dominal aortas appeared to be reduced by DES-thiouracil feeding, as compared to feeding of DES alone.

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Metabolism of Puromycin Aminonucleoside in the Normal, "Pre-Nephrotic," and Nephrotic Rat.* (32061)

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Administration of the aminonucleoside of puromycin (PA)† to the rat produces a nephrotic syndrome characterized by proteinuria, hypoalbuminemia, hypercholesterolemia, edema and ascites(1-4). Electron microscopic examination of kidney tissue revealed damage to the glomerular epithelial cell, the earliest manifestation being swelling of the foot processes, and the tubules(5). The biochemical lesion underlying aminonucleoside induced nephrosis is unknown. The rat is unique in its susceptibility to the nephrotoxic action of PA. The human and monkey are mildly susceptible while the guinea pig and mouse are resistant to the nephrotoxic action of this drug. The differential species toxicity may be related to differences in the metabolism of the drug in the various species. It was shown that the major urinary metabolites of PA are the monodemethylated analog, MMPA, and allantoin(6,7). Since certain drugs when administered chronically influence their own metabolic degradation by induction of enzymes of the hepatic microsomes(8), and experimental nephrosis by PA is generally produced by chronic administration of low doses (15 mg/kg), it was of interest to study the metabolism of PA at different time periods during the course of its chronic administration. It is possible that in non-susceptible species, enzymes may be induced which appreciably alter the metabolism of PA, qualitatively or quantitatively, such as to render it non-toxic. Alternatively, toxic metabolites may accumulate in susceptible species as a result of such enzyme induction. This report describes the metabolism of 8-C^{14}-PA in a rat, a susceptible species, at 3 different times during the course of administration of standard doses of PA, *i.e.*, at the normal, "pre-nephrotic" and nephrotic stages.

Materials and methods. Purification of PA. 8-C^{14}-PA was very kindly made available to us by Dr. Ralph Barclay, Sloan-Kettering Institute for Cancer Research, New York. Two-dimensional chromatography of the 8-C^{14}-PA

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† Abbreviations: PA, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl) purine; MMPA, 6-monomethylamino-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.

on Silica Gel $_{HF}$ (Merck, Brinkmann) plates with chloroform-methanol-2.5 N NH_4OH (250:100:15, v/v) (CMN) solvent(9) along with some non-radioactive monodemethylated derivative, MMPA(10), and counting of the UV absorbing spots, revealed an impurity with the same mobility as MMPA, which contained 8.1% of the radioactivity.‡ Therefore, the 8-C^{14}- PA was purified by one-dimensional chromatography in the thin-layer chromatographic system described above. Subsequent 2-dimensional analysis of the purified material with chloroform-methanol-1 N NH_4OH (250:100:50, v/v, lower layer)§ and CMN solvents showed PA 98.8%, MMPA 1.0%, and dimethyladenosine 0.2%.

Carrier compounds. The monodemethylated analog of PA, MMPA, was prepared by demethylation of PA with rat liver microsomes (10). APA was a gift from Dr. N. Gerber, IPA from Lederle.

8-Hydroxy-6-aminopurine (8-OH-6-AP) and 2,8-dihydroxy-6-aminopurine (2,8-OH-6-AP) were synthesized according to the procedure of Cavalieri and Bendich(15). 8-Hydroxy-6-methylaminopurine (8-OH-6-MAP) and 2,8-dihydroxy-6-methylaminopurine (2,8-OH-6-MAP) were prepared from 6-methylaminopurine (CalBioChem, Los Angeles, Calif.) by action of xanthine oxidase(16). All others were obtained from commercial sources.

Animal. A 100 g male rat (Simonson Laboratories, White Bear Lake, Minn.) was housed in a glass metabolism cage (Delmar Scientific Co., Maywood, Ill.) which provided for the separate collection of urine, feces, and expired CO_2. Urine was collected under toluene. Cage rinsings were combined with the collected urine, filtered and stored at 4°. The cage was rinsed further with about 200 ml of water and the feces were likewise rinsed with lesser amounts of water.

Ion-exchange fractionation of urine. The collected urine was filtered, adjusted to pH 1 with conc. HCl, and fractionated on BioRad AG 50W-X4 by stepwise pH gradient elution (Fig. 1) using a slight modification of the procedure of Wilson *et al*(6). The elution of known compounds was determined by UV analysis of the fractions with the exception of allantoin and urea which were located with phenol-hypochloride reagent(17).

