

Vestibular histofluorescence could be due to accumulation of both the antibiotic and its derivative, streptidine, after acute streptomycin treatment in the guinea pig

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Summary. Acute treatment with 300 mg/kg of pigmented guinea pigs with streptomycin sulfate induces an elevation of endogenous fluorescence in vestibular ampullary cristae. Fluorescence accumulates in all compartments of the epithelium, i.e., vestibular sensory and supporting cells and nerve fibers of the stroma and it was very intense 1 and 12 hours after its administration. Fluorescence decreased to control levels 24 hours following streptomycin injection. Fluorescence levels were very low either in untreated animals or in animals injected with saline physiological solution. To investigate whether this fluorescence was an intrinsic property of the antibiotic or whether it was due to a derivative of it, or both, an *in vitro* fluorescence spectrum was performed with 100 μ M solutions of streptomycin or streptidine, or both, dissolved in various buffer solutions at 488 nm of excitation.

A discrete level of fluorescence was observed in the spectrum regardless of media when separate solutions of both streptomycin or streptidine were studied. Fluorescence notably increased at 522-532 nm when the solutions contained both streptomycin and streptidine together.

These results suggest that streptidine putatively derived from streptomycin may contribute to the observed fluorescence accumulation in vestibular preparations after acute treatment. Thus, these metabolic properties of the inner ear which transform streptomycin into streptidine, something never considered earlier, could be claimed as partially responsible for converting a therapeutic agent into a compound which could be as harmful as STP to the inner ear.

Key words: Histofluorescence, Aminoglycosides Rodents, Ototoxicity

Introduction

The preferential damage of inner ear structures by aminoglycoside antibiotics may be explained by the fact that after their accumulation they are eliminated more slowly from inner ear fluids than from the blood (Federspil et al., 1976; Tran Ba Huy et al., 1981)

Specific deterioration of vestibular function has been described in patients suffering from tuberculosis treated with streptomycin (STP) (Igarashi and Jensen, 1981) and numerous papers investigating the cellular target of STP and other structurally related aminoglycosides (AG) have been published (Duvall and Wersäll, 1964; Aran et al., 1982; Bareggi et al., 1986; Meza et al., 1989, 1996; Fermin et al., 1998). Autoradiographical studies after administration of radioactive dihydrostreptomycin (DHSTP) have shown aminoglycoside accumulation in the cytoplasm of sensory cells in the cochlea (Von Ilberg et al., 1971), whereas histochemical studies conducted after systemic administration of DHSTP have shown a non-specific pattern of distribution of dihydrostreptomycin within inner ear structures (Hawkins, 1981). Presently, what seems to be clear is that long-term administration of STP damages the sensory cells of the vestibular organs without any alteration of nerve fibers or auditory function (Meza et al., 1989, 1996). Whether this preference is established from the beginning of exposure to AG or whether these latter exert their action structurally complete or broken down to a derived metabolite is unclear.

Bareggi et al. (1986) reported that when a very large dose of STP (750 mg/kg) was intraperitoneally administered to guinea pigs, all inner ear structures, both in the cochlea and the vestibular cristae showed a

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remarkable level of fluorescence when examined 1 hour after injection, which suggests that STP has already arrived to the ear via the blood vessels and reached all the structures in the ear. Whether this fluorescence may be attributed to physical properties of STP or for how long the AG remained in the inner ear complete or modified is presently undetermined.

As our observations of specific sensory cell destruction by STP were in chronic experiments using 300-400 mg/kg of the antibiotic respectively in guinea pigs and rats, (Meza et al., 1989, 1996), we decided to perform acute experiments undertaking fluorescence observations of frozen sections of cristae ampullaris of guinea pigs 1 hour after intramuscular STP injection and extended the observations to 12 and 24 hours after STP administration. This extended experimentation was performed because it is known that STP accumulates in the inner ear perilymph and is eliminated from the body within 24 hours of its injection (Anderson and Jewell, 1945; Voldrich, 1965). Thus, the presence or absence of fluorescence could be an indication of the antibiotic permanence in or its clearance from the ear structures.

