weight of recipients (Table III). In 5 of 7 experiments the ratio of effectiveness of cells when transferred to 1-kg recipients, to that found on transfer to neonatal recipients, ranged from 2.2 to 4.9. In the other 2 experiments this ratio was 10 and 24, respectively.

In a study by Dixon and Weigle(10) lymph node cells obtained from donor rabbits injected 2 hours earlier with *Shigella*-trypsin filtrates were transferred subcutaneously or intramuscularly into 5-day-old recipient rabbits. Agglutinins failed to appear in sera of 5-day-old rabbits, although agglutinins were found subsequently in sera of adult control recipients. A possible basis for this difference in findings may be that in our experiments lymph node cells were incubated *in vitro* with the antigen, and were transferred by intracardiac injection, and that neonatal recipients had been irradiated. However, in a more recent study Dixon and Weigle did not observe substantial differences in peak titers of neonatal recipients whether they were irradiated or not, or whether lymph node cells were given by subcutaneous or intracardiac injection (11).

In other studies involving transfer to neonatal rabbits of cells of rabbit lymph or spleen, Sterzl(12) transferred to 5-day old rabbits, by intraperitoneal injection, spleen cells which had been incubated *in vitro* with *S. paratyphi* and found agglutinins subsequently in sera of recipients, in range of titers of 8 to 128. Also, Holub(13) transferred to 2-5-day-old rabbits, by the same route, cells collected from the cisterna chyli or spleens of adult rabbits and incubated *in vitro* with *Brucella suis* or *Salmonella paratyphi B*. Agglutinins appeared in these recipients in range of 2-64.

Summary. 1. Newborn rabbits were injected with leucocytes obtained from adult rabbits which were prospective donors of lymph node cells. When antigen-incubated lymph node cells from these donors were transferred to young rabbits 1-2½ months later, agglutinins failed to appear, or did so in low titer. This suppression of transferred lymph node cells indicated that tissues of newborn rabbits had reacted to antigens of leucocytes of donor animals. 2. Transfer of lymph node cells incubated *in vitro* with *Shigella*-trypsin filtrate to uninjected newborn rabbits (1-11 days of age) resulted in the appearance of anti-*Shigella* agglutinins.

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Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. I. Metabolism of Tritiated Aminonucleoside.* (25295)

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Structural similarity of adenosine and the aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β - D - ribofuranosyl)-purine, suggests that the mechanism by which the lat-

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ter induces nephrosis in the rat may well involve interference with adenosine metabolism at the nucleoside, nucleotide, or nucleic acid level. Thus, for example, incorporation of aminonucleoside into molecular structures closely resembling those of adenosine nucleotides, adenosine-containing cofactors (such as diphosphopyridine nucleotide and coenzyme A), ribonucleic acids, or deoxyribonucleic acids, might result either in fouling of normal metabolic pathways by which such compounds are biosynthesized or in blocking of metabolic processes in which the aminonucleoside-containing structures fail to substitute for their adenosine counterparts. Proof that aminonucleoside is metabolized to any one of the indicated structures would, of course, furnish support for such a mechanism; and in addition serve as a guidepost to subsequent investigation. With this in mind, studies were undertaken of the metabolism of aminonucleoside labeled with tritium by the Wilzbach procedure. The present report focuses attention primarily on the chemical nature of tritium activity in an acid-soluble nucleotide fraction obtained from kidney tissue of rats rendered experimentally nephrotic with the aminonucleoside. In addition, it presents data on distribution of radioactivity in body fluids and urine, as well as in kidney and liver nucleic acids.