Feces, expired CO_2 and radioactive counting. The expired CO_2 was collected in 200 ml of 20% NaOH for 4 hours after the initial administration of 8-C^{14}-PA. A 5 ml aliquot was acidified with H_2SO_4 under N_2 and the released CO_2 carried by a stream of N_2 through a H_2SO_4 drying trap into 10 ml of 1 N Hyamine-OH. One ml of the Hyamine-OH was counted.

The feces were kept frozen until assayed. After thawing, they were dried in a vacuum oven at 70° for 24 hours, ground with a mortar and pestle, and a 30 mg sample combusted in a Schöniger flask. 10 ml of N Hyamine-OH was added and a 0.5 ml aliquot counted.

Radioactivity was measured with a Nuclear-Chicago Liquid Scintillation Spectrometer. A dioxane-methanol-xylene solvent was used to dissolve the aqueous samples(18). Corrections for quenching were made either by internal standard or by the channels-ratio procedure.

Experimental. Administration of 8-C^{14}-PA. 8-C^{14}-PA (4.42×10^6 dpm/mg) was administered subcutaneously to a 100 g male rat in a glass metabolism cage according to the following protocol:

1. Day 0. The rat is defined as normal since it had no previous dose of PA and if drug administration is discontinuel with this single dose, nephrosis does not develop.
2. Day 4. The rat is defined as "pre-nephrotic." No damage is apparent upon electron microscopic examination(5).
3. Day 8. The rat is defined as nephrotic, since a considerable amount of protein is

‡ Since dealkylation of alkylamines is now belived to proceed by free-radical mechanisms(11,12), this product could have arisen by radiation self-decomposition of 8-C^{14}-PA. We did not observe this impurity in this preparation previously(13). In the review by Bayly and Evans(14) on radiation self-decomposition, no mention was made of this possibility of radiolabeled alkylamines being dealkylated in this manner.

§ This nucleoside solvent system was developed by F. Shirota of this laboratory; CMN solvent is a monophasic modification.

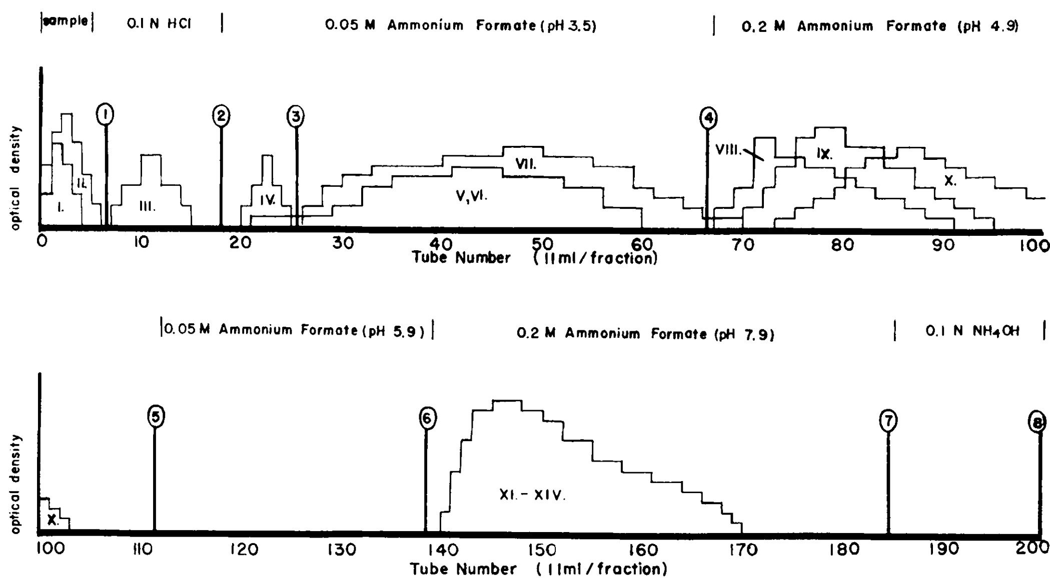

FIG. 1. Fractionation of possible metabolites. The 0.05 M ammonium formate (pH 3.5) was prepared by dissolving 0.05 mole of ammonium formate and adjusting the pH to 3.5. The other buffers were prepared by using formic acid as in the molar quantity stated and adjusting to pH with NH_4OH. Tubes were combined into fractions (1-8) as indicated by the numerals. I = allantoin; II = uric acid; III = xanthine; IV = hypoxanthine; V, VI = 2,8-OH-6MAP, 2,8-OH-6AP; VII=8-OH-6MAP, 8-OH-6AP; VIII=ademic; IX=6-MAP;X=6DMAP;XI-XIV=amino-nucleosides, (PA MMPA, APA, IPA).

being excreted in the urine and swelling of the glomerular foot-processes can be seen in electron micrographs(5).

