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DISTRIBUTION OF GENTAMICIN AMONG SUBCELLULAR FRACTIONS FROM RAT RENAL CORTEX

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Abstract—A substantial amount of data is available to suggest that lysosomal sequestration of aminoglycoside antibiotics plays a role in the pathogenesis of aminoglycoside-induced renal tubule cell injury; however, relatively little information is available on the subcellular distribution of aminoglycosides in the kidney during treatment protocols of the type that ultimately go on to produce extensive lethal renal tubule cell injury and acute renal failure in experimental animals. This study assessed the distribution of gentamicin and subcellular membranes on a discontinuous sucrose density gradient after *in vivo* exposure of rats to four daily 100 mg/kg doses of gentamicin as compared to *in vitro* exposure of normal rat renal cortex to gentamicin during tissue homogenization at drug levels comparable to those seen after *in vivo* treatment. After both *in vivo* and *in vitro* exposure, major localization of gentamicin, the lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase (NAG), and the endoplasmic reticulum marker enzyme NADPH-cytochrome *c* reductase, occurred in a very light membrane fraction. Within this membrane fraction, gentamicin was more closely associated with the NAG than with the NADPH-cytochrome *c* reductase. The results could not be explained by complete lysosomal disruption during subcellular fractionation after *in vivo* gentamicin. These data provide additional insights into both the possibilities for subcellular interactions of aminoglycosides in the kidney, and into the methodology required to optimally assess such interactions.

The recognition that a large fraction of the high levels of aminoglycoside antibiotics which accumulate in the renal cortex in treated patients and in animal models of aminoglycoside nephrotoxicity is sequestered within lysosomes has been a major advance in the understanding of the pathogenesis of aminoglycoside-induced renal tubular cell injury. However, the most unequivocal data on subcellular distribution of aminoglycosides after prolonged courses of exposure have been obtained not in kidney or in a kidney-derived tissue model, but in cultured fibroblasts [1, 2]. Subcellular fractionation and autoradiographic studies have indeed provided definitive evidence that lysosomal sequestration does occur in the kidney *in vivo* acutely after tracer doses of gentamicin [3–7], but little is known about the subcellular distribution of aminoglycosides during prolonged courses of exposure of the type associated with significant nephrotoxicity [3, 8–10]. Such information is of substantial importance in assessing the relevance of extralysosomal versus lysosomal events in the pathogenesis of aminoglycoside-induced renal tubular cell injury and nephrotoxicity. In this regard, recent data have indicated that a number of prominent extralysosomal events are demonstrable in the early development of gentamicin-induced renal tubular cell injury: (1) functional and structural defects are present in isolated mitochondria and brush border membranes [3, 9–13], (2) acidic phospholipids are specific binding sites for the aminoglycoside on brush border membranes [11], and (3) aminoglycoside treatment appears to produce early alterations in the acidic phospholipid content of non-lysosomal cellular membranes [13].

Furthermore, *in vitro* gentamicin binding to a variety of subcellular membranes has been documented [3, 11, 14–16]. The present study was designed to obtain more information on the possible subcellular distribution of gentamicin in the kidney after an *in vivo* course of treatment of sufficient magnitude to result in substantial nephrotoxicity in the rat but at a time prior to the occurrence of advanced renal tubular cell injury and necrosis. Cellular fractionation techniques previously reported to be effective in assessing the subcellular distribution of gentamicin in isolated fibroblasts [1, 2] and the subcellular distributions of cationic amphiphilic drugs which are concentrated in hepatic lysosomes were utilized [17–20].

MATERIALS AND METHODS

Male Sprague–Dawley rats (Harlan) weighing 275–300 g and maintained on a standard lab diet (1% calcium) were used for all studies. Gentamicin treatment was with a single daily subcutaneous dose of 100 mg/kg gentamicin as gentamicin sulfate (Sigma, St. Louis, MO) for 4 consecutive days. Rats were killed 24 hr after the last dose.

