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INHIBITORY EFFECTS OF KANAMYCIN ON GLYCOLYSIS IN COCHLEA AND KIDNEY—POSSIBLE INVOLVEMENT IN THE FORMATION OF OTO- AND NEPHROTOXICITIES

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Abstract—Effect of kanamycin (KM) on activities of Embden–Meyerhof (EMP) and hexose monophosphate (HMP) pathways was investigated in the cochlea, kidney, liver and brain of the guinea pig. In the organ of Corti, the activities of hexokinase (HK) and phosphofructokinase (PFK) were reduced significantly, whereas those of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were not altered following the administration of KM. On the other hand, the activities of these enzymes in a combined preparation of stria vascularis and spiral ligament remained unchanged. In the kidney the administration of KM also induced significant fall in the activities of HK and PFK without altering those of G6PDH and 6PGDH. In contrast, no alteration of activities of these four enzymes was found in the liver and brain. The conversion of [1-^{14}C]glucose to $^{14}CO_2$ was slightly higher than that of [6-^{14}C]glucose in both cochlea and kidney, yielding (C-1)/(C-6) ratios of 1.21 and 1.33, respectively. The administration of KM, exhibited a similar extent of inhibition on the oxidation of both [1-^{14}C] and [6-^{14}C]glucose, thus the (C-1)/(C-6) ratio remained essentially unchanged.

The distribution of KM following the intramuscular injection to the guinea pig revealed that the elimination of this drug from the perilymph and kidney is slow and these tissues maintain a high level of KM for the longer periods of time than that in the liver and brain.

Present results indicate that KM inhibits selectively the activity of the EMP pathway in the organ of Corti and kidney without altering that of the HMP pathway. Possible pharmacological implications of these findings in the formation of oto- and nephrotoxic effects of KM are briefly discussed.

It has been shown that kanamycin inhibits the respiration of bacteria [1] and of membranous cochlea [2–4]. In kanamycin-treated animals, inhibition of succinic dehydrogenase activity in the organ of Corti [5] as well as degeneration of mitochondria in kanamycin-damaged hair cells [6] was also reported. The above evidence indicates that inhibitory effects of kanamycin on tissue respiration and/or carbohydrate metabolism involve, at least in part, the inhibition of activities of TCA cycle. However, little is known whether or not the alteration in activities of the Embden–Meyerhof (EMP) and hexose monophosphate (HMP) pathways may involve in the occurrence of ototoxicity with kanamycin.

The present investigation is designed to evaluate the effect of kanamycin on the EMP and HMP pathways of cochlea, kidney, brain and liver. Studies of the metabolism via glycolysis were based on the determination of the activities of regulatory enzymes; hexokinase (HK) and phosphofructokinase (PFK) in EMP, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in HMP respectively, and on the measurement of the conversion of differentially labelled [^{14}C]glucose [C-1] and [C-6] to $^{14}CO_2$.

METHODS

In all experiments young healthy guinea pigs were used. Kanamycin sulfate (KM, 400 mg/kg/day) was given intramuscularly for 10 successive days and sacrificed the following day. Above schedules for KM administration were chosen because electrophysiological measurements indicated that significant changes in the cochlear microphonics due to the damages of cochlea occurred under these experimental conditions.

(*A*) *Measurement of activity of various enzymes*

(1) *Cochlea.* After decapitation, the temporal bone was immediately frozen with dry ice–ethanol (−78.5°) and freeze-dried for 2–3 days. After removal of the bony capsule, the membraneous cochlea was dissected under stereomicroscope and the organ of Corti and the lateral wall of the cochlear duct were obtained. Preparations of the organ of Corti included the outer and inner hair cells and supporting cells, while that of the lateral wall included the vascular stria, spiral ligament and spiral prominence. The dry weight of each sample was determined by means of the electric balance (minimal detectable range: 500 ng) and each sample was then transferred into the fluorometer tube containing 10 μl of 0.2% Lubrol in distilled water (chilled at 0° for 30 min) in order to solubilize the enzymes. For the estimation of the enzymatic activities of hexokinase (HK), phosphofructokinase (PFK), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), 500 μl of the complete reaction mixture responsible for each enzymatic assay (see Table 1) was added to each tube

Table 1. Assay conditions for the measurement of activities of selected enzyme in the cochlea

