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# STUDIES ON GENTAMICIN-INDUCED LABILIZATION OF RAT KIDNEY LYSOSOMES *IN VITRO*

## POSSIBLE PROTECTION BY SELENIUM

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**Abstract**—The labilizing effect of gentamicin, an aminoglycoside antibiotic, on isolated rat kidney lysosomes was investigated. The light-scattering behavior of lysosomal suspensions and the release of lysosomal acid hydrolase enzymes (acid phosphatase, β-glucuronidase and muramidase) from incubated lysosomal suspensions, in the presence of gentamicin, were used as indices of lysosomal membrane labilization. Gentamicin was found to cause a decrease in light absorbance and a release of lysosomal acid hydrolases, which indicate lysosomal membrane swelling. In the presence of selenium, in the form of potassium selenate, the decrease in light absorbance of lysosomal suspensions and the release of lysosomal acid hydrolases from isolated lysosome particles were reduced markedly. This suggests that selenium protects against gentamicin-induced lysosomal membrane labilization. The possible mechanisms of protection by selenium are discussed.

Gentamicin, an aminoglycoside antibiotic, has been reported to be nephrotoxic to laboratory animals and man and to accumulate particularly in the renal lysosomes of proximal tubular cells in the rat 24 hr after the administration of the drug [1] and to form myeloid bodies in humans [2]. Lysosomes play an important role in pathogenesis in various tissue and cellular processes. These cellular organelles contain numerous acid hydrolases which, when released into the extracellular environment, are capable of digesting macromolecules of many categories in the whole organism [3–6].

Selenium has been shown to interact with various toxic chemical agents to prevent damage to tissue and cellular systems. For example, laboratory animals intoxicated with necrogenic substances have usually developed various forms of lesions that are characterized by progressive damage to plasma, and other cell membranes [7] and which result in changes in some blood and urine enzymes associated with specific cellular regions [8, 9]. The administration of selenium, either parenterally or as a dietary supplement, under such toxic conditions has been shown to reduce or even prevent such changes [7, 10], this suggests that selenium may stabilize the various membranes.

The present investigation was carried out to determine whether selenium protects against gentamicin-induced lysosomal damage in the isolated rat kidney lysosomes. The variables studied included: (a) light absorbance behavior of isolated lysosomes in the presence of (1) gentamicin alone and (2) both gentamicin and selenium, and (b) the release of lysosomal acid hydrolases in the presence or absence of these chemical agents.

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### MATERIALS AND METHODS

*Micrococcus lysodeikticus* (freeze-dried), NADH and 4-nitrophenyl β-D-glucuronide were purchased from the Sigma Chemical Co., London, England, and 4-nitrophenylorthophosphate was obtained from British Drug Houses, Poole, England. All other reagents used were of analytical grade and were prepared in double-distilled water.

Male Albino rats were obtained from the breeding stock at the Faculty of Health Sciences, University of Ife.

*Preparation of lysosomes.* Six male rats were used. The animals were killed by decapitation and their kidneys were quickly removed, decapsulated, weighed and placed in ice-cold 0.25 M sucrose. Lysosomes were prepared in the cold room (0–4°), as described earlier [11], and modified using an MSE high speed refrigerated centrifuge, purity was checked by enzyme assays and fluorescence microscopy [11]. The tissue was cut finely with clean pre-cooled scissors, and portions were homogenized in 0.25 M sucrose using a TRI-R STIR-S model K43 homogenizer. This initial suspension was diluted to a final concentration of 10% (w/v) with 0.25 M sucrose, and an aliquot was placed in a clean pre-cooled tube for enzyme assay and protein measurement. The remaining portion was then centrifuged at 3000 *g* for 15 min in the cold room to sediment nuclei and cell debris; the resulting supernatant fraction was centrifuged at 15,000 *g* for 20 min to sediment a large granule fraction containing lysosomes. The lysosomal pellets were then resuspended by directing a stream of sucrose solution from a pipette onto them. This method yielded a suspension of undamaged lysosomes as revealed by electron microscopy. The lysosomal pellets were then finally suspended in 0.25 M sucrose containing 0.05 M Tris–

HCl buffer (pH 7.4). A sucrose–buffer solution (2 ml) was added to every gram of kidney used. This final suspension was used for subsequent determinations.