Kidneys were rapidly removed and placed in ice-cold 0.27 M sucrose. Then the cortices were dissected, minced and homogenized. For control (C) and *in vivo* gentamicin-treated (G) groups, the homogenizing solution consisted of 0.27 M sucrose,

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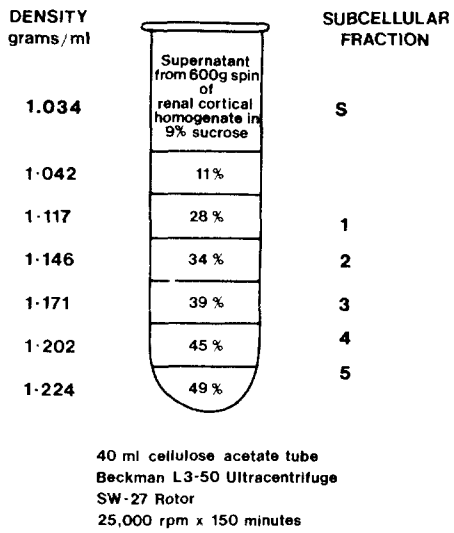

Fig. 1. Graphic summary of the discontinuous sucrose density gradient utilized. The subcellular fraction designations indicate areas of the gradient selected for detailed analysis in Tables 1 and 2.

1 mM EGTA*, 5 mM Tris–HCl, pH 7.4. For control groups exposed to gentamicin *in vitro* during homogenization, Tris–gentamicin (pH 7.4) was added to this homogenizing solution at a concentration estimated to approximate the level present after *in vivo* gentamicin treatment. The homogenate was spun at 600 *g* for 10 min to remove nuclei and nonhomogenized cellular debris.

For simultaneous subcellular fractionation into multiple membrane components, 10 ml of the 600 *g* postnuclear supernatant fraction was layered onto a discontinuous sucrose gradient in a 40 ml cellulose acetate tube, as illustrated in Fig. 1. The gradient was then centrifuged at 25,000 rpm using an SW-27 rotor in a Beckman L3-50 ultracentrifuge. For some studies, samples of major regions of the gradient were taken carefully from the top of the tube with a pipetter; for others, the entire gradient was fractionated into 1-ml samples using a Gilson fraction collector.

Activities of *N*-acetyl-β-D-glucosaminidase (NAG), alkaline phosphatase, rotenone-insensitive NADPH-cytochrome *c* reductase, Na^+-K^+-ATPase and cytochrome oxidase were assayed as previously detailed [13].

Gentamicin levels were measured by RIA (New England Nuclear) on samples solubilized with 0.15% Triton X-100.

Proteins were assayed by the method of Lowry *et al.* [21].

All reagents used were of the highest grade commercially available. All organic reagents were obtained from Sigma.

Statistical tests utilized are detailed in Results.

* Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; Tris, Tris(hydroxymethyl)aminomethane); NAG, *N*-acetyl-β-D-glucosaminidase; and NADPH, nicotinamide-adenine dinucleotide phosphate.

RESULTS

In the first series of experiments, gradients were sampled at several discrete points which preliminary studies had suggested would provide the best definition of various subcellular membranes as identified by enzyme markers. Table 1 summarizes the results of these studies as regards enzyme composition of each fraction, predominant membranes present as deduced from the enzyme composition, and gentamicin level factored for mg protein. The G group had a mean ± S.E. homogenate gentamicin level of 8.8 ± 0.3 μg/mg protein while the homogenate gentamicin level of the C + G group was 10.2 ± 0.1 μg/mg protein. As can be seen in Table 1, the major enrichment of gentamicin activity occurred in fraction 1 which was also enriched in the lysosomal marker, NAG, and in the endoplasmic reticulum marker, NADPH-cytochrome *c* reductase. Furthermore, both group G (*in vivo* gentamicin exposure) and group C + G (*in vitro* gentamicin exposure) exhibited generally similar patterns of gentamicin distribution on the gradient.

The sampling methodology employed in the first series of gradient studies did not allow for assessment of extent of recovery of enzymes and gentamicin off the gradient and could have missed differences between closely adjacent areas, or in non-sampled areas. For this reason, another series of experiments was done using the same type of sucrose gradient and the same groups, C, G, and C + G but sampling the entire gradient in 1-ml aliquots and analyzing each for protein, enzyme activity, and gentamicin level. Recoveries for all parameters measured were 85–110% of the amount layered on the gradients. The G group had a mean ± S.E. homogenate gentamicin level of 7.6 ± 0.6 μg/mg protein while the homogenate gentamicin level of the C + G group was 8.4 ± 0.3 μg/mg protein. Representative gradients from each group are illustrated in Fig. 2. As in the initial series of gradients, there were prominent localizations of NAG, NADPH-cytochrome *c* reductase and gentamicin to light membranes coming off in gradient fractions 10–19. This occurred similarly in the *in vivo* group (G) and in the *in vitro* group (C + G).