Additionally, as STP may give rise to streptidine (STD) by enzymatic hydrolysis (breakage of the $\alpha 1 \rightarrow 4$ glycosidic bond, Meza and Granados, 1999) (Fig. 1) in order to investigate whether this fluorescence is due to STP itself or to STD, that can accumulate and fluoresce, or both, we performed a series of *in vitro* experiments seeking for a fluorescence signal derived from STP or STD through analyzing by spectrofluorometry solutions of 100 mM of STP or STD or both dissolved in 0.85% NaCl, phosphate buffer, buffered cacodylate or paraformaldehyde solutions. These experiments were designed to imitate the salt milieu to which STP is exposed, during perfusion of the animal to prepare the specimens for fluorescence microscopy.

Materials and methods

Treatment

Twenty young male pigmented guinea pigs (*Cavia cobaya*) weighing around 100 g were used in this work. Fifteen animals received one intramuscular injection of commercial STP (Pisa Laboratories, México, 300 mg/kg) dissolved in commercial physiological saline solution (SPS), in the thigh of either hindleg. The remaining five animals were either untreated or received injections of SPS in the same conditions as the experimental ones.

Fixation and embedding procedures

One, 12 or 24 hrs after STP administration, animals in each time interval group (n=5/group) were deeply anesthetized with 1.5 g/kg chloral hydrate (Sigma) and transcardial perfusion was immediately started with isotonic saline solution (0.85% NaCl Sigma), followed by 4% paraformaldehyde (Polysciences Inc.) in 0.1M

sodium cacodylate buffer (Electron Microscopy Sciences) (SCB) pH 7.3 (fixative solution). After perfusion, the cranium was opened and auditory bullae were dissected and placed in cold fixative; bullae were opened under a dissecting microscope to expose vestibular organs which were postfixed in the same fixative as above for 2 hrs at 4 °C under slight agitation. Animals that were either injected with SPS and sacrificed 1 hour later or animals that were not injected at all served as controls and were subjected to the same procedure as those treated with STP.

Regardless of the experimental group, ampullary cristae extracted from the bulla of only one ear of each animal were immediately placed in SCB at 4 °C. Cristae were then washed 3 times for 10 minutes in the same fixative under the same conditions, and were transferred to cold (4 °C) 10% sucrose (Sigma) in which they remained for 3 hours; organs were then placed in 30% sucrose and left for 24 hrs at 4 °C. Ampullary cristae were embedded in tissue freezing medium, sectioned in 15 μ m slices and mounted on glass slides. Sections were then briefly immersed in water to remove the embedding medium and mounted in Permaflour Aquous Mountant.

Confocal microscopy

Preparations that were kept in the cold for no more than one week were observed for fluorescence. The scanning head of a Bio-Rad 1025 confocal microscope equipped with an Ar-Kr laser (Bio-Rad, UK) and coupled to a Nikon TMD 300 inverted microscope was used. Fluorescent light was collected with a Nikon 10x, 0.25 N.A. objective. The laser intensity of the confocal microscope was set to 30% and the iris opening was of 6.0 units providing lateral or axial resolution of about 2 μ m, respectively. The excitation wavelength was selected using an interference filter centered at the 488 nm line of the Ar-Kr laser. Emitted fluorescence was filtered at 522/32 nm. Image acquisition was performed

