery of administered radioactivity was 91 per cent, radioactive CO_2 was again found in the expired breath. Of interest are the observations that the kidneys contained very little radioactivity (0·3 per cent of the dose) and the plasma was essentially devoid of activity. The radioactivity found in the stomach and its contents in these guinea pigs was due to the ingestion of feces that became lodged in the metabolism cages.

TABLE 1. DISTRIBUTION AND EXCRETION PATTERNS OF AMINONUCLEOSIDE-8-^{14}C IN THE GUINEA PIG (8 HR)

Organ	Per cent of administered dose		
	Expt. I	Expt. II	Expt. III
Urine	40·7	42·4	52·2
Feces, large intestine and contents	24·1	31·1	27·7
Stomach and contents	1·1	1·0	4·7
Small intestine and contents	0·6	1·8	0·6
Whole blood	1·1	0·4	—
(plasma)	(0·0)	—	—
Liver	1·5	1·8	3·9
Kidneys	0·3	0·3	—
Lungs	0·2	—	—
Spleen	0·1	—	—
Heart	0·0	—	—
Psoas muscle	0·0	—	—
Adrenals	0·0	—	—
CO_2	1·8	0·0	1·9
Total	71·5	78·8	91·0

Carrier experiments revealed that 60–66 per cent of the urinary radioactivity was unchanged PA, and this amounted to 24–28 per cent of the administered dose (Table 2).

TABLE 2. EXCRETION OF UNCHANGED AMINONUCLEOSIDE-8-^{14}C IN THE URINE BY THE GUINEA PIG

	Collection period	Radioactivity excreted	Unchanged aminonucleoside	
	(hr)	(% of dose)	(% in urine)	(% of dose)
Expt. I	0–8	40·7	60·4	24·6
Expt. II	0–8	41·6	66·0	27·5

Comparison of the metabolism of PA *in the rat and guinea pig*

The quantitative metabolic fate of PA as reflected by urinary metabolites is compared in the rat, a susceptible species, and in the guinea pig, a species not susceptible to PA-nephrotoxicity, in Table 3. It can be seen that allantoin (isolated from the non-basic fraction) constituted the major urinary metabolite of PA in both species, whereas uric acid was only a minor metabolite. These two degradation products of PA accounted for better than 81 per cent of the radioactivity of the non-basic fraction of rat urine and guinea pig urine; the remainder was unaccounted for.

The free-base fraction of both rat and guinea pig urine contained the least amount of radioactivity, which amounted to no more than 10 per cent of the total urinary radioactivity. Some radioactivity was associated with carrier 6-dimethylaminopurine, 6-methylaminopurine, adenine, and hypoxanthine. In the guinea pig, urea accounted for 1·5 per cent of the total metabolite of PA appearing in the urine.

TABLE 3. METABOLIC FATE OF AMINONUCLEOSIDE IN THE RAT AND GUINEA PIG

	Guinea pig urine %		Rat urine* %	Rat bile* %
	Expt. II	Expt. III		
Non-basic fraction	(17·3)	(14·7)	(26·6)†	(12·6)
Uric acid	1·2	2·0	0·8	—
Allantoin	15·7	9·9	21·8	7·1
Unidentified	0·5	2·8	4·0	5·5
Free-base fraction	(5·4)‡	(8·4)	(9·9)	(30·5)
6-Dimethylaminopurine	0·7	1·2	2·0	1·9
6-Methylaminopurine	1·3	0·9	1·8	4·3
Adenine	0·4	0·3	0·7	1·9
Hypoxanthine	1·0	1·3	—	2·4
Xanthine	—	—	—	6·6
Urea	—	1·5	—	—
Unidentified	2·0	3·2	5·4	13·4
Nucleoside fraction§	(76·3)	(71·0)	(63·6)	(49·2)
6-Dimethylaminopurine	66·9	65·2	39·8	10·7
6-Methylaminopurine	6·1	5·6	21·2	23·1
Adenine	—	0·0	0·9	4·9
Hypoxanthine	—	0·0	—	1·1
Unidentified	3·3	0·2	1·7	9·4

* The rat is the same here.

† The numbers in parentheses represent the per cent of total urinary (or biliary) radioactivity initially found in that fraction before addition of carriers.

‡ The carrier purine bases were added as crystalline solids and partially purified by repeated recrystallizations. Since losses were considerable by this procedure, the final concentrations of the purified carriers were determined by ultraviolet spectrophotometry.

§ Purine bases determined after hydrolysis; see Methods.

The fraction of urine with the highest content of radioactivity was the nucleoside fraction and this amounted to 64 per cent in rat urine and 71–76 per cent in guinea pig urine. After paper chromatography and hydrolysis of the nucleosides, the purine bases associated with the nucleosides were identified as mostly 6-methylaminopurine, while adenine was detected in trace amounts. Translated in terms of their respective purine aminoribosides, unmetabolized PA appears to be the major urinary excretion product after administration of PA to rats or guinea pigs. It is noted that agreement between the amount of unchanged PA in the guinea pig urine of Expt. II, determined by a degradative procedure (67 per cent, Table 3) and by inverse isotope dilution directly on urine (66 per cent, Expt. II, Table 2), is excellent.

