reactions to egg albumin existed. Inhibition did not occur when early (3-6 hr) edematous reactions to skin testing existed, when significant amounts of circulating antibody to egg albumin were demonstrable. Thus the ability of antigen to inhibit cell migration in tissue culture has been confirmed with peritoneal exudate cells for tuberculin hypersensitivity and this *in vitro* characteristic has been shown to apply also to the delayed hypersensitivity that develops after immunization with a protein antigen in Freund's adjuvant.

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Distribution and Excretion of Aminonucleoside-8-C^{14} in Normal and Nephrotic Rats.* (27842)

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The discovery by Frenk *et al.*(1) and subsequently elaborated by others(2) of the ability of the aminonucleoside (PA) of the antibiotic puromycin to induce the classic nephrotic syndrome in rats as typically seen in the spontaneous disease of children has spurred a large number of investigators to study its mechanism of action in the hopes of understanding the etiology of the natural disease. Rats of different strains were susceptible to the action of this drug whether given subcutaneously or orally(3,4), but mice, guinea pigs, rabbits and dogs were found to be resistant to short term treatments(3). The nephrotoxicity of PA in primates (Rhesus monkeys)(5) and in humans(6) has been recorded.

Bartlett and Shelata using tritiated PA(7) and later with the aid of the more stable carbon-14 labeled material(8) have studied the incorporation of the label into nucleic acids and acid soluble nucleotides of the rat liver and kidney. Wilson *et al.*(9) have compared

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the metabolism of PA in the rat, rabbit and guinea pig, and despite the difficulty of isolation and identification of metabolites in the absence of tracer methodology, succeeded in demonstrating considerable differences in the disposition of the drug in the species studied. However, the precise metabolic handling which confers species susceptibility or resistance to the drug remains to be worked out. The availability of carbon-labeled PA greatly facilitates the study of the quantitative fate of this drug, and to this end, we have initiated such studies by determining the distribution and excretion of C^{14}-PA in the rat, a susceptible species.

Materials and methods. Aminonucleoside-8-C^{14}. A sample with a specific activity of 0.54 mC/mmole was prepared from a high activity sample† of aminonucleoside-8-C^{14} by appropriate, intimate mixing with unlabeled PA in distilled water followed by lyophilization to recover the product. Samples spotted on Whatman No. 1 paper were subjected to ascending chromatography in formic acid: abs. ethanol:water (15:80:15 by volume) (solvent 1) or n-butanol saturated water: conc. NH_4OH (100:1) (solvent 2). On viewing the chromatograms under a mercury lamp (Mineralite, Model R51), only a single fluorescent quenching spot, R_f 0.68 (solvent 1), R_f 0.79 (solvent 2), corresponding in position to unlabeled aminonucleoside which was run simultaneously, was visible on each chromatogram. Exact coincidence of this fluorescent quenching spot with the single radioactive peak recorded when the strips were scanned in a windowless paper chromatogram scanner (Atomic Accessories, Model RSC-5A) indicated the absence of any ultraviolet absorbing and/or radioactive contaminants. The resolution on the chromatograms was such that with solvent 1, adenosine and 6-dimethylaminopurine gave R_f of 0.31 and 0.81, respectively; while with solvent 2, adenosine had an R_f of 0.65. To increase the sensitivity of the radioactive scan near the region with peak radioactivity, the radioactive spot was cut out and the remaining paper strips joined and scanned at a higher gain setting, but only background levels were obtained.

Schedule of administration of labeled PA. The C^{14}-PA was made up as a 0.50% solution in distilled water and 0.60 ml corresponding to 3 mg or approximately 1.2×10^6 cpm was administered subcutaneously at day 1, day 5 and day 14 to each of 3 female albino rats of the Holtzman strain weighing 200 ± 10 g. The day 14 experiment was later repeated to confirm the somewhat unexpected results found with the nephrotic rat. The rats received one daily dose of 3 mg of unlabeled PA on each of the days preceding the radioactive dose, except for that rat which received the radioactive compound at day 1. To minimize any diurnal variations in the physiological disposition of the drug, the radioactive dose was always administered at midnight. The individual rats were housed in an all-glass metabolism cage which allowed for food and water when desired and permitted separate collection of urine, feces, and expired CO_2. For the 8-hour studies, food and water were withheld and expired CO_2 was trapped in 40% sodium hydroxide solution. Airflow to the cage of 600 ml/min insured adequate ventilation under these conditions.

For the 48-hr urine excretion study the rat was alternated between 2 glass metabolism cages, one supplied with food and water, and the other not. The animal was deprived of food and water during the first and alternate 8-hr periods except between the 24th and 48th hrs when food and water were allowed *ad libitum*. This permitted the cages to be rinsed and cleaned during the unoccupied periods. When a rat was housed continuously for 48 hours in a single cage much of the excreted radioactivity was lost in the intractable material which adhered to the glass surface of the cage.