On the other days through day 11, non-radioactive PA was administered. The protocol is described in Fig. 2; a typical urine protein excretion curve over this period for a group of rats of the same age is also included. We wish to stress the fact that no rat on this standard dose regimen of PA (15 mg/kg × 12 days) has ever escaped nephrosis, and the present experimental rat was no exception.

Results. Urinary excretion of radioactivity. The % of dose-C^{14} excreted in the urine over the course of the experiment is tabulated in Fig. 3A. The assumption was made that after each dose of 8-C^{14}-PA, the radioactivity excreted was due solely to that dose. Almost 90% of the dose-C^{14} that is to be excreted is excreted as a plug in 8 hours. It can be estimated from a probable excretion curve, modeled after the excretion of dichlorovinyl-cysteine(19), that about 60% of that excreted in 8 hours would have been excreted within 1 hour.

Fraction 1; Allantoin, uric acid. Fraction 1 contained, among other things, uric acid and allantoin. The fraction was divided into 2 aliquots. Carrier allantoin was added to one neutralized aliquot and crystallized. Carrier uric acid was added to the other aliquot

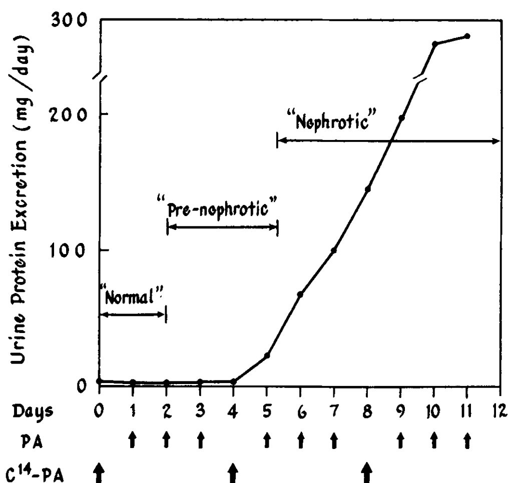

FIG. 2. Protocol in experimental nephorosis. PA or C^{14}-PA was administered subcutaneously (15 mg/kg) as indicated by the arrows. The urine protein curve is the mean curve of a group of rats of the same age and weight as the experimental animal.

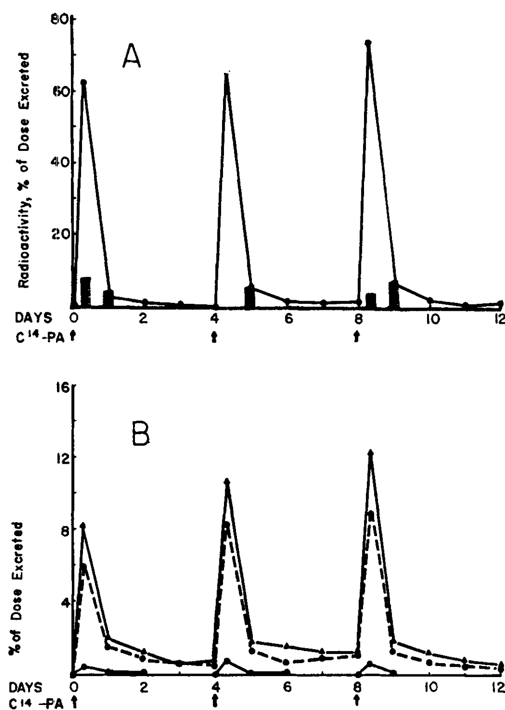

FIG. 3. A. Urinary and fecal excretion of radioactivity after 8-C^{14}-PA administration. O———O urine. hatched lines feces. B. Urinary excretion of uric acid and allantoin after 8-C^{14}-PA administration. ▲——▲, % of dose excreted as fraction 1, O - - - - O % of dose excreted as allantoin, O——O, % of dose excreted as uric acid.

and crystallized. Both were recrystallized from water. The identity of allantoin was confirmed by its decomposition point, uric acid by UV spectra. As can be seen from Fig. 3, allantoin was a principal urinary metabolite, and with time became the principal urinary metabolite.

Fraction 2, xanthine. Carrier xanthine was added to fraction 2, crystallized, and recrystallized. Xanthine accounted for less than 0.02% of the dose in 8-hour urine.

Fraction 3, hypoxanthine. Carrier hypoxanthine was added to fraction 3 and then separated by paper chromatography on Whatman 3 MM with solvent "C" of Wilson *et al*(6). The identity was confirmed by UV spectra. Hypoxanthine accounted for less than 0.04% of the dose in 8-hour urine.