The purity of the lysosomal suspensions was further checked visually by fluorescence microscopy with acridine orange. By this method, the lysosomes appear bright orange, and the mitochondria appear green. Microscopy showed the preparation to be satisfactory, and this was confirmed by the enzyme assays.

*Enzyme and protein measurements.* Aliquots of the crude kidney homogenate with or without Triton X-100 (0.1% final concentration) were used for enzyme and protein determinations. Acid phosphatase, β-glucuronidase, muramidase and proteins in the lysosomal pellets were measured in the presence of Triton X-100 (0.1%). An acid phosphatase assay was carried out as previously described [12]; muramidase activity was determined following the method of Litwack [13]; and β-glucuronidase was estimated as described by Furuya [14]. Aryl sulfatase, glutamate dehydrogenase and glucose-6-phosphatase activities were determined as previously described [15]; D-amino acid oxidase activity was assayed by the method of Robinson *et al.* [16]; and alkaline phosphatase activity was estimated as described earlier [17]. Protein was determined by the biuret method [18]. The determinations were made at conditions found to be optimum for the present studies. A Pye Unicam SP 1800 double beam spectrophotometer was used for all the measurements.

*Stability of kidney lysosomes.* The method adopted was the same as described earlier [11, 19]. Briefly, suspensions of lysosomes in 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4), with or without gentamicin or selenium, were incubated at 25°. At appropriate time intervals, the available lysosomal acid hydrolase activities were determined. The soluble enzyme was determined by assaying the enzyme activity of the supernatant solution obtained after centrifuging the incubated lysosomal preparation for 10 min at 15,000 $g_{av}$.

*Light absorbance measurements.* The stability of the lysosomes was also determined by following changes in the light-scattering behavior of lysosomal particles suspended in 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) [11, 20, 21]. The initial extinction value was designated 100% absorbance, and any changes in extinction at 520 nm ($E_{520}$) [11] were expressed as a percentage of this value. Two concentrations of gentamicin, made up in sterile physiological saline (3.0 and 5.3 mM), and selenium in the form of potassium selenate, made up in sterile physiological saline (0.3 mM), were added to the test lysosomal suspensions at the start of the experiment at 25° in the cuvette of a Pye Unicam SP 1800 double beam spectrophotometer with a chart recorder attached.

## RESULTS

The efficiency of the lysosomal preparation adopted in these studies was tested by determining the extent of contamination of the lysosomal suspensions with some known non-lysosomal enzymes and comparing the extent in kidney homogenates with that in isolated lysosomal pellets (Table 1). Table 1 also shows the ratios of lysosomal to homogenate enzyme activities. The enrichment factors depended largely on which enzyme was measured and on the homogenization procedure adopted, as well as the many variables and possible contributions by cellular organelles such as the golgi components. The specific activities of the acid hydrolases found in lysosomes (Table 1) were much higher than the specific activities of those found in the homogenate fractions, and the ratios of lysosomal to homogenate enzyme activities showed the preparation to be reasonably pure. In an ideally pure lysosomal preparation, other lysosomal associated enzymes such as the cathepsins, β-galactosidases, acid ribonucleases, phospholipase A, β-*N*-acetyl-glucosaminidases and acid lipases would be expected to show a similar distribution pattern. The non-lysosomal enzymes measured in this investigation were detected in minute amounts in the isolated lysosomal fractions. Their presence may be related to some degree of contamination during the centrifugation procedure.

The light absorbance of isolated lysosomal suspensions was not altered markedly by the various concentrations of selenium (Fig. 1). When selenium or gentamicin and the three lysosomal enzymes studied were incubated for up to 30 min at 25°, no inhibitory effect was observed.

