Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. II. Metabolism of Aminonucleoside-8-C^{14}.* (27011)

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Study of the metabolism of tritiated aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, has failed to provide evidence for the conversion of this nephrotogenic agent into molecular structures closely resembling those of the adenosine-containing nucleosides, nucleotides or cofactors (1). Certain other observations (2,3), however, have raised a number of questions concerning the validity of these findings. Littlefield and Dunn (2), for example, have reported the presence in trace amounts of both the 6-dimethylamino- and the 6-methylamino-purine in rat liver microsomal ribonucleic acid; and, Wilson *et al* (3) were able, in a dialyzed yeast enzyme system, to demonstrate nucleotide formation from 6-amino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine and the 6-methylamino and 6-dimethylamino derivatives thereof. Rate of conversion of the latter to nucleotide, however, was only 1/10th that of the 6-methylamino derivitive and only 6/100th that of the 6-amino compound (3).

Considering these observations in conjunction with the possibility that the sensitivity of our tracer experiments may have been seriously impaired by labilization of tritium from the labeled aminonucleoside and subsequent exchange of the isotope with the hydrogens of body water (1), it seemed highly desirable to reinvestigate the metabolism of aminonucleoside using carbon-14 as a tracer in the 8-position of the purine ring. Use of this labeling would not only increase the tracer sensitivity of the experiments through elimination of the loss of labilized tritium, but would also offer the additional advantage of limiting labeled metabolic products to those containing the purine-8-C^{14} moiety. The present report is concerned with the results of such studies and includes, in addition, observations on the chemical nature of carbon-14 activity found in both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), isolated from kidney and liver tissue residues remaining after perchloric acid extraction of the acid-soluble nucleotides.

Materials and Methods. Radiochemical purity of aminonucleoside-8-C^{14}. Establishment of the radiochemical purity of carbon-14 labeled aminonucleoside‡ was accomplished by chromatography of samples of labeled aminonucleoside, unlabeled aminonucleoside, and a mixture of the two on Whatman No. 1 paper, as described by Hotchkiss (4). Following descending development in the solvent system, n-butanol saturated with water, the results of which indicated substances moving with identical R_f values, the chromatogram of the labeled aminonucleoside was cut into 10 mm segments and each segment extracted with distilled water. Ultraviolet absorption at 268 mμ and carbon-14 activity were determined on each extract. Correspondence of carbon-14 activity with ultraviolet absorption and agreement of R_f data were considered indicative of the radiochemical purity of the labeled material.

Measurement of radioactivity. Sample vials were prepared as previously described (1) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314. All specific activity data were corrected for quenching effects determined by utilization of an internal standard. The overall counting efficiency for standard C^{14}-labeled benzoic acid in the system was 43.5%.

Types of metabolic experiments. Two types of experiments were conducted. In one of these, the nephrotic syndrome was induced in each of of 3 Sprague-Dawley virgin female rats by essentially the procedure of Fiegelson, Drake, and Recant (5). The rats were maintained in separate metabolism cages throughout the ex-

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periment and fed Rockland rat checkers *ad libitum*. Each rat was injected subcutaneously with 5 mg aminonucleoside-8-C^{14} per day for a period of 10 days. This level of treatment corresponded to approximately 16.5 μmoles of aminonucleoside per day and 1 μcurie of carbon-14 per day. The material was injected in the form of a 2% solution of the nucleoside in isotonic saline. Twenty-four hours after final injection, the rats were sacrificed by ether anesthesia and exsanguination. In the other type of experiment, no attempt was made to induce the nephrotic syndrome. Metabolism of aminonucleoside-8-C^{14} was studied following a single dose of 10 mg of labeled material containing 2.3 μcuries of carbon-14 administered 3 hours prior to sacrifice.

Preparation of acid-soluble nucleotide fraction and isolation of BaRNA and BaDNA. Kidney and liver tissue were immediately removed and either frozen on dry ice and stored at -15°C or processed immediately by the procedure of LePage and Heidelberger(6) for acid-soluble nucleotides and the barium salts of the nucleic acids. In general this procedure involves homogenization of the tissue in cold 4% perchloric acid, separation of the acid-soluble nucleotide fraction by centrifugation, removal of phospholipids from the tissue residue by alcohol-ether extraction, alkaline hydrolysis of the defatted tissue residue permitting separation of ribonucleotides from deoxyribonucleic acid, and finally, precipitation of the latter 2 fractions as the barium (Ba) salts.