Radioactivity measurements. All samples were assayed in duplicate in a Packard Tri-Carb Model 314 liquid scintillation spectrometer. Syringes after injections were usually rinsed twice with distilled water, the pooled washings counted, and the radioactive dose corrected for this loss. Cage washings were found to be radioactive and were therefore

† Very kindly made available to us by Dr. Ralph Barclay, Sloan-Kettering Institute for Cancer Research, New York.

TABLE I. Distribution and Excretion Patterns of C^{14}-PA during Induction of the Nephrotic Syndrome.

Organ	% of administered dose* Day 1	Day 5	Day 14	Day 14
Urine	65.3	69.8	8.10	30.1
Feces & contents of large intestine	26.4	15.2 †	8.38†	12.5
Whole blood	2.61	3.77	4.80	3.54
(Plasma)	(.01)	(.00)	(1.37)	(.38)
Liver	2.17	2.55	12.2	9.46
Large intestine	.91	.91	3.17	1.68
Small intestine & contents	.67	.68	4.83	3.67
Kidneys	.22	.22	4.07	3.69
Lungs	.15	.21	.95	.53
Spleen	.11	.07	.14	.16
Heart	.04	.04	.23	.12
Psoas muscle	.02	.00	.13	.04
Adrenals	.00	.00	.04	.02
Injection site	.16	—	1.01	—
Ascitic fluid	—	—	11.7	1.19
CO_2	.00	.00	—	.00
Total	98.8	93.5	59.8	66.7

* The day 5 and day 14 animals were pretreated with non-radioactive PA according to schedules described under *Methods*.

† Contents of large intestine only; no feces were excreted.

Figures in parentheses are not included in totals.

routinely added to the urine for counting. Eight hours after administration of the labeled PA, the animals were sacrificed by exsanguination from the aorta under ether anesthesia and blood was collected in a heparinized syringe. An aliquot was centrifuged to separate erythrocytes from plasma. Selected organs (Table I) were excised, minced and homogenized in an all-glass homogenizer with methanol to give a 10% suspension. Urine, ascitic fluid and plasma were diluted with 10 volumes of methanol, while blood was diluted serially with 10 volumes of water and 10 volumes of methanol. 1.0-ml aliquots of these samples were solubilized in 1.0 ml of a 1.3-1.4 M methanolic Hyamine hydroxide(10) in the sample counting vial (by warming to 75° when necessary), and then made up to counting volume by addition of 1.0 ml of methanol and 10.0 ml of a toluene-phosphor solution which was 0.4% in PPO and 0.005% in POPOP. Quenching effects were corrected for by addition of internal standards(11). These procedures for assaying radioactivity in tissue samples by liquid scintillation counting which were developed independently in our laboratories appear to be identical in principle to those developed by Herberg(12). The trapped CO_2 was liberated from the alkali with concentrated sulfuric acid and the evolved CO_2 was absorbed in Hyamine hydroxide for counting in the system just described.

Carrier technics. The amount of unchanged PA excreted in the urine was quantitatively determined by inverse isotope dilution(13) with carrier PA. A weighed amount of unlabeled PA was added to and intimately dissolved in the urine which had been assayed for total radioactivity. The urine sample was then adjusted to pH 8 with 2N ammonium hydroxide and the carrier was absorbed on 35 ml of moist Amberlite IRC-50 (H^+) resin in a batch operation. After equilibrating for 30 minutes with frequent shaking, the supernatant urine was drawn off and the resin washed with water until the washings were neutral. The carrier was eluted from the resin with 5 × 50 ml portions of 2N ammonium hydroxide. The last 200 ml were combined, reduced in volume on a rotating vacuum evaporator, and lyophilized. The reisolated carrier was recrystallized from absolute ethanol and further purified by subjecting it to paper chromatography on Whatman 3MM paper in solvent 1. The position of the PA carrier was located under ultraviolet light and the area cut out and eluted with water. Lyophilization of the eluate followed by recrystallization of the residue once or twice from absolute ethanol-ether usually rendered the carrier constant in specific radioactivity. Conversion of the purified carrier to its triacetyl derivative(14) at this stage without change in specific radioactivity attested to its radiochemical homogeneity. A typical result and the method of calculation can be found in Table III.

The rat used in the carrier study was rendered nephrotic by administration of 12 daily doses of unlabeled PA and the radioactive dose was not administered until the 14th day to allow 48 hours for excretion of the unlabeled PA.

