Bismuth ions are metabolized into autometallographic traceable bismuth-sulphur quantum dots

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Bismuth – sulphur quantum dots can be silver enhanced by autometallography (AMG). In the present study, autometallographic silver enhanced bismuth-sulphur nanocrystals were isolated from unfixed cryo-sections of kidneys and livers of rats exposed to bismuth (Bi^{207}) subnitrate. After being subjected to AMG all the organic material was removed by sonication and enzymatic digestion and the silver enhanced Bi-S quantum dots spun down by an ultracentrifuge and analyzed by scintillation.
The analysis showed that the autometallographic technique traces approximately 94% of the total bismuth. This implies that the injected bismuth is ultimately captured in bismuth-sulphur quantum dots, i.e., that Bi-S nanocrystals are the end product of bismuth metabolism

Key words: heavy metals, autometallography (AMG), quantum dots, nanocrystals, Bi-207.

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For more than a century the sparsely soluble bismuth salts have been used as a remedy for maladies as different as gastrointestinal disorders, syphilis, and hypertension. Bismuth subnitrate is still used for medical treatment of gastric and duodenal ulcers, alone or together with an antibiotica, and as an additive to oral cytotoxica. Bismuth is further, due to its radio-opacity, added to various catheters, bone implants, and surgical instruments in order to make them detectable by X-rays and computed tomography, CT (for a review see Stoltenberg, 2004). Recently, use of polymer-coated Bi_2S_3 nanoparticles as a CT imaging agent has been suggested to be superior to the commonly used CT contrast agents based on iodinated molecules (Rabin *et al.,* 2006).

A quantum dot (QD) is a nanocrystal just a few nanometers in diameter, thus the QDs hold intriguing potentials for future biological studies (Bruchez *et al.,* 1998; Warburton *et al.,* 2000; Banin and Millo, 2003; Michalet *et al.,* 2005). Such nano-sized imaging probes include among others semiconductor quantum dots, magnetic and magnetofluorescent particles, colloidal gold particles, and bismuth sulphur nanoparticles.

Metallic gold and silver nanoparticles, bismuth selenium/sulphur and the commercially available semiconductor quantum dots can all be silver enhanced by autometallography (Danscher, 1981, 2002; Danscher *et al.,* 2000; Danscher and Stoltenberg, 2006; Stoltenberg *et al.,* 2007). The autometallographic technique works by reducing silver ions adhering to the surface of the catalytic quantum dots to metallic silver atoms. The reduction is caused by electrons released from reducing molecules likewise adhering to the quantum dot. After a period of time the quantum dot is silver enhanced to dimensions that can be seen respectively in the electron microscope or the light microscope (Danscher and Stoltenberg, 2006; Stoltenberg *et al.,* 2007).

The aim of the present study was to determine whether bismuth ions, injected into rats as bismuth subnitrate, ends up as bismuth-sulphur quantum dots within one week.

Materials and Methods

Chemicals

Bismuth subnitrate (BiSN, Merck 1878). Radioisotope Bi-207, chemical form $Bi(NO_3)_3$ dissolved in 4 M HNO_3 (Oak Ridge National Laboratory, Oak Ridge, TN 37831-6426).

Animals

Four male Wistar rats, weighing 400 g (Møllegaard Breeding Center Ltd., Ejby, Denmark) were used. They were given one intraperitoneal injection of 54.3 kBq Bi^{207} and 500 mg/kg bismuth subnitrate dissolved in distilled water. Temgesic was used as an analgeticum prior to injection.

The animals were housed in plastic cages under the following conditions: 12 h light/dark cycle, 22±2°C, and 50±10% relative humidity. Food (Altromin No. 1314, Altromin Spezialfutterwerke, Germany) and tap water were given ad libitum. The study was performed in accordance with the Danish guidelines for animal welfare.

The rats were allowed to live for 7 days; hereafter the animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight) and decapitated. The liver and kidneys were excised and frozen with gaseous CO_2.