Chemical nature of carbon-14 activity in the acid-soluble nucleotide fraction. The 4% perchloric acid extracts containing the acid-soluble nucleotide fraction were cooled in an ice-salt bath and carefully neutralized to pH 6-7 with 5 N KOH using phenol red as an internal indicator. Perchlorate thus precipitated as the potassium salt was removed by centrifugation and the supernatant filtered. The pH of the filtrate was then adjusted to 6 for chromatography on 20 x 1 cm Dowex 1-X10 columns (formate) as described by Hurlbert *et al*(7). Elution of the nucleotide fractions from the column was followed by measuring the 260 mμ absorption in the Beckman DU Spectrophotometer. Carbon-14 activity was determined in each peak and fractions found to contain radioactivity were pooled and concentrated by lyophilization. Residues were then dissolved in water, the pH adjusted to 6, and the resulting solution rechromatographed on 10 x 1 cm Dowex 1-X10 columns with the modified ammonium formate system. Carbon-14 containing peaks obtained in rechromatography were identified by spectral characterization and chromatography on Whatman No. 1 paper with Solvent System III (8).

Chemical nature of carbon-14 activity in BaRNA and BaDNA fractions isolated from kidney and liver tissue. Samples ranging from 10 to 50 mg of the barium salts of the ribonucleotides or DNA were dissolved in 1 N HCl and heated for 1 hour in a boiling water bath. Barium ion was then removed by adding a slight excess of sulfuric acid and separation of the barium sulfate precipitate by centrifugation. Hydrolysates thus prepared were placed on 90 x 9 mm Dowex 50-X12 columns and chromatographed as described by LePage and Heidelberger(6). Eluate peaks found to contain carbon-14 activity were identified by position in the eluate, wave lengths of maximum extinction, and 250/260 and 280/260 absorbance ratios.

TABLE I. Distribution of Carbon-14 Activity* in Acid-Soluble Nucleotide and Nucleic Acid Fractions of Kidney and Liver Tissue.

Fraction	Activity	
	Kidney	Liver
	Counts/min/g tissue extracted	
Acid-soluble nucleotide (4% Perchloric acid extract)	17,280	6400
	Counts/min/mg barium salt	
BaRNA	46	129
BaDNA	2	57

* Total activity administered in 10-day period = 8.05 x 10^6 counts/min. Carbon-14 activity excreted in urine in 10-day period = 3.31 x 10^6 counts/min.

Results. Table I summarizes the distribution data for carbon-14 activity in the acid-soluble nucleotide and nucleic acid fractions of both kidney and liver tissue obtained from rats subjected to an experiment in which aminonucleoside was administered for a period of 10 days. In agreement with the earlier experiments(1), the acid-soluble nucleotide fraction obtained from kidney tissue was much more highly labeled than the comparable fraction