Table 1. Purity of "marker" enzymes in the lysosomal preparation and the kidney homogenates, and ratios of lysosomal to homogenate enzyme activities

| Enzyme | Specific activities | | |
|---|---|---|---|
| | Homogenate | Lysosome | (ratios) |
| Acid phosphatase | 32.1 ± 7.2* | 447.7 ± 22.3* | 13.9 |
| β-Glucoronidase | 33.7 ± 3.54 | 136.7 ± 13.4 | 4.0 |
| Aryl sulfatase | 1.9 ± 0.6 | 40.1 ± 5.0 | 21.1 |
| Muramidase | 7.1 ± 1.5 | 115.1 ± 12.8 | 16.2 |
| Glutamate dehydrogenase | 27.4 ± 4.9 | 3.7 ± 0.8 | 0.14 |
| D-Amino acid oxidase | 36.3 ± 6.6 | 0.9 ± 0.3 | 0.02 |
| Glucose-6-phosphatase | 126.3 ± 9.3 | 0.2 ± 0.01 | 0.002 |
| Alkaline phosphatase | 187.6 ± 13.5 | 6.2 ± 2.2 | 0.03 |

* Results are expressed as specific activities in nmoles per min per mg protein ± S.E.M. of six rats.

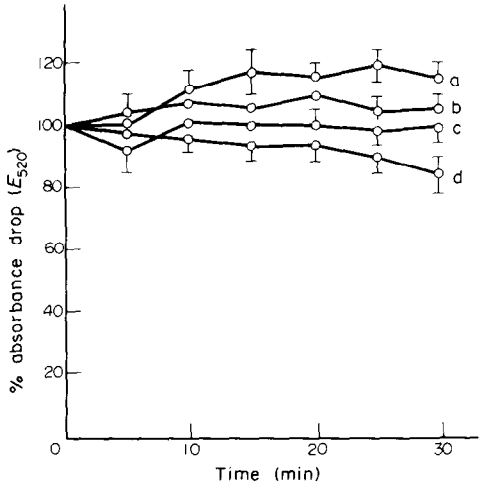


Fig. 1. Effects of various concentrations of selenium on light absorbance by lysosomal suspensions. Aliquots of lysosomal suspensions in 0.25 M sucrose containing 0.05 M Tris–HCl buffer (pH 7.4) were incubated for various periods of time. The initial extinction value was designated 100% absorbance, and any changes in extinction at 520 nm were expressed as a percentage of this value ± S.E.M. Six rats were used. Key: (a) lysosomes plus selenium (0.3 mM), (b) lysosomes plus selenium (0.18 mM), (c) lysosomes plus selenium (0.12 mM), and (d) lysosomes alone.

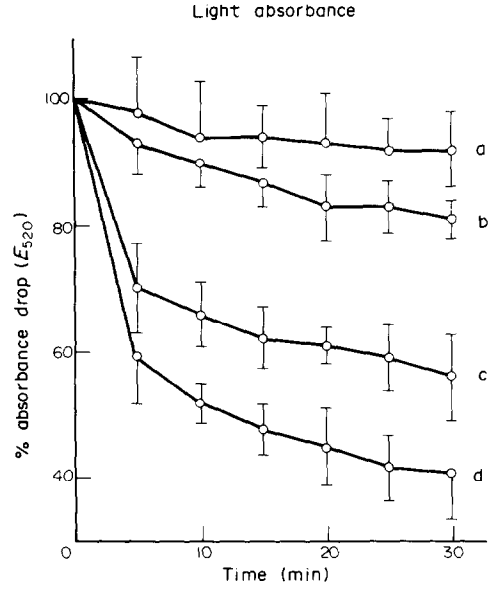


Fig. 2. Light absorbance of lysosomal suspensions in the presence of gentamicin (3.0 and 5.3 mM) with or without selenium (0.3 mM). Aliquots of lysosomal suspensions in 0.25 M sucrose containing 0.05 M Tris–HCl buffer (pH 7.4) were incubated with gentamicin in the presence or absence of selenium. The initial extinction value was designated 100% absorbance, and any changes in extinction at 520 nM were expressed as a percentage of this value ± S.E.M. Six rats were used. Keys: (a) lysosomes alone, (b) lysosomes plus gentamicin (3.0 mM) plus selenium (0.3 mM), (c) lysosomes plus gentamicin (3.0 mM), and (d) lysosomes plus gentamicin (5.3 mM).