Biliary excretion of PA *metabolites in the rat*

The fecal route of elimination of s.c. administered PA-8-^{14}C appeared to be quite considerable (27–30 per cent) in the rat[8] as well as in the guinea pig (Table 1). It was

therefore of interest to examine the bile for the presence of metabolites, since the liver is probably the major organ site for metabolism of PA. Bile collected for 8 hr from a rat treated with PA-8-^{14}C contained metabolites similar to that found in the urine (Table 3). The degradation products of the purine base 6-dimethylaminopurine, namely, 6-methylaminopurine, adenine, hypoxanthine, xanthine and allantoin, were found in significant quantities. In addition, the nucleoside fraction gave, after hydrolysis to the purine bases, 6-dimethylaminopurine, 6-methylaminopurine, and, to a lesser extent, adenine and hypoxanthine, indicating the presence of the aminoribosides of these purine bases in the bile. Approximately 28 per cent of the radioactivity of the bile remained unaccounted for.

DISCUSSION

Although the present experiments do not delineate the metabolic pathway of the 3-amino-3-deoxy-D-ribosyl portion of PA due to the location of the ^{14}C label on the 8 position of the purine ring, the degradation of the 6-dimethylaminopurinyl portion of the molecule was followed completely, e.g., to CO_2 in the case of the guinea pigs. Wilson *et al.*,[11] lacking radiolabeled PA, were restricted to the study of the catabolism of PA only to metabolites with an intact ribosidic linkage. The metabolic disposition of PA in the rat and guinea pig is summarized in Fig. 2.

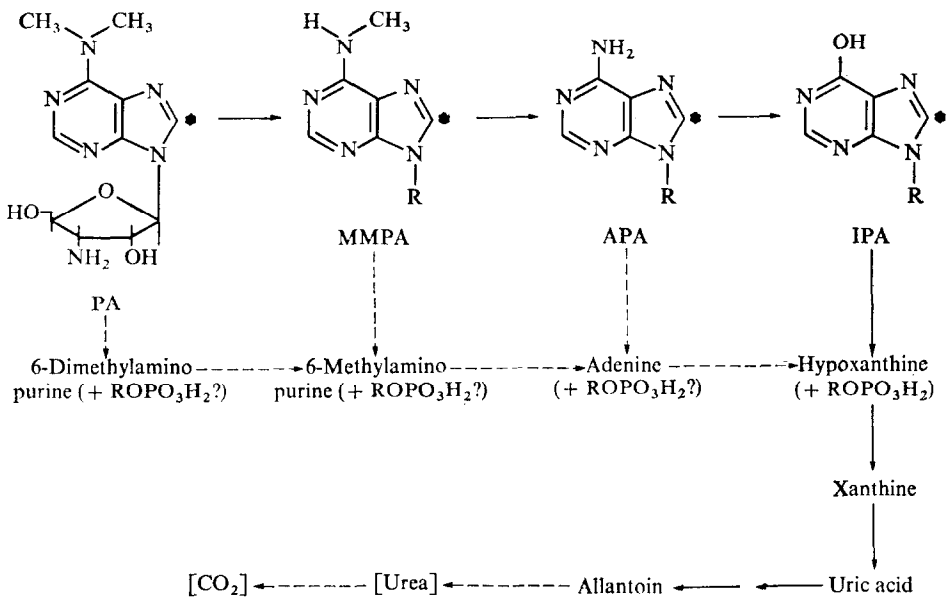

FIG. 2. Metabolic degradation path of PA in the rat and guinea pig. ——→ Major pathway;— — —→ minor or possible pathways; position of ^{14}C-label; R = 3-amino-3-deoxy-β(orα)-D-ribosyl-; [ ] established for guinea pig only.

The enzyme systems responsible for the metabolism of PA can readily be rationalized from published data *in vitro*. PA is demethylated by liver microsomes[12–14] to the monodemethylated analog (MMPA) and is presumably further demethylated to the adenosine analog (APA) by the same microsomal enzyme. Deamination to the inosine analog (IPA) followed by enzymatic cleavage of the aminoribosidic linkage would give

hypoxanthine, the latter then taking the normal catabolic pathway to allantoin. It has been shown that APA is deaminated by adenosine deaminase,[15] and that IPA is cleaved to hypoxanthine by a nucleoside phosphorylase from rat liver,[16] although at a rate only 1/5 that for inosine itself. The rate-limiting step in the metabolic degradation of PA appears to be the demethylation of the monomethyl analog MMPA, since this compound is the major nucleoside metabolite found in the urine of both rats and guinea pigs. In the biosynthetic preparation of this anlog by fortified rat liver microsomes,[14] the adenosine analog APA was not formed under the conditions of the experiment indicating that the second demethylation step is probably slower than the first. Furthermore, the corresponding 6-methylaminopurine riboside is demethylated by rat liver microsomes at a rate only 1/4 that for 6-dimethylaminopurine riboside,* in line with the known fact that secondary amines are dealkylated at a slower rate than tertiary amines.[17]

No decision can be made as to how the free purine bases, 6-methylaminopurine, adenine, and hypoxanthine have arisen, since they can be derived from their respective aminonucleosides by enzymatic hydrolysis of the ribosidic linkage or by the successive demethylation of 6-dimethylaminopurine followed by deamination. At best, these represent minor pathways of metabolism.