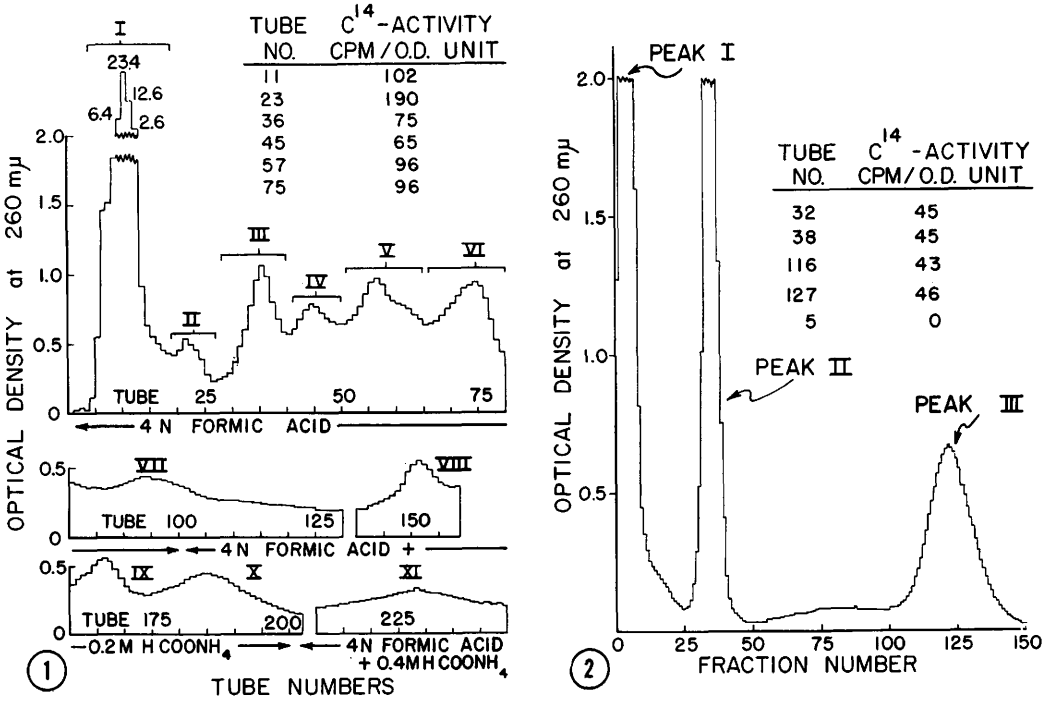

FIG. 1. Chromatography of acid-soluble nucleotide fraction of kidney. Chromatography on a 20×1 cm Dowex 1-X10 (formate) column of the acid-extract of 9.9 g pooled kidney tissue obtained from 6 rats, each of which had been injected subcutaneously with 10 mg aminonucleoside-8-C^{14} (2.3 μcuries) 3 hrs. prior to sacrifice. A total of 464 five ml fractions were collected. Eleven peaks, indicated by Roman numerals I-XI, were separated. Tubes, indicated by the bracket enclosures for each of the peak fractions, were pooled and concentrated by lyophilization. Optical densities recorded for tube Nos. 10, 11, 12, and 13 in Peak I were calculated from measurements made on suitable dilutions.

FIG. 2. Chromatography of acid-hydrolysate of liver BaRNA. Chromatography of acid-hydrolysate of 50 mg BaRNA on a 9×1 cm column Dowex 50-X12 cation exchange resin, developed with 2 N HCl at a flow rate of 0.8 ml/min. A total of 500 three ml fractions were collected. Pyrimidine nucleotides were eluted in Fractions 1-20. The purine base, guanine, was eluted in Fractions 28-42, and adenine in Fractions 105-140.

obtained from liver tissue. Results expressed in terms of counts/min./g tissue extracted, indicate 2.7 times as much carbon-14 activity in the acid-soluble nucleotide fraction of kidney tissue as was found in liver tissue. In contrast to this finding, however, are the specific activity data for kidney and liver RNA and DNA. In terms of counts/min./mg barium salt, liver RNA was 2.8 times as active as kidney RNA, and liver DNA 28 times as active as kidney DNA.

Detailed study of the chemical nature of carbon-14 activity in acid-soluble nucleotide fractions of both kidney and liver tissue revealed no difference between fractions obtained from tissues of rats sacrificed in as short a period as 3 hours after administration of a single large dose of aminonucleoside-8-C^{14} and similar fractions from rats given small daily injections of the material for periods as long as 10 days. Carbon-14 activity was found only in the nucleotides containing the purine bases adenine and guanine. Fig. 1 presents a typical chromatogram of the acid-soluble nucleotide fraction of pooled kidney tissue from 6 rats subjected to the acute experiment. Collection of 464 five ml fractions from the column revealed the presence of eleven 260 mμ-absorbing peaks. These were eluted from the column in the first 245 tubes. Carbon-14 activity determined on the peak tube from each of the 11 fractions indicated the presence of only 6 fractions labeled with the isotope. For purposes of comparison, the radioactivity of each of the peak tubes labeled with carbon-14 is presented in terms of total counts/min./optical density

unit. Rechromatography of lyophilized Fractions I through VI on Dowex 1-X10 columns developed with the modified ammonium formate system(7), followed by identification of carbon-14 containing peaks, as described under methods, revealed the presence of only adenosine and guanosine phosphates. With the exception of Fractions II and III, which upon rechromatography gave only one peak, all other fractions were resolved into 2 or more peaks. No evidence was obtained for conversion of either aminonucleoside or its 6-monomethylaminopurine derivitive into the corresponding nucleotide.

Fig. 2 presents a typical chromatogram of the acid-hydrolysate of 50 mg of barium RNA isolated from livers of rats treated for a period of 10 days with aminonucleoside. Carbon-14 activity was found only in peaks II and III. Table II summarizes the spectral characterization data. For purposes of comparison, similar spectral data are included for adenine, guanine, 6-dimethylaminopurine and 6-methylaminopurine. Considered in conjunction with positions of elution of known pyrimidine nucleotides and the purine bases(6,9), it was concluded that peak II was guanine and peak III adenine. No evidence was obtained for the presence of carbon-14 labeled 6-methylamino- or 6-dimethylamino-purine in the acid-hydrolysates of either kidney or liver RNA or DNA.

TABLE II. Spectral Characterization Data for Purine Bases in HCl.

Base	Wave length		Absorbance ratios	
	Max.	Min.	250/260	280/260
	mμ	mμ		
Peak II	248	222	1.33	.64
" III	262	230	.93	.46
Adenine	264	228	.76	.37
Guanine	245	225	1.37	.74
6-Methylaminopurine*	267	232	.59	.75
6-Dimethylaminopurine	275	242	.82	1.07

* Values obtained from ultraviolet absorption spectrum given by Adler *et al* (9).