	Enzyme	Substrate (mM)	Coenzyme (mM)	Auxiliary enzyme (μg/ml)	Buffer	Other additions	Reagent volume μl	Incubation time (min)	Standard used
HMP	G6PDH	G6P 2	NADP 0.3	6PGDH 20	AMP_2-HCl 100 mM (pH 9.3)	BSA 0.05% EDTA 0.5 mM	510	40	$NADPH_2$
HMP	6PGDH	6PG 3	NADP 0.5	None	Tris–HCl 100 mM (pH 8.2)	BSA 0.05% EDTA 1 mM	510	30	$NADPH_2$
EMP	HK	Glucose 1	NADP 0.03 ATP 1	G6PDH 1.5	Tris–HCl 50 mM (pH 8.2)	BSA 0.02% $MgCl_2$ 5 mM Mercapto-ethanol 2 mM	510	40	$NADPH_2$
EMP	PFK	F6P 1	NADH 0.01 ATP 0.5	Aldolase 30 Triose-p-isomerase 10 α-GOPDH 5	Imidazol-HCl 50 mM (pH 7.0)	Potassium acetate 150 mM $MgCl_2$ 5 mM 5 AMP 0.1 mM Ammonium acetate 3 mM	510	60	$NADH_2$

Abbreviation used; AMP_2: 2-amino-2-methyl-1.3-propanediol, BSA: bovine serum albumin, α-GOPDH: α-glycerophosphate dehydrogenase.

and incubated at 25° for different lengths of time (see Table 1). For each assay, complete reaction mixture without the tissue samples was prepared and used as a tissue blank. After incubation, content of $NADPH_2$ or $NADH_2$ was measured by its native fluorescence. All these micro-methods have been adapted from the procedures described by Lowry *et al.* [7, 8] for the study of brain and retina and by Thalman *et al.* [9] for the study of the cochlea, respectively.

(2) *Kidney, brain and liver.* All tissues were homogenized with nine parts of ice cold isotonic KCl (0.15 M–KCl neutralized by $KHCO_3$, pH 7.0) in a glass homogenizer.

Hexokinase activity in the homogenate was determined spectrophotometrically according to the method of Galton *et al.* [10]. The reaction mixture contained 70 mM Tris–HCl buffer (pH 7.4), 10 mM glucose, 0.55 mM NADP, 7.4 mM $MgCl_2$, 3.7 mM ATP, 5 mM mercaptoethanol and 2.5 μg/ml of glucose-6-phosphate dehydrogenase. Phosphofructokinase activity in the homogenate was determined by the same method described as in the case of cochlea. For the estimation of the enzymatic activities of G6PDH and 6PGDH, the homogenate was centrifuged at 3000 g for 10 min at 4° and the supernatant was dialysed for 12 hr at 4° against the same buffer containing 0.6 mM EDTA. The dehydrogenase activities of the dialysate was determined fluorospectrophotometrically according to the method of Lowry *et al.* [11]. The reaction mixture consisted of 0.05 mM NADP, 1 mM EDTA and 0.05 mM Tris–HCl (pH 7.6 for G6PDH and 9.0 for 6PGDH, respectively). The reaction for all these enzyme assays was initiated by the addition of an appropriate amount of tissue samples. The protein concentration in each sample was measured by the method of Lowry *et al.* [12] and the results were expressed as specific activity per protein.

(*B*) *Measurement of kanamycin levels in blood, perilymph and various organs*

At 1, 3, 6, 12, 24 hr after the injection of kanamycin sulfate (400 mg/kg, i.m.), the chests of the animals were opened under ether anesthesia and blood was aspirated from the heart. After decapitation, the temporal bone, brain, liver and kidney were removed. In preparations of temporal bone, the tympanic bulla was opened and thus the bony cochlea was made accessible. For collecting perilymph, a thin glass capillary was introduced through the oval and round window into perilymphatic space under stereomicroscope. The contamination of aspirated perilymph by blood was examined under microscope and the contaminated samples were discarded. The brain, liver and kidney were homogenized and the homogenate and whole blood were diluted with 0.1 M phosphate buffer to the suitable antibiotic concentration. The agar diffusion method was applied to determine the concentration of kanamycin in the form of a cup-test for blood and tissue samples, and of a disk-test for perilymph. *Bacillus subtilis*, American Type Culture Collection (ATCC) 6633, was used as test bacterium.