The presence of derivatives of PA or MMPA or of both that are 'substituted' in the 3′-amino nitrogen in the urine of rats and gunea pigs treated with PA as reported by Wilson *et al.*,[11] could not be completely confirmed with the methods used in this study, although some radioactivity was associated with the areas corresponding to these metabolites on paper chromatograms. However, since the structures of these substituted derivatives are not known, extensive efforts were not made in this direction.

The observed degradation of the radioactive label in the 8 position of PA in part to $^{14}CO_2$ in 2 of 3 guinea pigs, but not in the rat, deserves comment since purines are known not to be completely degraded to CO_2 in mammals.[18] It is likely that the bacterial flora of the intestine of the guinea pig, which may be different from that of the rat, is responsible for this degradation.

It is evident from these studies (Table 3) that the rat demethylated PA to its monomethyl analog, MMPA, to an extent nearly 4-fold greater than did the guinea pig (21·2 per cent vs. 5·6 per cent of dose in 8 hr). This is in quite good agreement with the data *in vitro* of Mazel *et al.*,[13] who showed that rat liver microsomes demethylate PA at a rate 2·9 times faster than liver microsomes from guinea pigs. MMPA, which is a competitive inhibitor of adenosine deaminase,[14] has been shown to be nephrotoxic to the rat by Wilson *et al.*,[3] although only enough of the sample to treat a single rat was isolated from the urine. Although corroboration of MMPA toxicity is still necessary,† it is interesting to speculate that perhaps the differential toxicity of PA to the rat and the guinea pig might be related to the excessive production of this metabolite in the rat.

* H. T. Nagasawa, K. F. Swingle and C. S. Alexander, unpublished observations.

† Chemical synthesis of this metabolite is in progress.

REFERENCES

1. S. FRENK, J. M. ANTONOWICZ, J. M. CRAIG and J. METCOFF, *Proc. Soc. exp. Biol. Med.* **89**, 424 (1955).
2. E. B. FEIGELSON, J. W. DRAKE and L. RECANT, *J. Lab. clin. Med.* **50**, 437 (1957).

3. S. G. F. WILSON, D. B. HACKEL, S. HORWOOD, G. NASH and G. HEYMANN, *Pediatrics, N.Y.* **21**, 963 (1958).
4. A. F. MICHAELS, JR., H. D. VENTERS, H. G. WORTHEN and R. A. GOOD, *Lab. Invest.* **11**, 1266 (1962).
5. J. H. BURCHENAL, *Curr. Res. Cancer Chemother.* **4**, 17 (1956).
6. D. A. KARNOFSKY and B. D. CLARKHAM, *Pharmac. Rev.* **3**, 392 (1963).
7 .J. P. PETERS and P. S. VAN SLYKE, *Quantitative Clinical Chemistry*, vol. 2, p. 682. William and Wilkins, Baltimore (1932).
8. C. S. ALEXANDER, H. T. NAGASAWA and D. FILBIN, *Proc. Soc. exp. Biol. Med.* **111**, 521 (1962).
9. C. C. IRVING and R. F. WILLARD, *Cancer Res.* **24**, 77 (1964).
10. S. HELF, in *Liquid Scintillation Counting* (Eds. C. G. BELL, JR. and F. N. HAYES), p. 96. Pergamon Press, Oxford (1958).
11. S. G. F. WILSON, W. HEYMANN and D. A. GOLDTHWAIT, *Pediatrics, N.Y.* **25**, 228 (1960).
12. P. MAZEL and J. F. HENDERSON, *Biochem. Pharmac.* **14**, 92 (1965).
13. P. MAZEL, A. KERZA-KWIATECKI and J. J. SIMANIS, *Biochim. biophys. Acta* **114**, 72 (1966).
14. N. DICKIE, L. NORTON, R. F. DERR, C. S. ALEXANDER and H. T. NAGASAWA, *Proc. Soc. exp. Biol. Med.* **123**, 421 (1966).
15. J. G. CORY and R. J. SUHADOLNIK, *Biochemistry* **4**, 1729 (1965).
16. L. GOLDMAN, J. W. MARSICO and M. J. WEISS, *J. mednl. Chem.* **6**, 410 (1963).
17. R. E. MCMAHON, *J. pharm. Sci.* **55**, 457 (1966).
18. J. S. FRUTON and S. SIMONDS, *General Biochemistry*, 2nd edn., p. 856. John Wiley, New York (1958).