Results. The patterns of distribution and excretion of radioactivity 8 hrs after subcutaneous administration of C^{14}-PA to a nor-

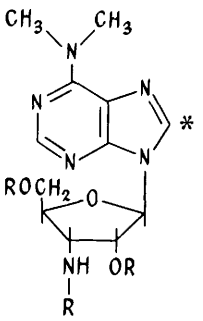

FIG. 1. Aminonucleoside of puromycin (R = H); triacetyl derivative (R = CH_3CO–); position of carbon-14 label (*).

mal rat, to a rat which had received 4 previous doses of PA, and to 2 rats with the full-blown nephrotic syndrome are tabulated in Table I. Especially noteworthy are the gross similarities of the day 1 and day 5 animals. Thus, 65-70% of the radioactivity appeared in the urine within this time, while 15-26% could be accounted for by the contents of the large intestine, *i.e.*, chiefly feces. Since the animals were not restricted in the cages, the possibility of the feces being contaminated with some urine cannot be entirely discounted; however, the intestinal contents of the 2 rats (day 5 and day 14) which did not excrete any feces within the observed 8-hr period contained appreciable radioactivity suggesting that the biliary pathway may be important in the metabolic disposition of the drug. As expected the liver contained an appreciable fraction of the radioactivity (2-3% of dose), but surprisingly the kidneys accounted for less than 0.25%. Even on a relative weight basis the radioactivity content/g of kidney was half that of the liver. It is evident that the blood cells contained a considerable quantity of radioactivity whereas the plasma contained practically none. This suggests concentration of PA or its metabolites by erythrocytes which may serve as a storehouse for the unexcreted portion of the drug. The absence of radioactive CO_2 in the expired air confirms the well-known fact that in mammals the purine ring remains intact and is not further degraded.

Markedly contrasted to the above are the distribution and excretion patterns of the animals made nephrotic by 13 daily injections of unlabeled PA followed by the labeled PA at day 14. The alarmingly low recovery of total radioactivity (60%) prompted a repeat study on another nephrotic animal, but the results were essentially similar. The radioactivities of the kidney, liver and intestines were especially elevated, although blood levels were only slightly increased. On the basis of reduced urine output (average of 3.5 ml/8-hr period compared to 4.3 ml/8 hr for the day 1 rat) only a 20% decrease in excretion of radioactivity would be expected. The remainder may be attributed to (1) delayed or altered metabolism of the drug and/or (2) distribution of PA into an expanded pool of extra- and intracellular body water characteristic of edematous states. The latter explanation may be considered to apply to the 14-day rats with comparable nephrosis except that water retention was less pronounced in the first rat than in the second (*e.g.*, 10 ml *vs* 25 ml ascitic fluid respectively). Thus, even allowing for the difference in urine output (4.7 *vs* 2.3 ml), the first rat excreted twice as much radioactivity in the urine. This suggests that the failure to excrete more of the administered radioactivity was due to its retention in a larger exchangeable pool of body water.

The major portion of the administered radioactivity appearing in the urine of normal rats is excreted within the first 8 hrs with only a small fraction (2% of dose) being excreted in the subsequent 40 hrs. This is clearly shown for the rat described in Table II where the urine collection was extended to 48 hrs. Taken together with the data of Table I, the reasonable conclusion can be reached that greater than 90% of the admin-

TABLE II. Urinary Excretion of Radioactivity after Subcutaneous Administration of C^{14}-PA (1.30 × 10^6 cpm).

Interval, hr	Urine vol, ml	Total counts × 10^{-6}	Administered dose excreted (%)	
			Interval	Cumulative
0- 8	5.5	9.42	72.6	72.6
8-16	2.0	.070	.54	73.1
16-24	5.1	.102	.79	73.9
24-48	12.1	.061	.47	74.4
Total		9.65	74.4	74.4

TABLE III. Quantitation of PA in Urine by Inverse Isotope Dilution with Carrier.

Successive purification procedure	S.A. of carrier cpm/mg	cpm/μmole
Elution from Amberlite IRC-50 followed by recrystallization	1678	—
Paper chromatography & elution from paper	1466	—
Recrystallization	1510	444
Conversion to triacetyl derivative	1126*	473*
Recrystallization of triacetyl derivative	1142*	479*

* S.A. of triacetyl derivative.

Administered dose: 9.82×10^5 cpm. Total radioactivity in urine: 7.24×10^5 cpm. Carrier PA added: 251.7 mg.

$$\% \text{ unchanged PA in urine} = \frac{\left\{\begin{array}{c}\text{Avg S.A. of}\\ \text{purified carrier}\\ \text{\& derivative}\end{array}\right\}\left\{\frac{1000}{\text{M.W. PA}}\right\}\left\{\begin{array}{c}\text{mg}\\ \text{carrier}\\ \text{added}\end{array}\right\}\left\{100\right\}}{\{\text{Total radioactivity in urine}\}}$$

istered dose is excreted collectively in the urine and feces within the first 8 hrs.