To better quantify the average behaviors of membrane enzymes and gentamicin in this series of gradients, the data were analyzed to determine the mean percent of total enzyme and gentamicin on the gradient found in each gradient region. Based on enzyme characteristics, values for the 1-ml aliquots were pooled to give results for gradient regions approximately corresponding to the areas sampled in the first group of gradients (Table 1). However, in this series of gradients, no area remained unsampled, the identified regions are contiguous and cover the entire gradient, and a fraction rich in mitochondria (No. 5 in Fig. 1) which was not sampled in the first series of gradients is reported. These data are summarized in Table 2.

Somewhat more cytochrome oxidase activity was present in the area defined as fraction 1 in the second series of gradients (Table 2), but otherwise the overall behavior of marker enzyme activity was similar to that in the initial series of studies (Table 1). It is

Table 1. Enzyme profiles and gentamicin levels of sucrose gradient fractions*

	Relative specific activities of marker enzymes†																Gentamicin level‖ (μg/mg protein)	
	N-Acetyl-β-D-glucosaminidase			NADPH-cytochrome *c* reductase			Alkaline phosphatase			Na^+-K^+-ATPase			Cytochrome *c* oxidase					
Fraction‡	C	G	C + G	C	G	C + G	C	G	C + G	C	G	C + G	C	G	C + G	Membranes§	G**	C + G**
S	0.05 ± 0	0.09 ± 0.01	0.11 ± 0.02	0.87 ± 0.29	0.45 ± 0.05	0.35 ± 0.03	0.15 ± 0.01	0.21 ± 0.04	0.20 ± 0.02	ND	ND	ND	ND	ND	ND	Cytosolic proteins	2.0 ± 0.10	5.2 ± 0.20
1	2.18 ± 0.36	3.07 ± 0.13	2.15 ± 0.08	3.98 ± 0.28	4.21 ± 0.58	6.68 ± 0.30	0.83 ± 0.07	0.71 ± 0.11	0.62 ± 0.08	1.24 ± 0.17	0.84 ± 0.11	2.81 ± 0.47	ND	ND	ND	"Light" lysosomes + Endoplasmic reticulum	18.0 ± 3.0	30.0 ± 2.4
2	0.53 ± 0.02	0.93 ± 0.05	0.38 ± 0.02	1.81 ± 0.14	2.46 ± 0.15	0.93 ± 0.15	3.43 ± 0.05	4.20 ± 0.37	4.10 ± 0.21	3.35 ± 0.41	3.25 ± 0.25	2.61 ± 0.48	0.23 ± 0.02	0.17 ± 0.01	NM	Plasma membranes	4.4 ± 0.3	6.2 ± 0.4
3	0.77 ± 0.04	1.28 ± 0.21	1.40 ± 0.08	1.36 ± 0.09	1.71 ± 0.08	0.96 ± 0.11	2.86 ± 0.54	3.99 ± 1.32	0.74 ± 0.19	0.99 ± 0.08	1.19 ± 0.22	0.69 ± 0.06	2.39 ± 0.20	1.89 ± 0.46	NM	Mitochondria + Plasma membranes	6.5 ± 0.4	4.6 ± 0.5
5	2.40 ± 0.21	2.21 ± 0.08	6.61 ± 0.22	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	"Heavy" lysosomes	12.7 ± 2.7	14.3 ± 4.8

* All values are means ± S.E. for N = 3–5. Abbreviations: ND = activity below accurately quantifiable limits, NM = not measured, C = control, G = *in vivo* gentamicin, and C + G = *in vitro* gentamicin.

† Relative specific activity is the ratio of enzyme activity in fraction divided by the enzyme activity in renal cortex homogenate prior to centrifugation.

‡ Location of fraction on gradient indicated by symbol in this column corresponds to indicated area in Fig. 1.

§ Predominant membranes present in fraction as determined by enzyme activity profile.

‖–** Overall significant differences within group by ANOVA: $P < 0.01$. In both G and C + G groups, levels of gentamicin were significantly greater in fraction 1 than in every other fraction.