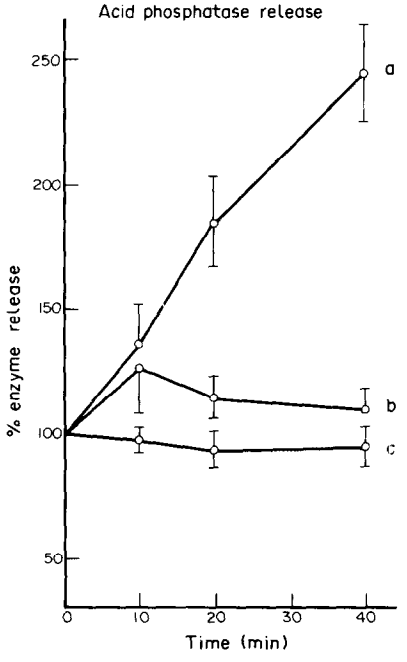


Fig. 3. Lysosomal acid phosphatase release in the presence of gentamicin with or without selenium. Aliquots of lysosomal suspensions in 0.25 M sucrose contaioning 0.05 M Tris–HCl buffer (pH 7.4) were incubated with gentamicin in the presence or absence of selenium at 25°. At appropriate time intervals, the available lysosomal acid phosphatase activity was measured. The soluble enzyme was determined by assaying the enzyme activity of the supernatant solution obtained after centrifuging the incubated lysosomal preparation for 10 min at 15,000 $g_{av}$. Results are expressed as percent enzyme released with time ± S.E.M. Six rats were used. Key: (a) lysosomes plus gentamicin (3.0 mM), (b) lysosomes plus gentamicin (3.0 mM) plus selenium (0.3 mM), and (c) lysosomes alone.

Figure 2 shows the light absorbance of isolated lysosomal particles in the presence of two concentrations of gentamicin. The higher concentration of this antibiotic compound (5.3 mM) resulted in an absorbance drop of nearly 60% compared to the control, after 30 min of incubation. The light absorbance behavior of lysosomal particles in the presence of gentamicin (3.0 mM) and selenium (0.3mM) is also shown in Fig. 2. The light absorbance decrease was greatly minimized in the presence of selenium compared to the level when gentamicin alone was employed.

During enzyme release studies, 3.0 mM gentamicin was used. This concentration gave a more consistent and reproducible response using the conditions adopted in this investigation. The release of acid hydrolase enzymes from isolated lysosomal particles was reduced markedly when the lysosomes were incubated in the medium containing gentamicin and selenium. In the medium containing gentamicin alone, acid phosphatase release was linear up to 148% of control level after 40 min of incubation (Fig. 3), but the release was greatly reduced (*ca.* 20%) in the presence of selenium. β-Glucuronidase release was fairly linear in the incubation medium containing