Procedures. Tritiated Aminonucleoside. One g of aminonucleoside† was exposed to 2.5 curies of tritium at 27°C and 0.4 atmos for 2 weeks. The labeled material was dissolved in water, which was later removed with vacuum at room temperature. This operation was repeated twice. The material was then lyophylized from distilled water 5 times, until percentage of total activity labilized and exchanged with hydrogens of water/lyophylization had reached a constant value. Samples of labeled aminonucleoside, unlabeled aminonucleoside, and a combined sample of labeled and unlabeled material yielded identical R_f values when chromatographed on Whatman No. 1 paper by descending development with the solvent system, n-butanol saturated with water. *Radiochemical purity* was established by cutting the chromatograph of the labeled aminonucleoside into 10 mm segments, extracting each segment with distilled water and determining the ultraviolet absorption and tritium activity of each extract. Radioactivity was found only in the segment from the aminonucleoside spot. The final material had a specific activity of 10.3 μc/ml. To study distribution of tritium activity in the labeled material, the aminonucleoside was hydrolyzed with 0.5 N sulfuric acid, and the 6-dimethylaminopurine separated as the silver salt from the 3-amino-3-deoxyribose moiety. Determination of the tritium activity of each fraction indicated 5.9% of the activity in the 6-dimethylaminopurine and 94.1% in the 3-amino-3-deoxyribose. *Measurements of radioactivity.* Sample vials were prepared by adding in the following order: 1 ml molar Hyamine, 5 ml absolute ethanol, 0.5 ml or less of sample solution, volume of water required to bring total volume of sample and water to 0.5 ml, and 10 ml of a counting solvent solution. The latter was composed of 1 g of 2,5-diphenyloxazole (PPO) and 0.025 g of 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) in 500 ml toluene. All samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314 long enough to give a 99% confidence interval with a 2% error for the counting rate. All data are corrected for quenching effects and background. The overall counting efficiency for water in our system was 2.4%. *Induction of experimental nephrosis.* The procedure for inducing nephrosis in normal adult Sprague-Dawley virgin female rats was essentially the same as that described by Fiegelson, Drake, and Recant(1). Each of 3 rats, maintained in separate metabolism cages and fed Rockland rat checkers *ad lib,* was injected subcutaneously with the tritiated aminonucleoside, 1.5 mg (about 5 μmoles)/100 g body weight/day for 10 days. The material was injected in the form of a 1.5% solution in isotonic saline. Twenty-four hour urine collections were made for each animal and checked daily for development of proteinuria. Trace proteinuria, normally found in most rats, in-

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creased progressively after the 5th or 6th day of aminonucleoside administration and was very marked in all animals by the 10th day. Twenty-four hours after final injection of labeled aminonucleoside, the rats were sacrificed by ether anesthesia and exsanguination. At sacrifice considerable ascites was present. Samples of both blood and ascitic fluid were obtained for tritium assay. *Isolation of BaRNA and BaDNA.* Kidney and liver tissues were immediately removed and either frozen on dry ice and stored at −15°C or processed immediately by the procedure of LePage and Heidelberger(2) to isolate the barium salts of the ribonucleotides (BaRNA) and deoxyribonucleic acid (BaDNA). In general, this involves removal of acid-soluble nucleotides by repeated homogenization of the tissues in cold (0-3°C) 4% perchloric acid, separation of the 4% perchloric acid extracts by centrifugation, extraction of the tissue residue to separate the ribonucleotides from deoxyribonucleic acid, and finally precipitation of the ribonucleotides and deoxyribonucleic acid as barium salts. *Study of chemical nature of tritium activity in acid-soluble nucleotide fraction.* Acid-soluble nucleotides in the 4% perchloric acid extract were adsorbed on Norit A and then eluted with 25% ethanol containing 0.5% ammonia. Following adjustment of the pH to 1.5, the eluate was extracted with ice-cold ether to remove ultraviolet absorbing non-nucleotide material, and then aerated to remove excess ether from the aqueous solution. The pH of the solution was adjusted to 7.5 and the solution chromatographed on a Dowex 1-X4 column, according to the procedure of Pontis and Blumson(3). Ten ml fractions were collected and the elution of the nucleotides followed by measuring the absorption at 260 mμ in a Beckman DU Spectrophotometer. Absorbance ratios were determined for 250/260 and 270/260 mμ measurements, and complete ultraviolet spectra run to obtain characterizing wave lengths of maximum and minimum absorption for each peak eluted. Aliquots of each peak were assayed for tritium activity as described above. Final identification of radioactive peaks was accomplished by isolating the nucleotide as the calcium salt, decomposing the latter with ammonium oxalate, and chromatographing an aliquot of each nucleotide solution along with known nucleotide solutions on Whatman No. 1 paper developed with System III(4).