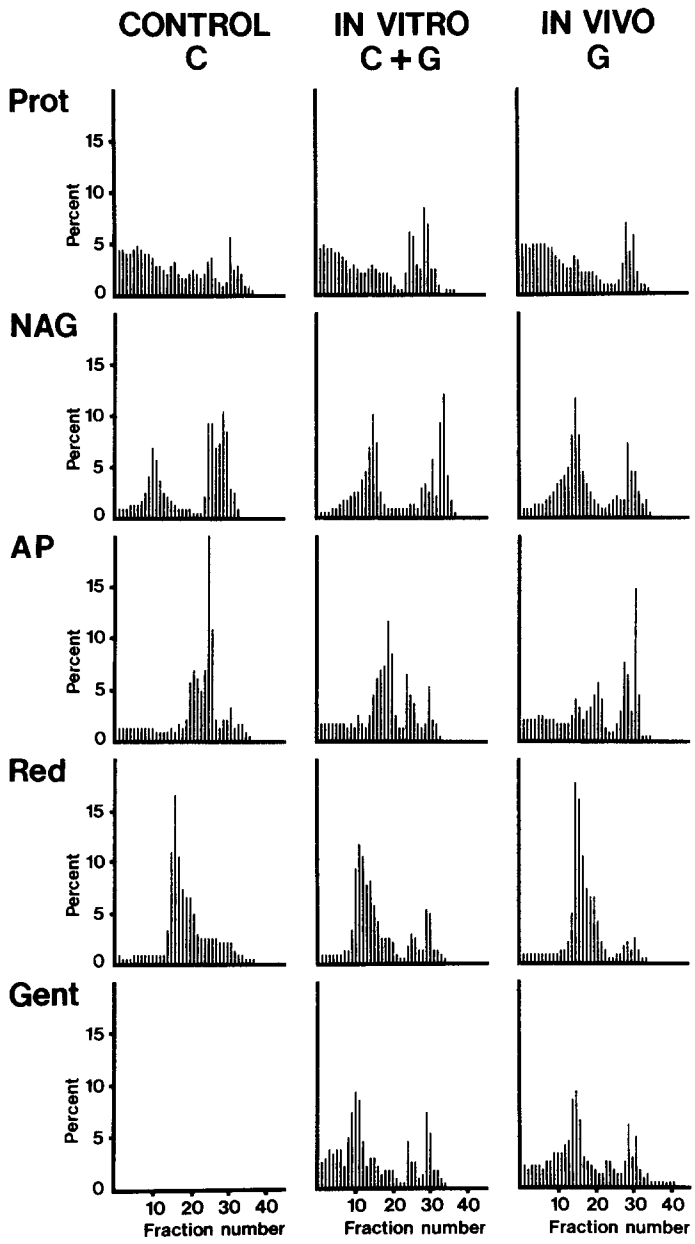

Fig. 2. Profiles of the distributions of protein, gentamicin and subcellular membrane associated enzymes on representative gradients from a control rat (C), a rat treated with gentamicin *in vivo* (G), and a control rat whose renal cortex was homogenized *in vitro* with gentamicin present (C + G). Each bar indicates a single 1-ml aliquot of the gradient with fraction number 1 corresponding to the top of the gradient (S in Fig. 1) and fraction 40 being the bottom of the gradient. For each aliquot, the percent of total activity of the measured parameter found in it is graphed. Abbreviations: protein (Prot), *N*-acetyl-β-D-glucosaminidase (NAG), alkaline phosphatase (AP), NADPH-cytochrome *c* reductase (Red), and gentamicin (Gent).

evident from Table 2 that, as in the first series of gradients, gentamicin preferentially migrates in fraction 1 which also contains the most NAG and NADPH-cytochrome *c* reductase activities. Additionally, as was seen in the first series of experiments, gentamicin behaved similarly in G (*in vivo*) and C + G (*in vitro*) groups.

The analysis summarized in Table 2, however, does not optimally quantitate the enzyme activity and gentamicin in the unsedimentable cytosolic protein fraction because particulate areas intermix variably with the lower several milliliters of this area of the gradient. To better assess the amount of unsedimentable activity without this confounding factor and, thereby, to gain some estimate of whether changes in fragility of subcellular organelles after *in vivo* or *in vitro* gentamicin substantially affected the results, the amounts of protein, enzyme activity, and

Table 2. Percent of total gradient content of protein, enzymes and gentamicin in individual fractions*