Tissue handling for radioisotope activity quantification

Samples from each animal were taken from the left kidney and right lobe of the liver. Two hundred, 30 µm thick, cryo-sections were cut and placed in vials; the autometallographic developer was poured onto them. After 60 minutes of development the developer was replaced with a 5% thiosulphate solution for another 10 minutes (Danscher *et al.*, 2000). Then the sections were rinsed three times with distilled water before being homogenized and exposed to proteinase K for digestion of the organic material.

After 2 hours at 60°C the solution was poured into a centrifuge tube and placed in a centrifuge (Beckman L8-M) for 10 minutes at 1.000 rpm. The pellet and the supernatant were then analyzed in a gamma counter.

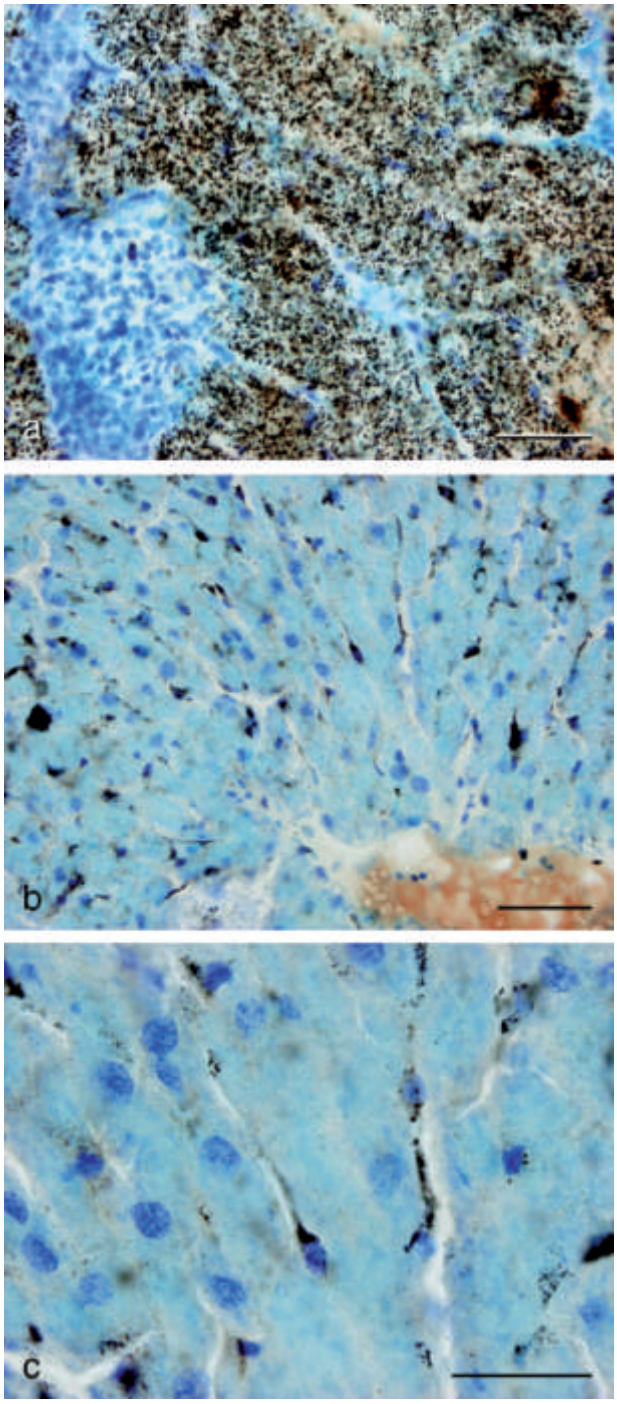

Figure 1. Photograph of a 30 µm thick cryo-section of rat kidney and liver. Autometallographic developed for 60 min and counterstained with toluidine blue. a) The intense staining of the proximal tubules contrasts the complete renal corpuscle void of autometallographic silver enhanced Bi-S quantum dots. Bar = 50 µm. b, c) Silver enhanced Bi-S nanocrystals is primarily seen in Kuppfer cells. Bars = 50 µm (b) and 30 µm (c).

The radioisotope activity in the samples was counted in a well-crystal scintillation detector (Cobra II, Packard, USA) to a statistical accuracy of 5%.