TABLE I. Distribution of Tritium Activity* in Body Fluids.

Fluid	Activity
	Counts/min./ml plasma
Protein-free filtrate of plasma (trichloroacetic acid precipitated)	3860
	Counts/min./ml
Plasma water†	2895
Ascitic fluid	4335
Ascitic fluid water	4020
	Counts/min./10 day collection
Urine	1.27×10^6
" water	2.85×10^5

* Total activity administered over 10-day period $= 3.82 \times 10^6$ counts/min.

† Water samples obtained from body fluids by evaporation of samples of fluid in a Petri dish at room temperature and condensation of the water vapor on the dish cover cooled with dry ice.

Results. Table I summarizes a typical distribution of tritium activity in body fluids of one of the rats after the 10-day period of treatment with labeled aminonucleoside. The data indicate that approximately ⅓ of administered activity was excreted in the urine, and that 22.4% of urinary activity was in the form of water. The activity in plasma and ascitic fluid respectively was 75% and 95% due to labeled water. Study of the chemical nature of radioactivity in a protein-free filtrate of urine from this animal, concentrated on a Celite-Norit A column, eluted with 50% aqueous ethanol, and chromatographed on an Amberlite IR-50-Type 2 column developed with 2 N NH_4OH, revealed the presence of only one radioactive ultra-violet absorbing component. Absorbance ratios and wave lengths for maximum and minimum absorption identified this material as aminonucleoside.

Distribution of tritium activity in various fractions obtained during isolation of the barium salts of the ribonucleotides (BaRNA) and deoxyribonucleic acid (BaDNA) follows the same general pattern in both kidney and liver tissue (Table II). A detailed study of the chemical nature of radioactivity in the

TABLE II. Distribution of Tritium 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	7280	5884
	Counts/min./mg/barium salt	
BaRNA	18	25
BaDNA	1	3
Tissue residue	None	None

acid-soluble nucleotide fraction obtained from pooled kidney tissue of all 3 rats, revealed that, out of a total of 8 nucleotide peaks separated by column chromatography, only 2 were significantly labeled with tritium. Table III summarizes absorbance data and wave lengths of maximum and minimum absorption for each of these peaks, referred to simply as Peaks 1 and 2. It is readily apparent that absorbance data for these peaks corresponds respectively with similar data for cytidine and adenosine nucleotides. Final identification of Peak 1 as cytidine diphosphate and of Peak 2 as adenosine monophosphate was completed by means of paper chromatography. The calcium salt of cytidine diphosphate had a specific activity of 24 counts/min./mg, and that of the adenosine monophosphate 106 counts/min./mg.

Discussion. The present studies fail to provide any evidence for metabolism of labeled aminonucleoside into molecular structures closely resembling those of adenosine nucleotides or adenosine-containing cofactors such as diphosphopyridine nucleotide. This negative result would seem to eliminate the possibility of aminonucleoside nephrosis being induced by a mechanism involving biosynthesis of counterfeit or fraudulent nucleotides or cofactors which foul or inhibit normal metabolic pathways requiring their adenosine counterparts.

The observation of tritium activity in adenosine and cytidine nucleotides, however, provides positive information concerning metabolism of the aminonucleoside. In this respect, calculations clearly indicate that labeling to the extent observed in either group of nucleotides can not be accounted for in terms of an exchange mechanism involving hydrogens of body water. This is true whether one assumes that all the hydrogens of each nucleotide can be exchanged with those of tritium-labeled body water, or that only the hydrogens attached to the amino group in the 6 position of the purine or pyrimidine moieties and those of the hydroxyl groups of adenosine and cytidine are exchangeable. Thus, for example, water in plasma, which for all practical purposes can be assumed representative of body water, counts 2895 counts/min./ml or 27 counts/min./mg hydrogen. Assuming all the hydrogens of the adenosine nucleotide to be replaceable, an activity of 3400 counts/min./mg body water hydrogen would be required to label the nucleotide to the extent of 106 counts/min./mg calcium salt. Similar calculations for the labeling of the cytidine nucleotide indicate an activity of 811 counts/min./mg body water hydrogen as a requirement. If one assumes only the hydrogens of the hydroxyl and amino groups to be exchangeable, then body water hydrogen of still higher activity is required.