Fraction†	Protein			*N*-Acetyl-β-D-glucosaminidase			NADPH-cytochrome *c* reductase			Alkaline phosphatase			Cytochrome *c* oxidase			Gentamicin		Membranes¶
	C	G	C + G	C	G	C + G	C	G	C + G	C	G	C + G	C	G	C + G	G**	C + G**	
S	29.9 ± 3.6	43.4 ± 1.5	33.8 ± 2.6	6.2 ± 1.4	13.1 ± 0.8	8.4 ± 1.8	4.8 ± 0.3	9.2 ± 1.0	6.3 ± 0.4	9.1 ± 1.3	18.6 ± 1.7	11.0 ± 0.6	0	0	0	21.6 ± 1.1	24.9 ± 2.0	Cytosolic proteins
1	24.5 ± 2.1	19.4 ± 2.4	18.3 ± 0.8	39.6 ± 3.6	48.6 ± 1.0	30.7 ± 2.1	37.2 ± 2.8	56.6 ± 2.0	53.6 ± 4.9	10.2 ± 1.9	17.6 ± 2.5	16.0 ± 3.8	1.0 ± 0.3	17.6 ± 2.4	8.3 ± 2.9	38.7 ± 1.8	37.0 ± 2.0	"Light" lysosomes + Endoplasmic reticulum
2	9.2 ± 0.2	7.2 ± 4.1	9.4 ± 1.5	7.7 ± 1.0	8.4 ± 1.8	6.0 ± 1.2	33.5 ± 2.4	22.0 ± 3.0	16.8 ± 4.6	14.8 ± 2.3	15.8 ± 4.5	27.0 ± 7.4	4.0 ± 0.6	7.5 ± 1.4	13.5 ± 9.2	8.6 ± 0.9	9.5 ± 0.5	Endoplasmic reticulum + Plasma membranes
3	16.6 ± 0.9	16.8 ± 4.0	14.2 ± 1.5	10.0 ± 1.1	18.0 ± 3.4	15.0 ± 3.4	14.6 ± 1.2	6.8 ± 1.3	9.5 ± 0.9	54.1 ± 4.4	33.2 ± 9.0	30.1 ± 5.5	30.7 ± 6.2	28.8 ± 2.1	32.0 ± 2.5	17.1 ± 2.0	10.5 ± 0.7	Mitochondria + Plasma membranes
4	15.4 ± 0.9	12.9 ± 1.0	21.4 ± 0.7	14.5 ± 3.1	11.9 ± 0.7	28.3 ± 3.0	6.9 ± 0.4	4.6 ± 0.9	10.6 ± 1.2	9.7 ± 0.2	13.8 ± 2.7	14.0 ± 1.2	56.4 ± 4.4	45.1 ± 5.9	44.2 ± 9.9	11.0 ± 1.5	13.3 ± 1.2	Mitochondria
5	4.1 ± 0.1	0.6 ± 0.2	3.5 ± 1.4	21.7 ± 3.9	1.5 ± 0.6	11.5 ± 4.7	1.8 ± 0.1	0.4 ± 0.2	2.6 ± 1.0	1.9 ± 0.3	0.4 ± 0.1	1.6 ± 0.7	7.7 ± 2.9	0.4 ± 0.1	3.0 ± 1.4	3.8 ± 1.2	3.7 ± 1.9	"Heavy" lysosomes

* All values are mean percent ± S.E. C = control, N = 3; G = *in vivo* gentamicin, N = 4; C + G = *in vitro* gentamicin, N = 3.

† Location of fractions on gradient indicated by symbol in this column corresponds to area indicated in Fig. 1.

**–‖ Overall significant differences within group by ANOVA: $P < 0.01$. In both G and C + G groups, the percent of gentamicin found in fraction 1 was significantly greater than in every other fraction. G and C + G groups did not differ significantly from each other in the distribution of gentamicin.

¶ Predominant membranes present in fraction as determined by enzyme profile.

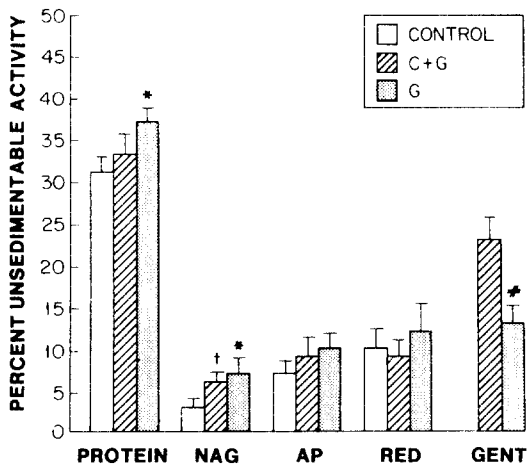

Fig. 3. Unsedimentable enzyme and gentamicin activities. Values given are the mean percentages ± S.E. of enzyme activity originally in the 600 *g* postnuclear supernatant fraction which remained in the supernatant layer of the gradient after centrifugation and which were, thus, unsedimentable. Details of how these values were calculated are given with the Results. G = *in vivo* gentamicin. C + G = *in vitro* gentamicin. Statistically significant ($P < 0.05$ or better) differences by paired *t*-test are indicated as follows: (*) G vs control; (#) G vs C + G; and (†) control vs C + G.

gentamicin in the first 5 ml of the gradient were multiplied by 2 in order to extrapolate to the 10 ml volume of 600 *g* supernatant fraction originally layered on each gradient so as to assess what fraction of each enzyme component of the original postnuclear (600 *g*) supernatant fraction was nonsedimentable. These data were available for both types of gradient studies done and were pooled. The results are summarized in Fig. 3.