The amount of unchanged PA appearing in the urine after administration of the C^{14}-labeled drug to normal and nephrotic rats was quantitatively determined by inverse isotope dilution with carrier PA (Table III, see also under *Methods*); the results are given in Table IV. The normal and nephrotic rats of the 0-8-hr series of Table IV were different from the animals of Table I and the results are remarkable not for the slight individual variations observed in the urinary excretion rates, but for the gross similarities of pattern. About half of the urinary radioactivity from the normal rats could be accounted for as unchanged PA, the figure being slightly less on the basis of administered dose. In contrast, only slightly more than 1/3 of the 8-hr urinary radioactivity from a nephrotic rat was unchanged PA, and since the excretion rate was low for this animal, this amounted to only 5% of the administered dose. One major difference between the urine composition of the day 1 rat and of the day 14 rats, of course, was the high protein content of the latter which conceivably could have resulted in binding of excreted PA and thus prevented it from behaving in the same manner as the added carrier. This possibility seems unlikely, however, in view of the observation that PA was not bound to plasma proteins.

TABLE IV. Unchanged Aminonucleoside in Urine of Normal and Nephrotic Rats.

	Collection period, hr	Radioactivity excreted, % of dose	Unchanged PA excreted % in urine	Unchanged PA excreted % of dose
Normal	0– 8	76.4	54.9	41.9
"	0–48	74.4	49.0	35.3
Nephrotic	0– 8	15.6	36.1	5.63

Discussion. Bartlett and Shelata in an initial attempt to quantitate urine and plasma levels of PA after its administration have utilized a tritium labeled sample in which 94% of the activity resided in the amino sugar moiety rather than in the purine portion of the molecule(7). However, they found the tritium label to be extremely labile biologically, the major fraction of the radioactivity of urine and body fluids having been accountable as tritiated water formed by isotope exchange. A carbon-14 label in the purine ring of PA which is stable to degradation except by actual metabolism would be less subject to artifacts of the type encountered with tritio-PA, and the present data with 8-C^{14}-PA would thus supersede the earlier results.

The extremely rapid rate of excretion of the drug when given to clinically normal animals is a point of great interest since, in any discussion of the mechanism of its nephrotoxic action, factors such as tissue localization, tissue concentration and urinary and fecal excretion rates should be taken into consideration. On the other hand, the high retention of the drug in nephrotic animals may considerably enhance its toxicity by prolonging the stay of the drug at its site of action. Thus, PA as a result of its unique action *prevents* its own excretion and thus may alter its rate of metabolic detoxication. Whether PA itself is the nephrotoxic agent in question or whether a metabolite is in fact responsible has not been completely clarified, although the results of Wilson *et al.*(9) suggest that some of the urinary metabolites of PA may also be toxic in this respect.

In agreement with an earlier work based on

spectrophotometric measurements, we found that rats excreted a large fraction of the administered dose as unmetabolized PA. The guinea pig, a species which is resistant to the nephrotoxic action of PA, reportedly excretes even more of its dose unchanged, while the rabbit, likewise resistant, metabolizes a major fraction of PA to a "substituted" product of unknown structure(9). The species susceptibility(3) and structural specificity(15) exhibited by PA are intriguing facets with mechanistic implications, and comparative metabolism studies may prove to be useful investigational probes for the elucidation of its mechanism of action.

Summary. The quantitative distribution and excretion of aminonucleoside-8-C^{14} was studied in normal and nephrotic rats. In the 8 hours following subcutaneous administration the normal rat excreted 65-70% of the dose in the urine, half of which was unchanged PA, and 15-26% in the feces. Despite being the target organ, the kidney contained less than 0.25% of the dose, while the liver contained considerably more. Nephrotic rats excreted much less of the radioactivity–total as well as in the form of unchanged PA. The decrease in excretion of total radioactivity was attributed to its retention in an expanded pool of body water characteristically seen in the nephrotic syndrome.

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Effect of Pilocarpine on Acid-Soluble Phosphate Compounds of Rat Submaxillary Glands. (27843)

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Recent work(1,2) indicates that the rate of transfer of Ca^{45} into rat salivary glands is relatively slow and is speeded by parasympathetic stimulants. For example, pilocarpine administration increases the rate of equilibration of Ca^{45} *in vivo* by 3-fold(1). Both uptake and loss of Ca^{45} by rat parotid glands are increased by pilocarpine or acetylcholine when studied *in vitro*(2). Rat salivary glands also contain more calcium than other soft tissues and approximately half of this calcium is lost when secretory activity is stimulated maximally by pilocarpine administration(3). A number of mechanisms can be postulated to explain some of these findings. For example, intracellular acid-soluble phosphate compounds could bind calcium(4) or furnish energy for the transfer process(5). Hence, changes in concentration or rates of incorporation of P^{32} are of interest.

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