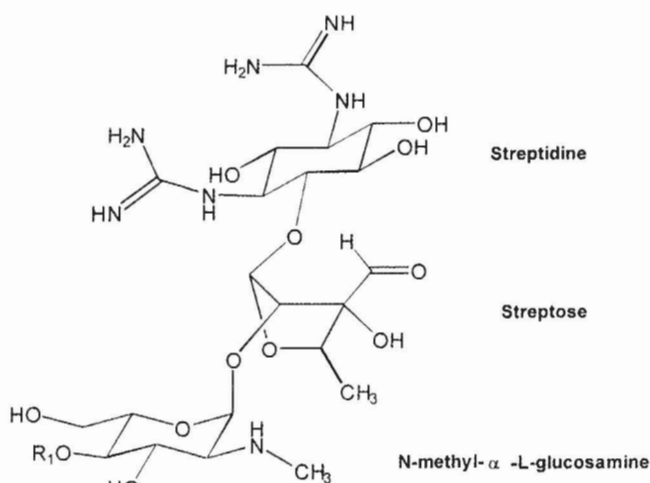


Fig 1. Chemical structure of streptomycin showing its components.

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using the MLP 1024 (Bio-Rad); captured images (Kalman averaged, $n=9$) were converted to TIFF format using Confocal assisted 4.02 software by Todd Clark Brelje, 1994. Fluorescence intensity determination of each image was conducted as follows: a small ($15\ \mu\text{m}$) circular area of interest was defined and mean fluorescence intensity values were measured by moving the circle over the sensory epithelia, or nerve fibers of the vestibular cristae. In addition, two measurements away from the vestibular cristae were performed to determine the background fluorescence level (Image-Pro Plus; media Cybernetics). This value was subtracted from measurement of tissue. Statistical analysis of specific fluorescence between different groups (ANOVA) was conducted using StatView 4.5 (Abacus Concepts Inc.) and plot bars in Figure 3 were drawn with Origin 3.78 (Microcal software). Final editing and lettering of the images was performed using Paintshop Pro 5.01 (Jasc Software).

Fluorescence spectrum determination of STP, STD or STP-STD solutions

Individual experiments with solution concentration of $100\ \mu\text{M}$ STP or STD or both, in 0.85% NaCl pH 7.0; 0.1M sodium cacodylate buffer (SCB) pH 7.4; 0.1 M sodium phosphate (SPB) buffer, pH 7.4 or 4% paraformaldehyde (PAF) in SCB, pH 7.4 were scanned from 520 to 560 nm emission under 488 nm of excitation in a Spee Fluoromax Spectrofluorometer either at $22\ ^\circ\text{C}$ or at $37\ ^\circ\text{C}$. The basal line value for each buffer was automatically subtracted from measurements.

Elemental analysis

Purity of STP (Pisa Laboratories, México) was determined by elemental analysis of C, N, H, S and O in an EA 1108 Elemental Analyzer (Fisan Instruments) using sulfanilamide as standard of calibration. STD that was prepared from STP as already described (Barba-Bahrens et al., 1990) was also checked for purity by the same elemental analysis methodology.

Results

Elemental analysis of the purity of STP or STD

Percentage of elements (C, H, N, S) constituting these compounds showed that they were essentially pure, with hydrating content of 6 water molecules for STP and 1 H_2O for STD (Table 1). These results are of paramount importance, since observations and conclusions drawn from experiments either in vivo or in vitro could be entirely attributed to properties of STP or STD and not to any impurity that these compounds may carry with them.

Vestibular cristae fluorescence after STP injection

All ampullary cristae of animals injected with STP

and observed under a confocal Laser Microscope (Fig. 2 A-D) showed some degree of fluorescence. However, its intensity varied with time: 1 hour after injection, a high level of fluorescence was present in all structures, sensory and supporting cells and fibers of the stroma (Fig. 2B), and decreased significantly within 12 hours after injection (Fig. 2C). In vestibular cristae derived from animals 24 hours after STP injection, intensity of fluorescence notably decreased in all structures (Fig. 2D) to reach the control level (Fig. 2A). Calculations of fluorescence intensity in sensory epithelium and nerve fibers as expressed in arbitrary units is presented in Fig. 3A,B. It can be observed that fluorescence accumulated at a higher level in the sensory epithelium (Fig. 3A) than in nerve fibers of the stroma at all the time intervals observed and it had the same disappearance rate in both compartments.