The percent nonsedimentable activity found in the supernatant fraction was similar to that reported in other density gradient studies to the extent that comparable data are available [1, 2, 17, 18]. Group G had slightly but significantly more nonsedimentable protein than group C and groups G and C + G had more nonsedimentable NAG than controls, suggesting the occurrence of some increased organellar fragility with gentamicin, but the differences were small. Unsedimentable gentamicin was significantly higher in group C + G than in group G indicating that some constraints on redistribution of gentamicin after *in vivo* treatment, probably related to *in vivo* sequestration, were retained during the separatory procedures.

Table 3. Associations between gentamicin and membrane marker enzymes in gradient fraction 1*

	In vitro		*In vivo*	
Expt. No.	NAG	RED	NAG	RED
1	0.882	0.320	0.824	0.342
2	0.241	0.198	0.933	0.166
3	0.914	0.201	0.924	0.182
4			0.965	0.278
Mean	0.679	0.240	0.912	0.242
S.E.	0.179	0.033	0.026	0.035

* Correlation coefficients between levels of gentamicin and NAG and gentamicin and NADPH-cytochrome *c* reductase (RED) in the seven to nine 1-ml fractions comprising "Fraction 1" of the sucrose density gradient after *in vitro* and *in vivo* gentamicin.

The second group of gradient studies in which all fractions were analyzed (Table 2) provided the opportunity to correlate levels of gentamicin with levels of NAG and NADPH-cytochrome *c* reductase *within* the 7–9 fractions comprising region 1 of the gradient, the area of most prominent gentamicin localization, to ascertain with which enzyme the gentamicin was most closely associated. These data are summarized in Table 3. They indicate that, after *in vivo* gentamicin, an excellent correlation between gentamicin and NAG activity was uniformly present while gentamicin correlated poorly with NADPH-cytochrome *c* reductase activity. However, an identical pattern of gentamicin distribution was seen after *in vivo* gentamicin in two of three experiments.

DISCUSSION

The past several years have witnessed a substantial increase in the understanding of the cellular pathophysiology of aminoglycoside nephrotoxicity. Prominent among the advances in this area has been recognition of major lysosomal effects of aminoglycosides in renal proximal tubule cells. Multiple observations indicate the importance of lysosomes in the effects of aminoglycosides on renal tubular cells. Morphologic changes characterized by increases in lysosomal size and development of myeloid bodies have been well documented and, more recently, carefully quantitated in both animal models and humans [22–24]. Autoradiographic studies have provided evidence for lysosomal sequestration of labeled gentamicin [5–7]. Cell fractionation studies have demonstrated lysosomal localization of gentamicin after *in vivo* treatment with low doses in animal models [4] and across a wide dose range in cultured fibroblasts [1, 2]. Inhibitory effects of aminoglycosides on lysosomal phospholipases have been documented [25, 26] and increases in lysosomal phospholipid levels have been shown to contribute to the tissue phospholipidosis seen in renal cortex after aminoglycoside treatment [27]. Aminoglycoside-induced increases and decreases in stability of lysosomal membranes have been reported [15, 28, 29].

However, the available data on lysosomal effects of aminoglycosides do not yet provide a full explanation of the pathogenesis of aminoglycoside-induced renal tubule cell injury for several reasons: (1) the mechanisms by which lysosomal dysfunction results in acute lethal cell injury, despite much study in many models of injury, remain incompletely delineated and controversial [30, 31], (2) increases in size of lysosomes associated with increased uptake of slowly metabolized materials do not necessarily lead to either lysosomal dysfunction or to cell injury as illustrated by the benign nature of the so-called osmotic nephropathies resulting from administration of agents such as dextran [32], and (3) induction of a lysosomal phospholipidosis in the kidney as a result

of lysosomal sequestration of a cationic amphiphilic compound does not necessarily lead to acute lethal cell injury as illustrated by the effects of treatment with chlorphentermine. This cationic amphiphilic drug produces more widespread morphologic changes of lysosomal phospholipidosis in the kidney than do aminoglycosides but, to the extent that data are available, renal functional impairment is mild and acute lethal renal tubule cell injury is not prominent [33]. While certain properties of aminoglycosides may make them uniquely effective lysosomal toxins, substantial data are available to support the importance of cellular loci in addition to lysosomes as sites of aminoglycoside-induced renal tubular cell injury. Interactions of aminoglycosides with brush border membranes and mitochondria have been detailed with *in vitro* model systems [3]. Evidence for the *in vivo* occurrence of such interactions prior to the development of lethal cell injury has been reported [9, 10, 12]. Aminoglycoside inhibition of nonlysosomal phospholipases occurs [34, 35]. The phospholipidosis produced by aminoglycosides in the kidney is not necessarily limited to lysosomes [13]. A recent detailed reassessment of aminoglycoside uptake by radioautography has suggested the occurrence of an early cytoplasmic phase of aminoglycoside intracellular distribution [7].