Tissue handling and autometallographic staining of bismuth in morphologically intact tissue

Tissue that were not used for radioisotope activity quantification (vide supra) were processed for light and electron microscopy according to the previously established protocols (Danscher *et al.,* 2000; Danscher and Stoltenberg, 2006). In short: 30 µm thick, cryo-sections were cut and placed on 10% Farmer-rinsed glass slides. The newly prepared 26°C warm AMG developer (Danscher and Stoltenberg, 2006) is poured onto likewise heated glass jars containing slides. The jars are then placed in a water bath at 26°C, and the whole set-up placed in a light-tight cover, for 60 minutes. The development is finalized by replacing the AMG developer with a 5% sodium thiosulphate solution for 10 min. Finally the slides can be counterstained, e.g. with toluidin blue. For electron microscopy tissue block were embedded in Epon, and semithin (3 µm) plastic sections cut and autometallographic developed. A blank Epon block is glued by way of unpolymerized Epon on top of the section to be ultrastructurally analyzed. After polymerization, the block is removed by heating the glass slide to 90°C. The block is trimmed and ultrathin sections cut and counterstained with uranyl and lead.

Results

Bismuth tracing using AMG

Autometallographic developed liver and kidney sections from the exposed animals revealed an intense staining pattern. In the kidney the proximal convoluted tubules contained the most intensely stained cells while the renal corpuscles were completely void of autometallographic silver enhanced Bi-S quantum dots (Figure 1a). In the liver, silver enhanced Bi-S quantum dots were primarily seen in Kuppfer cells (Figure 1b, c). At the ultrastructural level, the bismuth-sulphur nanocrystals were found to be localized in lysosomes / residual bodies of the hepatic macrophages and the epithelial cells lining the renal tubules (Figure 2).

Amount of bismuth that has been metabolized into Bi-S quantum dots

The pellets of silver enhanced bismuth-sulphur nanoparticles from the kidney were found to contain 92.2% (SD 4.5%) of the total amount of Bi-207. The pellets from the liver contained 95.9% (SD 0.8%). The supernatant from both liver and kidney contained less than 2% of the total Bi-207 in the probe (Table 1).