(*C*) *Estimation of $^{14}CO_2$ production from [^{14}C]glucose*

(1) *Cochlea.* After decapitation, the temporal bone was immediately removed and the bullae were opened. The bony capsules of the cochlea were removed and transferred into the Krebs–Ringer Tris–HCl (pH 7.4) solution at 0°. Each membraneous cochlea was placed in a cell of an ultramicrorespirometer containing 0.3 ml of Krebs–Ringer 25 mM Tris–HCl (pH 7.4) solution containing 5 mM glucose. The [1-^{14}C]glucose or [6-^{14}C]glucose (1,050,000 cpm; sp act. 57 mCi/m-mole and 53.7 mCi/m-mole, respectively) was then added in the solution. Each cell was sealed by silicon rubber from which a folded filter paper, immersed in 25 μl of hydroxide of hya-

Table 2. Effect of kanamycin (KM) administration on selected enzymes in the cochlea

Enzyme	Organ of Corti		Lateral wall of the cochlear duct	
	Control	KM	Control	KM
Hexokinase	1.3 ± 0.1 (8)	0.9 ± 0.58 (8)*	0.85 ± 0.06 (15)	0.90 ± 0.05 (15)
Phosphofructokinase	3.3 ± 0.3 (4)	2.0 ± 0.2 (3)†	2.4 ± 0.3 (13)	1.9 ± 0.2 (18)
Glucose-6-P dehydrogenase	1.2 ± 0.1 (6)	1.2 ± 0.1 (5)	0.80 ± 0.04 (14)	0.72 ± 0.04 (14)
6-P-gluconate dehydrogenase	0.29 ± 0.04 (5)	0.27 ± 0.02 (6)	0.21 ± 0.02 (19)	0.18 ± 0.01 (22)

* $P < 0.02$; † $P < 0.05$, compared with each control value.
Activities are expressed as moles/kg dry wt/hr. Each value represents the mean ± S.E.M. obtained from the indicated number (in parenthesis) of samples.

mine for absorption of $^{14}CO_2$, was suspended. All cells were gassed from the side-arm with pure oxygen for 2 min and incubations were carried out for 30 min at 37.5°. After terminating the reaction by the injection of 50 μl of 4N perchloric acid through silicon rubber stopper, the cells were shaken for an additional 60 min to trap all the $^{14}CO_2$ evolved. The filter paper and 10 μl of incubation mixture from each cell were transferred into 20 ml of Bray's scintillation fluid mixture [13] and the radioactivity in these samples was determined in a Packard 3375 liquid scintillation spectrometer. In all experiments the blank cells without tissues were incubated and a minimal amount of $^{14}CO_2$ evaporated in the blank was subtracted from each experimental value.

(2) *Kidney*. After decapitation, kidney slices were made and approximately 100 mg wet wt of the slices were transferred to the conventional Warburg flask. Each flask contained 2.8 ml of Krebs–Ringer Tris–HCl (pH 7.4) solution containing 5 mM glucose. The [1-^{14}C]glucose or [6-^{14}C]glucose (550,000 cpm; sp act. 57 mCi/m-mole and 53.7 mCi/m-mole, respectively) was placed in the side arm. The centerwell contained a folded filter paper immersed in 0.2 ml of hydroxide of hyamine. The whole incubation procedure was carried out in the same manner as in the case of the membraneous cochlea, except the 15 min of preincubation was performed in this case prior to the addition of labelled substrate.

RESULTS

Effect of kanamycin on activities of enzymes related to EMP and HMP pathways in the cochlea. Table 2 shows the effect of kanamycin on activities of main enzymes in the Embden–Myerhof pathway (Hexokinase (HK) and phosphofructokinase (PFK)) and hexosemonophosphate pathway (glucose-6-phosphate (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH)) respectively. In the organ of Corti, the activities of HK and PFK were reduced significantly, while those of G6PDH and 6PGDH were not altered. On the other hand, these enzyme activities in a combined preparation of stria vascularis and spiral ligament (lateral wall of the cochlear duct) remained unchanged in kanamycin-treated animals.