To better understand the relationship between lysosomal and extralysosomal events in the cellular pathophysiology of aminoglycoside nephrotoxicity, it would be helpful to know more about the potential for gentamicin distribution intracellularly. The present study was designed to better ascertain the utility of subcellular fractionation procedures for obtaining this information under conditions of substantial but unequivocally, prelethal aminoglycoside-induced renal tubular cell injury. It has been stated that cellular fractionation studies after high or prolonged doses of aminoglycosides are fraught with difficulty [4], but no specific data have been reported to provide a basis for further investigation or improvement of methodology. The present study provides such information.

Prior subcellular fractionation studies of liver after treatment with cationic amphiphilic drugs which induced the formation of large numbers of myeloid bodies in hepatocytes have shown that these myeloid bodies can be isolated in enriched form, they have the enzymatic characteristics of lysosomes, they tend to equilibrate at lower gradient densities than do normal lysosomes, and they are the major intracellular sites of drug concentration [17–20]. Detailed studies of fibroblasts exposed to a range of concentrations of gentamicin and other aminoglycoside antibiotics have demonstrated similar dose-dependent effects of the aminoglycosides on lysosomes [1, 2].

One important consideration in cell fractionation studies after *in vivo* treatment is the possibility of redistribution of drug during the fractionation procedure. This is of particular importance for the aminoglycosides in view of their proclivity to bind to various subcellular membranes [11, 14–16]. Most of the available studies with the cationic amphiphilic drugs and aminoglycosides have not provided data on distribution of drug after *in vitro* exposure during subcellular fractionation. In the single study which carefully addressed this issue it was found that streptomycin exposure *in vitro* did not produce a preferential lysosomal drug distribution such as was seen after *in vivo* exposure to several aminoglycosides [1]. However, streptomycin is the least cationic of the commonly utilized aminoglycosides and, probably as a result of this, has the lowest *in vivo* uptake rate, the fewest documented subcellular effects in the kidney, and the least *in vivo* nephrotoxicity [3]. In contrast to the observations with streptomycin, appreciable *in vitro* binding of gentamicin to a light microsomal fraction from renal cortex has been described but enzymatic characterization of this fraction was not provided and concomitant studies of the distribution of gentamicin after *in vivo* administration were not reported [14].

The present study compared the distribution of gentamicin on a discontinuous sucrose density gradient 24 hr after four daily 100 mg/kg doses with the distribution of gentamicin added to the homogenizing solution used for normal renal cortex so as to produce levels similar to those found in the treated animals. The four dose protocol has been used extensively in our laboratory in a number of studies of *in vivo* gentamicin nephrotoxicity. It reproducibly results in widespread but uniformly prelethal proximal renal tubule morphological and functional changes [9, 10].

The gradient procedures utilized for the present studies were chosen, after evaluation of a variety of methods, to reproducibly separate subcellular organelles with the minimum number of steps, the least disruptive handling and without selectively losing and, thereby, failing to assess the contribution of any major subcellular component. As a result, individual subcellular elements are not nearly as purified as would result from procedures dedicated to isolating them uniquely.

The main findings of the present study were that: (a) In both control and *in vivo* and *in vitro* gentamicin preparations a major localization of the activity of the lysosomal enzyme NAG occurred in a very light membrane fraction also enriched in the endoplasmic reticulum enzyme NADPH-cytochrome *c* reductase. (b) After *in vivo* gentamicin treatment the distribution of gentamicin along the gradient also showed a major localization in the same light membrane fraction as NAG did and, within this gradient fraction, the distribution of gentamicin correlated very closely with the distribution of NAG. This observation, thus, is apparently consistent with previous reports on the behavior of lysosomes after treatment with cationic amphiphilic drugs or aminoglycosides in that lysosomes tended to migrate at lower densities and contained the drug [1, 2, 17–20]. (c) However, when gentamicin was added *in vitro* to the homogenizing solution used for renal cortices from control rats to levels approximating the gentamicin levels seen after *in vivo* treatment, the distribution of gentamicin on the gradient was very similar to that seen after *in vivo* treatment with respect to a major localization of gentamicin in the same light membrane fraction as NAG and a relatively close correlation of gentamicin with NAG within that fraction. In this regard, the results of

these studies of *in vitro* gentamicin addition are similar to those of the one previously reported study of exposure of subcellular membrane fragments [14] to gentamicin; however, the present study substantially extends that observation by enzymatically defining the nature of the light membrane fraction showing preferential gentamicin localization.