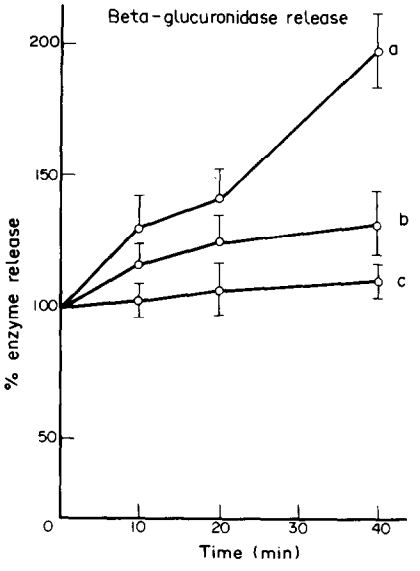


Fig. 4. Lysosomal β-glucuronidase release in the presence of gentamicin with or without selenium. Aliquots of lysosomal suspensions in 0.25 M sucrose containing 0.05 M Tris–HCl buffer (pH 7.4) were incubated with gentamicin in the presence or absence of selenium at 25°. At appropriate time intervals, the available lysosomal β-glucuronidase activity was measured. The soluble enzyme was determined by assaying the enzyme activity of the supernatant solution obtained after centrifuging the incubated lysosomal preparation for 10 min at 15,000 $g_{av}$. results are expressed as percent enzyme released with time ± S.E.M. Six rats were used. Key: (a) lysosomes plus gentamicin (3.0 mM), (b) lysosomes plus gentamicin (3.0 mM) plus selenium (0.3 mM), and (c) lysosomes alone.

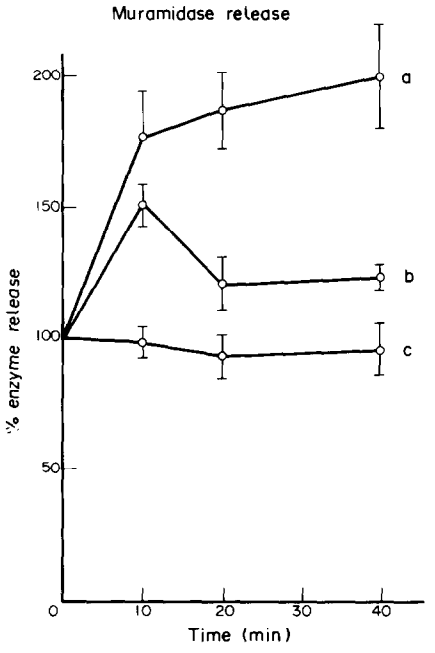


Fig. 5. Lysosomal muramidase release in the presence of gentamicin with or without selenium. Aliquots of lysosomal suspensions in 0.25 M sucrose containing 0.05 M Tris–HCl buffer (pH 7.4) were incubated with gentamicin in the presence or absence of selenium at 25°. At appropriate time intervals, the available lysosomal muramidase activity was measured. The soluble enzyme was determined by assaying the enzyme activity of the supernatant solution obtained after centrifuging the incubated lysosomal preparation for 10 min at 15,000 $g_{av}$. Results are expressed as percent enzyme released with time ± S.E.M. Six rats were used. Key: (a) lysosomes plus gentamicin (3.0 mM), (b) lysosomes plus gentamicin (3.0 mM) plus selenium (0.3 mM), and (c) lysosomes alone.

the drug alone, but it was greatly reduced (*ca.* 45%) in the presence of selenium (Fig. 4).

Figure 5 illustrates the release of lysosomal muramidase enzyme activity in the presence and absence of selenium. Within 10 min of incubating the lysosomal suspensions with gentamicin alone, an appreciable percentage of this enzyme activity had been released into the incubation medium. Up to 180% of muramidase activity was released after 40 min of incubation compared to the controls. In the presence of selenium, gentamicin did not appear to result in any appreciable amount of enzyme release. After an initial increase in muramidase activity during the first 10 min of incubation, probably reflecting the time lag during the interaction of selenium and gentamicin molecules, the enzyme release dropped to a much lower level for the rest of the incubation period.

## DISCUSSION

The biochemical and morphological purity of the lysosome preparations adopted in the present studies has been exhaustively investigated and agrees well with that published by other workers [11, 15, 22]. The degree of purity has further been shown by some correlation between light-scattering changes in a lysosomal suspension and the release of lysosomal acid hydrolases (Table 1 and Figs. 3–5). The calculated partial correlation coefficient between light absorbance decrease and the release of lysosomal acid phosphatase at a fixed time interval was 0.81.