Table 3. Effect of kanamycin (KM) administration on selected enzymes in kidney, liver and brain

Enzyme	Kidney		Liver		Brain	
	Control	KM	Control	KM	Control	KM
HK	56.6 ± 1.3 (3)	33.8 ± 1.3 (3)*	18.5 ± 0.1 (3)	18.3 ± 0.4 (3)	26.9 ± 1.3 (3)	26.7 ± 0.9 (3)
PFK	4.95 ± 0.27 (6)	3.23 ± 0.13 (6)*	4.19 ± 0.29 (5)	3.48 ± 0.38 (5)	6.10 ± 0.26 (5)	6.21 ± 0.42 (4)
G6PDH	27.8 ± 1.9 (7)	27.2 ± 1.0 (7)	4.64 ± 0.67 (4)	4.71 ± 0.96 (4)	9.12 ± 0.67 (5)	9.92 ± 0.69 (5)
6PGDH	28.2 ± 0.8 (7)	27.0 ± 0.5 (7)	9.78 ± 0.64 (4)	9.90 ± 0.24 (4)	17.3 ± 1.3 (4)	17.4 ± 0.6 (4)

* $P < 0.01$, compared with each control value.
Activities are expressed as moles/mg protein/min. Each value represents the mean ± S.E.M.obtained from the indicated number (in parenthesis) of samples.

Table 4. Effect of kanamycin (KM) administration on [1-^{14}C]glucose and [6-^{14}C]glucose oxidations in the preparations of cochlea and kidney

	Cochlea		Kidney	
	Control	KM	Control	KM
$^{14}CO_2$ from [1-^{14}C]Glucose	2124 ± 98 (5)	1622 ± 86 (4)*	8179 ± 640 (4)	5489 ± 867 (4)†
$^{14}CO_2$ from [6-^{14}C]Glucose	1660 ± 98 (4)	1313 ± 16 (3)†	6126 ± 309 (5)	4141 ± 539 (5)*
Ratio (C-1)/(C-6)	1.21	1.23	1.34	1.33

* $P < 0.02$; † $P < 0.05$, compared with each control value.
CO_2 formations are expressed as cpm/cochlea or dpm/mg protein of kidney. Each value represents the mean ± S.E.M. obtained from indicated number (in parenthesis) of samples.

Table 5. Kanamycin levels in the blood and perilymph

Hr after injection	0*	1	3	6	12	24
Blood (μg/ml)	0.54† ± 0.04 (6)	361.2 ± 28.8 (6)	83.8 ± 11.3 (6)	34.0 ± 20.3 (6)	2.2 ± 0.3 (6)	0.65 ± 0.10 (6)
Perilymph (μg/g)	nil‡ (12)	21.2 ± 5.4 (18)	28.3 ± 2.5 (22)	40.8 ± 4.2 (21)	40.0 ± 8.5 (24)	2.6 ± 0.6 (24)

* Indicate the values obtained from control animals.
† Represents non specific antibacterial activity of the blood, such as lysozyme activity.
‡ Below detection by the assay methods employed.
Kanamycin levels following the single injection of 400 mg/kg of kanamycin sulfate are presented. Each value represents the mean ± S.E.M. obtained from the indicated number (in parenthesis) of samples.

Effect of kanamycin on activities of enzymes related to EMP and HMP pathways in the kidney, liver and brain. In the kidney, the administration of kanamycin induced a significant fall in the activities of HK and PFK without altering those of G6PDH and 6PGDH (Table 3). In contrast, no alteration of activities of these four enzymes was found in the liver and brain.

Effect of kanamycin on $^{14}CO_2$ formation from [1-^{14}C] or [6-^{14}C]glucose. The effect of kanamycin administration on [1-^{14}C]glucose and [6-^{14}C]glucose oxidation in the cochlea and kidney was determined (Table 4). The conversion of [1-^{14}C]glucose to $^{14}CO_2$ was slightly higher than that of [6-^{14}C]glucose in both tissues, yielding (C-1)/(C-6) ratios of 1.21 and 1.33, respectively. These results indicate that the HMP pathway is operative slightly in these organs, because [6-^{14}C]glucose is oxidized only via EMP, while [1-^{14}C]glucose is considered to be oxidized via both EMP and HMP, and produces additional $^{14}CO_2$. The administration of kanamycin, however, exhibited a similar extent of inhibition on the oxidation of both [1-^{14}C]glucose and [6-^{14}C]glucose, thus the (C-1)/(C-6) ratio remained essentially unchanged. As previously shown (Table 2 and 3), kanamycin selectively inhibits enzyme activities involved in the EMP (HK and PFK) but those in the HMP (G6PDH and 6PGDH) are not affected. Considering these facts, it seems likely that observed inhibitory effects of kanamycin on the oxidation of [1-^{14}C]glucose do not mean direct inhibitory effects of this agent on the activity of HMP, but may be representative of the inhibition on enzyme activities related to but not involved in the HMP such as HK (an enzyme responsible for entering to the HMP pathway).