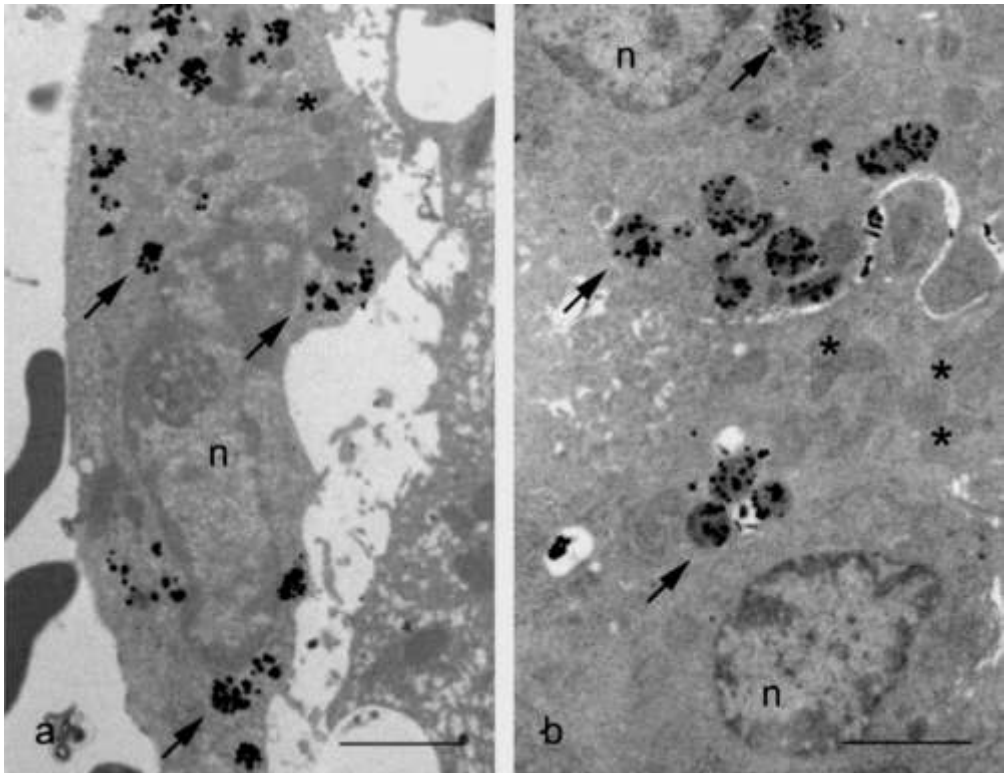

Figure 2. Electron micrograph of a bismuth loaded Kuppfer cell in the liver (a) and bismuth loaded cells lining the renal tubules (b). The autometallographic traced bismuth sulphur nanocrystals are located in lysosomes (arrows pointing at some examples); n = nucleus. Profiles of mitochondria (marked with *) is void of bismuth. The poor quality of the morphology is due to the use of unfixed tissue. Bars = 2 µm.

Table 1.

	Pellet	*Supernatant*	*AMG developer*	*Thiosulphate*
Kidney 1	0.870	0.022	0.058	0.050
Kidney 2	0.900	0.011	0.055	0.034
Kidney 3	0.960	0.031	0.007	0.002
Kidney 4	0.960	0.008	0.020	0.012
Mean	0.922	0.018	0.035	0.024
SD	0.045	0.010	0.026	0.021
	Pellet	*Supernatant*	*AMG developer*	*Thiosulphate*
Liver 1	0.958	0.000	0.030	0.012
Liver 2	0.960	0.005	0.028	0.007
Liver 3	0.968	0.019	0.012	0.000
Liver 4	0.949	0.010	0.033	0.008
Mean	0.959	0.008	0.026	0.007
SD	0.008	0.008	0.009	0.005

Table showing the gamma counts in kidney and liver as fraction of the total counts (background corrected).

By comparing the total gamma count in the sections before being subjected to AMG and enzymatic digestion with the total of the probes it was found that the autometallographic (developer and thiosulfate) procedure caused a loss of approximately 5% (5.9% and 3.3%; kidney and liver respectively).

Discussion

The finding that approximately 94% of the injected bismuth is metabolized into Bi-S quantum dots within a week suggests that the mammalian body has a rather efficient way to neutralize bismuth ions. Rats exposed to mercury chloride reveal an AMG pattern in kidney that is almost identical to the bismuth AMG pattern (Danscher and Moller-Madsen, 1985; Stoltenberg and Danscher, 2000; Danscher *et al.,* 2000), but the speed by which mercury is bound in Hg-S quantum dots seems to be much slower, as only 30% of the injected Hg-203 was captured in Hg-S quantum dots (Norgaard *et al.,* 1994).

It seems though that binding of bismuth and mercury ions in quantum dots is the way of neutralizing/detoxificating these rather dangerous metals. The quantum dot process seems to take place in lysosomes and most of these inert particles rest in the residual bodies in the cells. In the central nervous system such accumulations of bismuth or mercury quantum dots can remain for years (Danscher and Moller-Madsen, 1985; Schionning, 2000; Stoltenberg, 2004). The present data support the notion that mercury is more toxic to the mammals and fish than bismuth (Slikkerveer and de Wolff, 1989; Ross *et al.,* 1994, 1996; Schionning *et al.,* 1998; Schionning, 2000; Sorensen *et al.,* 2000; Clarkson *et al.,* 2003; Grandjean *et al.,* 2003; Pedersen *et al.,* 2003; Stoltenberg *et al.,* 2003a, b, c; Counter and Buchanan, 2004; Debes *et al.,* 2006). The distribution of the two metals in the organism and the places where they are ultrastructurally accumulated are not totally identical, but still very much alike (Schionning, 2000; Stoltenberg, 2004; Danscher and Stoltenberg, 2006).

The present study also reveals that autometallography is a very efficient technique for tracing bismuth in exposed organisms. Although not 100% of the injected bismuth ions could be traced by the technique after one week the results suggest that all bismuth eventually will end up in Bi-S quantum dots. The benefit of the technique is that it enables light and electron microscopically tracing of the exact location of the toxic metal, making AMG a tool for a better understanding of the toxicological and pharmacodynamic properties of this more and more widely used heavy metal.

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