STP and STD fluorescence in the various buffer solutions

To determine whether the observed fluorescence in tissue sections could be emitted by accumulated STP or STD as its derivative, or both present in vestibular structures, a fluorescence spectrum of $100\ \mu\text{M}$ solutions of STP, STD or STP-STD, dissolved in 0.85 NaCl, SPB, GASCB or SCB was obtained in a spectrofluorometer. Both compounds fluoresce regardless of the media but fluorescence signal of STD was higher than that of STP, at equimolar concentrations. Notwithstanding, when fluorescence was determined in a mixed STP-STD solution, its level was additive except for the phosphate buffer solution, in which case the fluorescence decreased

Table 1. Elemental Analysis of STP and STD Sulfate Salts

	PERCENT FOUND				H_2O Molecules
	C	H	N	S	
STP	32.71	6.30	12.57	6.29	6
STD	26.0	6.05	22.00	9.37	1

Percents were calculated considering minimal formulae:

STP: $(\text{C}_{21}\text{H}_{39}\text{N}_7\text{O}_{12})_2 (\text{H}_2\text{SO}_4)_3$

STD: $\text{C}_8\text{H}_{18}\text{N}_6\text{O}_4 \cdot \text{H}_2\text{SO}_4 \cdot \text{H}_2\text{O}$

Table 2. Level of streptomycin* - or streptidine* - derived fluorescence** in various salt solutions***.

	PHOSPHATES	NaCl	CACODYLATES	p-FORMALDEHYDE +CACODYLATES
STD	1319±21	192±17	236±22	333±29
STP	217±18	50±27	206±20	251±22
STP+STD	628±24	260±25	410±27	531±38

*: figures are mean values of c.p.s units for the fluorescence emitted between 522-532 nm±standard errors after excitation at 488 nm, of three independent experiments; **: concentration of either STP, STD or STP-STD was of $100\ \mu\text{M}$; ***: composition as described in Material and Methods.

(Table 2). These observations suggest that both chemical substances may contribute to the fluorescence observed in tissue sections.

Discussion

This paper presents evidence that histofluorescence in guinea pig ampullary cristae structures observed after acute treatment with STP is not only due to the accumulation of this antibiotic but also to the presence of a metabolite derived from it, which according to recent experiments from our group, could well be STD. (Meza and Granados, 1999). This statement is originated from the observation that fluorometric scanning of solutions of STP or STD in various buffers imitating the salt milieu to which this antibiotic is exposed in fixed inner ear structures resulted in fluorescence emitted at 522-532 nm increase when both compounds were together (Table 2); STD fluorescence signal in phosphate buffer, however, was higher than the mixture STD-STP probably due to a quenching effect. The wavelength of

emission of this *in vitro* signal is the same at which 15 μm cryostat sections of ampullary cristae fluoresce after STP administration. These observations are in line with and extend the reported histofluorescence produced 1 hour after 750 mg intraperitoneal injection of this antibiotic in inner ear structures of the guinea pig (Bareggi et al., 1986), which ascribed the observed fluorescence solely to accumulation of STP itself in inner ear structures. However, our interpretation is in disagreement with that of this group as some other possibilities may occur and are discussed below.

Histofluorescence is not due to any impurity of STP or STD

One interpretation of these results could be that observed fluorescence is not due to STP or STD but to an impurity present in either compound which accumulates in inner ear structures. This possibility was overruled when elemental analysis of STP or STD sulfate used showed that these compounds are