Discussion. Clearly these results and those previously reported(1) fail to provide evidence for the conversion of aminonucleoside to the corresponding aminonucleotide in the rat or for biosynthesis of nucleotides or other cofactors in which intact aminonucleoside, the 6-dimethylaminopurine moiety, or the 3-amino-3-deoxyribose moiety are substituted for normally occurring adenosine, 6-aminopurine, or ribose moieties. This would seem conclusively to eliminate from consideration a mechanism of action of aminonucleoside involving formation of abnormal or counterfeit nucleotides or cofactors which subsequently foul or inhibit metabolic pathways requiring the normally occurring adenosine compounds.

In the present studies no carbon-14 activity was found in either the pyrimidine nucleotides chromatographed from the acid-soluble nucleotide fractions of both kidney and liver tissue of aminonucleoside-nephrotic rats or in pyrimidine bases chromatographed from acid-hydrolysates of RNA and DNA isolated from the same tissues. Clearly labeling of cytidine nucleotide observed in metabolic studies with tritiated aminonucleoside(1) must have arisen as a result of cleavage of aminonucleoside at the glycosidic linkage and utilization of labeled ribose arising from the 3-amino-3-deoxyribose moiety in the biosynthesis of the cytidine nucleotide.

The observation of carbon-14 activity in adenine and guanine chromatographed from the acid hydrolysates of RNA and DNA clearly indicates metabolism of the 6-dimethylaminopurine moiety of aminonucleoside to precursor purine. Demethylation of the 6-dimethylamino group, accomplished either prior or subsequent to the splitting of the glycosidic linkage, would of course yield adenine, which could then be metabolized to guanine.

Although both the 6-methylamino- and the 6-dimethylamino-purine have reportedly been found in trace amounts in rat liver microsomal RNA(2), we were unable to detect, even with the assistance of the carbon-14 labeled purine moiety, the presence of either of the methylated bases in kidney or liver RNA or DNA isolated from the tissue residues remaining after extraction of the acid-soluble nucleotides with perchloric acid. While this observation might be explained in terms of differences in the purine base composition of microsomal RNA and that of RNA and DNA isolated as described, lack of evidence for the conversion of aminonucleoside to the corresponding aminonucleotide would seem to preclude incorporation of either of the labeled 6-methylamino- or 6-dimethylamino-purine moieties into nucleic acids.

Summary. Detailed studies were made of the chemical nature of carbon-14 activity found in acid-soluble nucleotide, ribonucleic acid, and deoxyribonucleic acid fractions isolated from kidney or liver tissue of rats rendered experimentally nephrotic with a series of 10 small daily injections of aminonucleoside-8-C^{14}, and, in similar fractions obtained from rats sacrificed as early as 3 hours after administration of a single large dose of the labeled compound. No significant differences were noted. Only the adenosine and guanosine phosphates of the acid-soluble nucleotide fractions and the adenine and guanine bases of RNA and DNA hydrolysates were labeled with carbon-14. No evidence was obtained either for conversion of the intact aminonucleoside to the corresponding nucleotide or for incorporation of the 6-dimethylamino- or 6-methylamino-purine moieties into ribonucleic acid or deoxyribonucleic acid of kidney or liver tissue.

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Effect of Adrenal Tissue on Desoxycorticosterone Hypertension.* (27012)

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Swingle and coworkers, comparing 7 adrenalectomized and 4 intact dogs, noted that a given amount of desoxycorticosterone produced a lesser degree of hypertension in the intact dogs(1). Soffer also found it difficult to produce hpyertension with desoxycorticosterone in patients with intact adrenals, even though it occurred readily in patients with adrenal insufficiency(2). In this study we wanted to find out whether the presence or absence of adrenal tissue has any effect on development of desoxycorticosterone hypertension in the rat. We carried out our study on 2 groups of rats, producing hypertension by administering a given amount of desoxycorticosterone to both groups. In one group both adrenals had been removed; in the other group, both adrenals were intact.

Methods. Forty male Wistar rats, of approximately 100 g, were each injected subcutaneously with 31 mg of desoxycorticosterone trimethylacetate. Four weeks later the same dose was repeated. Half of these rats were given bilateral adrenalectomies; the other half received sham operations with adrenal tissue left intact. All 40 were allowed to drink only .5% saline and were fed only Purina lab chow.

Between the sixth and seventh week after original injection, 3 blood pressure readings, at 2-day intervals, were taken on each rat, using the microphonic method(3). The average of these was considered the "true" blood pressure.

Results. Blood pressures were obtained on 18 rats in the adrenalectomized group and on 15 in the group receiving sham operations. The mean blood pressure of the adrenalectomized group was found to be 174 mm Hg. (S.D. ±9); that of the group whose adrenals were left intact was 178 mm Hg. (S.D. ±19). The

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