The data obtained in the present study are compatible with several interpretations:

(a) The similarity of gentamicin distribution after *in vivo* and *in vitro* exposure may be due to total disruption of lysosomes during homogenization of the renal cortex after *in vivo* gentamicin. Several observations argue against this as the only explanation for the findings: (1) the major features of enzyme distribution on the gradient in the present study were similar in control and gentamicin-treated rats, suggesting that substantial differences in organellar integrity were not produced by the *in vivo* gentamicin treatment as utilized. (2) The methodology for tissue homogenization used in this study was the same as that routinely described by other investigators [1, 2, 4, 17–20] and has, in our hands, produced highly functional mitochondria and highly enriched mitochondrial as well as heavy lysosome fractions by differential centrifugation. (3) Unsedimentable enzyme activity left in the supernatant layer after ultracentrifugation of the gradient (Fig. 3) was as low as has been reported in the literature [1, 2, 4, 17–20]. Furthermore, the protein content of the supernatant fraction was no higher than that in any of the other subcelluar fractionation studies referenced in this paper which provided evaluable data for this parameter. We did not measure "free" as opposed to latent enzyme activities in our gradient fractions. Although "free" enzyme activity is a valuable parameter for assessing lysosomal integrity, it may arise from unusually permeable as well as from severely damaged lysosomes and is, thus, a more equivocal measure of major lysosomal disruption than is unsedimentable activity. Furthermore, lysosomal membrane changes contributing to increases in "free" enzyme activity may occur *in vivo* during gentamicin treatment as well as during homogenization so that increased free levels do not necessarily mean that such changes are *in vitro* artifacts. (4) Unsedimentable gentamicin was significantly higher with *in vitro* addition than after *in vivo* treatment suggesting that gentamicin which was sequestered *in vivo* was not being totally released during subcellular fractionation.

(b) The high levels of gentamicin in the light membrane fraction in association with the lysosomal enzyme NAG may indicate that concentrative uptake of gentamicin by lysosomes can occur as a result of direct gentamicin lysosomal interactions without the requirement for adsorptive pinocytosis to the plasma membrane, the process which has been felt to account for most *in vivo* renal tubule cell uptake of gentamicin [3]. Such lysosomal sequestration of gentamicin upon direct exposure would be compatible with results of recent autoradiographic studies suggesting the occurrence of a cytoplasmic phase of gentamicin distribution acutely after renal tubule cell gentamicin uptake and prior to lysosomal sequestration *in vivo* [7].

(c) The similarity between the distribution of gentamicin on the sucrose gradients after *in vivo* and *in vitro* exposure may be due to a coincidence of two events. *In vivo* gentamicin may indeed be sequestered in light lysosomes while *in vitro* gentamicin becomes bound to membrane surfaces rich in acidic phospholipids. We have shown previously that the light membrane fraction which contained the most gentamicin after *in vivo* and *in vitro* exposure in the present study is particularly rich in one of the major phospholipid binding sites for aminoglycosides, phosphatidylinositol [13]. Binding to phosphatidylinositol does not, however, explain why, after *in vivo* exposure, the association of gentamicin with a lysosomal enzyme, NAG, is closer than with the other enriched membrane bound enzyme in that fraction, NADPH-cytochrome *c* reductase. Furthermore, fractions slightly heavier than the membrane fraction which is most enriched in gentamicin also contain relatively high acidic phospholipid levels [13] without showing similar degrees of enrichment of gentamicin.

The data in this study do not allow definitive evaluation of each of these hypotheses; however, with the phenomenon identified and reproducibly characterized it will be possible to design experiments to distinguish between the major mechanisms outlined. The available data do suggest that some caution must remain in attributing gentamicin renal tubule cell uptake exclusively to adsorptive pinocytosis followed by sequestration within lysosomes without opportunity to interact with intracellular structures. Though difficult and requiring careful interpretation, further studies of gentamicin distribution during prolonged and high dose models of the type that ultimately lead to significant renal functional impairment are likely to yield useful information for understanding the cellular pathophysiology of gentamicin-induced renal tubular cell injury.

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