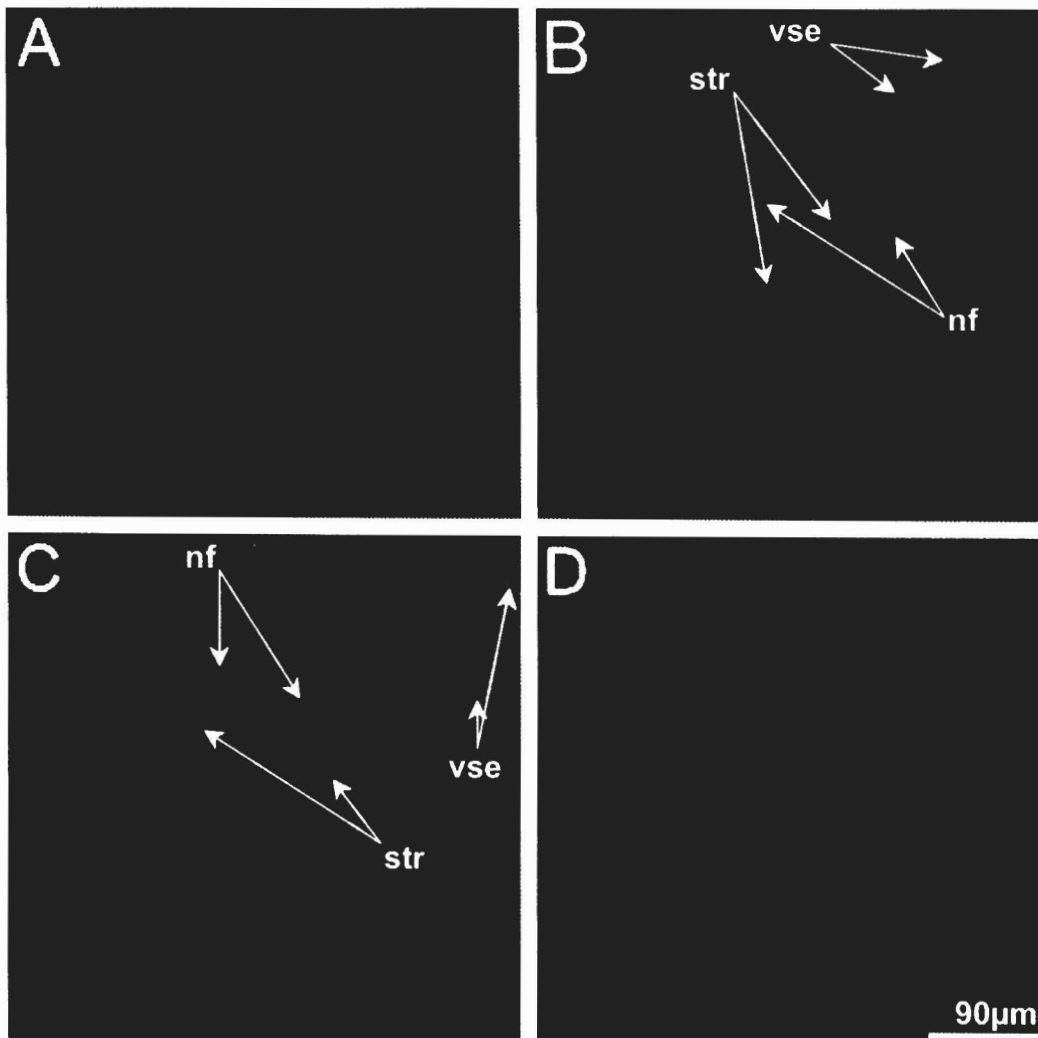


Fig. 2. Confocal microscopy images of 15 μm ampullary cristae sections of control (untreated) guinea pigs (**A**) or 300 mg/kg STP dissolved in saline physiological solution (SPS)-injected animals, one hour (**B**), 12 hours (**C**) or 24 hours (**D**) after injection. Fluorescence is uniformly distributed in the sensory epithelium (vse) and nerve fibers of the stroma (str) in both 1 hour- and 12 hour-treated animals (**A-C**) whereas 24 hours after injection, the vestibular cristae presented as low a fluorescence level as the control (**D**).