Distribution of kanamycin in the blood, perilymph, kidney, liver and brain. The distribution of kanamycin following the intramuscular injection to the guinea pig is presented in Tables 5 and 6. The peaks of kanamycin level in the blood, kidney, liver and brain appeared at 1 hr after the injection, whereas that in the perilymph was detected 6–12 hr after. These results suggest that permeation of kanamycin to the perilymph may be a rather slow process and may not be established by a simple equilibrium with that in the blood.

It is also noteworthy that the level of kanamycin in the kidney has been found to be the highest among the various organs tested and maintained at an enormously high level even 24 hr after the administration.

DISCUSSION

The distribution of kanamycin in the inner ear following the systemic administration revealed that this antibiotic penetrates slowly into the inner ear and the process of removal is also slower than that found in the blood. These results maintain essential agreements with previous reports by Voldrich [16] and Stupp *et al.* [17]. In the other organs tested, it was found that kanamycin is rapidly accumulated by the kidney to an enormously large extent and is eliminated slowly from this organ. In the kidney, more than one-third of the peak value was detected even 24 hr after administration. By contrast, the extent of distribution of kanamycin in the liver and brain was remarkably small compared with that in the kidney and the rate of elimination in the case of liver and brain was also extremely rapid. These facts suggest that one of the reasons for the well-known nephrotoxicity of this drug may be explainable by the large and prolonged accumulation in the kidney. In the present work it is shown that kanamycin inhibits selectively the activity of the EMP pathway in the organ of Corti and kidney without altering that of the HMP pathway therein. In addition such an inhibitory effect of kanamycin on the EMP pathway was not detected in the liver and brain. These facts suggest that the observed metabolic changes in the EMP pathway of the organ of Corti and kidney may be directly related, at least in part, to the occurrence of well-known ototoxic and nephrotoxic effects by this agent. Although it has been reported that in the liver a compensatory

Table 6. Kanamycin levels in the kidney, liver and brain

Hr after injection	0*	1	3	6	12	24
Kidney (μg/g)	0.89 ± 0.07† (6)	827.8 ± 47.6 (6)	695.9 ± 29.8 (6)	692.2 ± 147.3 (6)	516.0 ± 26.7 (6)	301.5 ± 0.2 (6)
Liver (μg/g)	0.41 ± 0.03† (6)	38.3 ± 5.2 (6)	14.7 ± 4.7 (6)	5.8 ± 0.7 (6)	6.1 ± 0.7 (6)	3.9 ± 1.0 (6)
Brain (μg/g)	0.34 ± 0.03† (6)	5.9 ± 0.6 (6)	1.9 ± 0.5 (6)	1.8 ± 0.7 (6)	0.73 ± 0.08 (6)	0.53 ± 0.23 (6)

* Indicate the values obtained from control animals.
† Represents non specific antibacterial activity of the blood, such as lysozyme activity.
Kanamycin levels following the single injection of 400 mg/kg of kanamycin sulfate are presented. Each value represents the mean ± S.E.M. obtained from the indicated number (in parenthesis) of samples.

activation of enzymes belonging to the HMP pathway induces various pathological conditions such as post-starvation [14] and hepatic damage [15], it is unlikely to cause this type of compensatory activation if the HMP is operative in the kanamycin-intoxicated kidney and inner ear. Since the metabolic and functional roles of the HMP in the cochlea and kidney have not been completely elucidated, the significance of this lack of compensatory activation in terms of occurrence of kanamycin toxicities is uncertain. However, it is possible that this lack of a compensatory activation in the HMP following the inhibition of the EMP pathway may be related to the occurrence of selective toxicity of kanamycin in these tissues. Although specific molecular mechanisms underlying differential susceptibility of enzymes in the HMP and EMP pathways to kanamycin remain to be elucidated, the fact that inhibitory effects of kanamycin on the EMP pathway in addition to well-known inhibitory effect on the activity of TCA cycle [5, 6] may be an important factor for inducing oto- and nephrotoxic effects of this drug should be emphasized.

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