Clearly then, the observed labeling of the nucleotides must be considered indicative of either direct or indirect metabolism of the tritiated aminonucleoside to labeled products subsequently utilized in the biosynthesis of acid-soluble nucleotides. A possible pathway, for example, might involve enzymatic splitting of the aminonucleoside at the glycosidic bond followed by demethylation of the 6-dimethylaminopurine to yield precursor adenine, and oxidative deamination of 3-amino-3-deoxyribose followed by reduction of the ketopentose to yield precursor ribose. Still another pathway—possibly more important in terms of adenosine metabolism and mecha-

TABLE III. Absorption Data for Acid-Soluble Nucleotides at pH 2.0.

Nucleotide	Absorbance		Absorbance ratios	
	Max	Min	250/260	270/260
	mμ	mμ		
Peak #1	278	242	.57	1.50
#2	257	230	.84	.69
Adenosine phosphates	257	230	.84	.71
Cytidine phosphates	280	241	.45	1.71
Aminonucleoside	268	233	.59	1.14

nism of action of aminonucleoside—might involve a reaction sequence in which the aminonucleoside is metabolized directly to adenosine without splitting at the glycosidic bond. Such a pathway, occurring at the nucleoside level of metabolism, would of course involve the same series of reactions suggested for the 6-dimethylaminopurine and 3-amino-3-deoxyribose moieties. Pertinent in connection with such a metabolic pathway are a number of experimentally determined facts which indicate that intactness of the aminonucleoside molecule is a requirement for the induction of experimental nephrosis in the rat. Thus, in our laboratory (unpublished observations) and in that of a number of other investigators (5), several substances closely related to the aminonucleoside have been tested for their nephrotoxic action. 6-Dimethylaminopurine, 3-amino-3-deoxyribose, a combination of these, 6-dimethylamino-9-(β-D-ribofuranosyl)-purine, and 3′-amino-3′-deoxyadenosine all failed to produce nephrosis under conditions similar to those described in the present report. Only the aminonucleoside and the antibiotic, puromycin, from which it was obtained, were active.

While these investigations with tritiated aminonucleoside were in progress, observations were reported from a number of other laboratories which, in conjunction with the present ones, focus attention on the possibility that aminonucleoside nephrosis may be induced by a mechanism in which unaltered aminonucleoside simply inhibits some key step in the normal metabolic sequence of nucleoside or nucleotide metabolism. In this context, observations that 6-dimethylamino-9-β-D-ribofuranosyl-purine and 6-amino-9(3′-amino-3′-deoxyribofuranosyl)-purine inhibit the conversion of 5-amino-4-imidazole carboxamide riboside to the corresponding ribotide(6), and that the aminonucleoside inhibits production of acid-labile phosphate (ATP) by a crude brewer's yeast system in the presence of adenosine and phosphate(7) suggest interference with phosphorylation mechanisms.

Summary. 1. The preparation and metabolism of radiochemically pure aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, labeled with tritium by the Wilzbach procedure, is described and discussed. Data are presented for the distribution of radioactivity in body fluids and urine as well as in kidney and liver nucleic acids. 2. Study of the chemical nature of tritium activity in an acid-soluble nucleotide fraction, prepared from pooled kidney tissue of rats rendered nephrotic with the tritiated aminonucleoside, indicates that the rat is capable of metabolizing the latter into labeled products utilizable in the biosynthesis of adenosine and cytidine nucleotides. 3. Labeling of these nucleotides, which cannot be accounted for in terms of an exchange mechanism involving the hydrogens of body water, is considered in terms of alternative metabolic pathways.

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