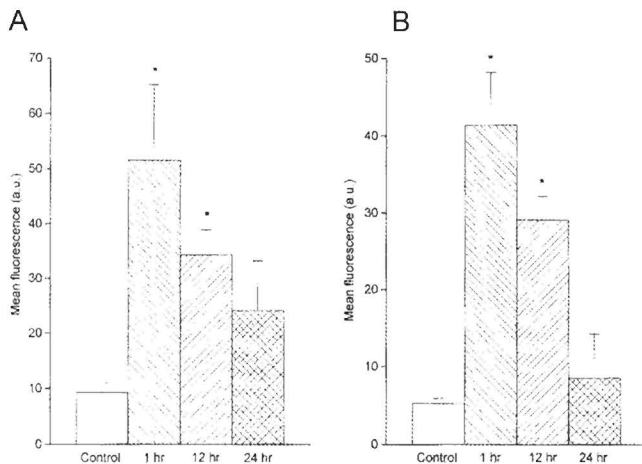


Fig 3. Fluorescence measurement in sensory epithelium (**A**) and nerve fibers of the stroma (**B**), as explained in Methods. Each column is the mean of 4 experiments with S.E.M. indicated by the bar. Significant differences ($p < 0.0001$) between control and STP-treated were as follows: In A control vs 1 hr; control vs 12 hr; 1 hr vs 12 hrs; 12 hr vs 24 hrs; 1 hr vs 24 hr; values of control vs 24 hr were not significant. In B: control vs 1 hr; control vs 12 hr; 1 hr vs 12 hr; 12 hr vs 24 hr; values of control vs 24 hr were not significant. When fluorescence in sensory epithelium and nerve fibers were compared, the levels of fluorescence were significantly lower in nerve fibers than in sensory epithelium at all times observed.

essentially pure.

Histofluorescence is not induced by stress

Another possibility could be that stress produced by the injection and handling of animals might be responsible for the accumulation of a fluorescent compound structurally unrelated to STP, in the inner ear. This phenomenon is not likely to occur since both Bareggi et al. and our group found a very low fluorescent level when observations were made in inner ear structures of untreated guinea pigs (Bareggi et al., 1986, results not shown in their paper, only described) or our own results in either untreated or injected animals with a volume of SPS equivalent to that used as the vehicle for the STP treatment and handled equally as the STP-treated ones (Fig. 2A).

Histofluorescence is due not only to STP but additionally to a fluorescent metabolite derived from it (streptidine) which may contribute to vestibulotoxicity or be the actual toxic agent

The rise of histofluorescence level observed in vestibullary structures over a period of one hour after injection of STP in doses equivalent to those used for medication in humans, and its decrease 12 hours after treatment partially agrees with reports (Voldrich, 1965; Anderson and Jewell, 1945) showing that STP specifically accumulates in the perilymph of the inner ear, 4 hours being the time of highest concentration of

the antibiotic, in contrast with our observations of high fluorescence intensity one hour after treatment, but it is in line with the same reports describing 24 hours the time of clearance. However, the level of fluorescence was significantly lower in nerve fibers than in the vestibular epithelium at all times observed. These results may explain the specificity observed with light microscopy in guinea pigs, showing some alterations of sensory cells of ampullary cristae and not of nerve fibers even at very short time periods after administration of the antibiotic (Meza and Iturbe, 1986). As both STP and its derivative STD may contribute to "in vitro" fluorescence, it follows that maybe after parenteral administration, STD derived from STP, by enzymatic hidrolisis (Meza and Granados, 1999) is accumulating in vestibular cristae structures together with STP and contributes to its toxicity. The disappearance of fluorescence after 24 hours of administration may mean that both compounds have been transported inside either the sensory cell or another compartment, whose identity is yet to be discovered.

Acknowledgements. We thank Dr. Anatoli Iatsimirski for invaluable advice in fluorescence spectroscopy methods, and Dr. Robert Fox (Manheim, PA, USA) for his careful review and correction of the manuscript. The technical expertise of Mr. Federico García Jandete, Miss Concepción José and Ms. Tatiana Fiordeliso in histology, dissection and statistics, respectively, is gratefully acknowledged, as is the expert secretarial assistance of Mrs Myriam Morales. Grant E 27876 from CONACyT (México) to G.M. partially financed this investigation.

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Accepted July 9, 2001