The data presented in these studies demonstrate that gentamicin labilized rat kidney lysosomal membranes *in vitro*. The two concentrations of the drug used in the light-scattering experiments resulted in an absorbance drop of the lysosomal suspensions over a time period (Fig. 2), indicating some degree of lysosomal membrane swelling. The incubation of lysosomes with gentamicin (3.0 mM) for a period of up to 40 min resulted in a marked release of lysosomal acid hydrolases into the incubation medium (Figs. 3–5). These observations indicate that this antibiotic compound labilized the rat kidney lysosomal membranes, thus confirming and extending the earlier reports of a gentamicin-induced lysosomal membrane alteration and instability shown to occur much earlier than the alteration of the functional properties of other renal membrane systems [23, 24], and the induction of a phospholipidosis observed in the rat renal cortical cell membranes following the administration of this antibiotic [25]. Gentamicin and other aminoglycosides have been reported previously to bind to the phospholipid receptor sites of the various cell membrane systems [26].

The data presented show that selenium (0.3 mM), in the form of potassium selenate, reduced, to a considerable extent, the labilizing effect of gentamicin. Protection by selenium was maximal at 0.3 mM. Lower concentrations did not show any consistent protective effect, and concentrations much higher than the one quoted tended to mask the response of lysosomal pellets with time. Various concentrations of selenium, ranging from 0.12 to 0.3 mM (Fig. 1), did not appear to affect the light absorbance behavior of isolated lysosomal suspensions to any appreciable extent. Selenium was also found to have no inhibitory effects on any of the lysosomal enzymes studied. Incubating lysosomal suspensions with gentamicin in the presence of selenium resulted in a significant reduction in the light absorbance drop (Fig. 2). Lysosomal acid hydrolase release was reduced considerably when lysosomes were incubated with gentamicin in the presence of selenium (Figs. 3–5). The release of lysosomal acid phosphatase (Fig. 3) correlated very well with time (correlation coefficient = 0.98 and significant at $P < 0.05$). Indeed, acid phosphatase usually shows a remarkably higher proportion in the insoluble membrane fraction compared to any other lysosomal enzyme after lysosomal membrane disruption [27].

The role of selenium in the stabilization of lysosomal membranes against thermal stress has been reported [7]. Selenium, acting as an antioxidant, has also been shown to reduce adverse oxidative reactions that most membrane phospholipid molecules undergo in cases of biochemical damage [28]. Lysosome leakage is known to lead to various forms of inflammatory disturbances resulting from acid hydrolase release. Acid hydrolases are mediators of inflammatory processes. Lysosome membrane stabilizers such as cephaloridine [20], vitamin E [7] or acetylsalicylic acid [29] are known to reduce damage caused by released acid hydrolases.

Although some previous workers have employed some reduced forms of selenium, such as the selenium analogs of sulfhydryl compounds [10, 30] or the selenite ions [7], to demonstrate the protective properties of selenium, the present investigation utilized selenate ions ($SeO_4^{2-}$), as the oxidized forms of selenium. It is possible and energetically feasible that the selenate ions can react with the free amino groups present in the molecules of gentamicin. This results in a reduced form of selenium ($SeO_3^{2-}$).

$$3SeO_4^{2-} + RNH_3^+ + 2OH^- \rightarrow 3SeO_3^{2-} + NO_2^- + ROH + H_2O$$

Oxidized selenium ions are known to undergo a ready conversion to the reduced forms under appropriate conditions [31].

From the results of the present studies, some propositions are possible to explain the observed protection by selenium against gentamicin-induced lysosomal membrane labilization. Selenium, acting as an antioxidant, may protect and stabilize the phospholipid components of the lysosomal membrane against any possible oxidative reactions that may occur from their interactions with gentamicin molecules. It is possible that selenium may also protect the protein components of the lysosomal membranes, since selenium binds to proteins in a manner that protects them from oxidative inactivation [30]. Selenium may also complex with gentamicin molecules, thus rendering these drug molecules ineffective in their lysosomal membrane labilizing actions.

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