Fraction 4, hydroxyaminopurines. Significant counts were obtained only in the 8-hour urines in which 3.0%, 2.8% and 1.2% of the dose was found in the urine excreted on day 0, 4, and 8, respectively. Carrier 8-OH-6-AP, 2,8-OH-6-AP, 8-OH-6-MAP, and 2,8-OH-6-MAP were added and then separated, after concentration *in vacuo*, by chromatography on Whatman 3MM with Solvent "C" of Wilson *et al*(6). The chromatograms were scanned with a Vanguard scanner and 4 radioactive peaks were detected with $R_{8\text{-OH-6-MAP}}$ and % radioactivity of 46 and 16%, 80 and 10%, 137 and 26%, and 163 and 41% in the first 8-hour urine. The individual compounds were then eluted and rechromatographed in the same system. With the exception of 2,8-OH-6-MAP, which is the principal metabolite of 6-MAP in the mouse(20) and the rat(21), and which accounted for 0.3% of the dose, less than 0.07% of the dose was found to be associated with any of the compounds. There were no significant changes with time.

Fraction 5, free bases. Fraction 5 accounted for 6.0%, 3.9%, and 4.8% of the dose, respectively, in the 8-hour urines excreted on day 0, 4, and 8. Carrier adenine, 6-MAP, and 6-DMAP were added and the bases separated first on thin-layer plates of Silica Gel HF with CMN solvent and then purified on Whatman 3MM with n-butanol-water-conc. ammonia (172:18:10, v/v). Adenine was less than 0.2%, 6-MAP less than 0.3% and 6-DMAP less than 0.6% of the dose on any day.

Fraction 6. There was 2.2%, 1.7%, and 1.5% of the radioactive dose in fraction 6 in the 8-hour urine excreted on day 0, 4, 8, respectively.

Fraction 7, aminonucleosides. Carrier aminonucleosides (PA, MMPA, APA, and IPA) were added. The solution was desalted by absorption on BioRad AG 50W-X4 and eluted with 0.1 N ammonia. The aminonucleosides were separated on 1 mm Silica Gel HF plates with CMN solvent. Each aminonucleoside band was eluted and rechromatographed on Whatman 3MM with solvent "C" of Wilson *et al*(6). The excretion is summarized in Table I,A. Although it appears that the excretion of PA was higher on day 8 1/3, if the change in excretion is considered and the results are calculated as % of the excreted

TABLE I. Aminonucleoside Excretion in Urine.

		A. % of dose			
Day	% Dose in Fraction 7	PA	MMPA	APA	IPA
⅓	40.9	32.4	8.4	.1	—
1	1.4				
4⅓	40.3	32.4	7.5	.3	—
5	2.3				
8⅓	51.8	43.9	7.7	.1	—
9	3.1				

		B. % of C^{14} excreted		
		% of C^{14} excreted as:		
Day	% C^{14} excreted	PA	MMPA	Allantoin
⅓	61.2	52.9	13.7	9.6
4⅓	65.5	49.5	11.5	12.6
8⅓	73.3	56.7	10.5	12.1

C^{14}, there was no significant change (Table I,B).

Fraction 8. Fraction 8 contained 0.5% and 1.0% of the dose in the 8-hour urine excreted on day 0 and 8, respectively.

Feces. Fig. 3A shows fecal excretion over the course of the experiment, which accounted for 10-13% of the dose.

Expired CO_2. No significant radioactivity was detected in the expired CO_2.

Discussion. It is apparent that the metabolism of PA did not change significantly over the time period examined. Thus, PA does not induce the formation of enzymes which result in an alteration of its own metabolism. Whether the metabolism is significantly different or enzymes are induced to either alter the metabolism or increase the rate of metabolism in resistant species remains to be seen. Preliminary results with mice indicate this is not the case(22).

The observation that the major urinary metabolites of PA in rat urine are MMPA and allantoin(7) has been confirmed. Oxidation of the free purine bases, 6-methylaminopurine and adenine, released in the metabolic degradation of PA, to the 2-hydroxy- and the 2,8-dihydroxy derivatives by xanthine oxidase, occurs only to a minor extent. The enzymes of the degradative pathway of PA in mammals have been discussed(7).

Summary. Approximately 80% of injected aminonucleoside-8-C^{14} is excreted in the urine and feces within 24 hrs by the rat. The principal urinary products are unchanged aminonucleoside, the monodemethylated analog, and allantoin. Daily administration of aminonucleoside for 12 days did not influence the course of its own metabolism.

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