Intracellular Accumulation of a Fluorescent Derivative of Paromomycin in Human Fibroblasts

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Human fetal lung fibroblasts grown in the presence of dansyl-paromomycin (DNS-Pm), a fluorescent derivative of the aminoglycoside antibiotic, paromomycin, probably accumulate DNS-Pm in the lysosomes. The intracellular concentration of DNS-Pm is proportional to the extracellular concentration and to the length of time cells are exposed to the compound. The accumulation of DNS-Pm by human fibroblasts continued to increase for several days, reaching a saturation after 7 days. The kinetic data are consistent with the establishment of a steady state in the cell between fluid-phase pinocytosis and exocytosis of DNS-Pm. About 80% of the intracellular DNS-Pm was released in 24 hr when fresh medium without the analogue was added. The residual 20% remained within the cells, suggesting that it may be irreversibly bound to the lysosomes, endoplasmic reticulum, or ribosomes. The uptake of paromomycin by cells in culture may be a useful means to study error propagation during growth and lifespan of cells in vitro.

Paromomycin (Pm) is an aminoglycoside antibiotic that causes phenotypic suppression of a variety of nonsense mutations in yeast [1–3]. Pm also stimulates misreading in cell-free protein synthesizing systems derived from various eukaryotic cells, such as yeast, wheat embryo, tetrahymena, and human fibroblasts [1–6]. This drug may therefore be of value as an error-promoting agent in mammalian cells and provides a possible means of testing error theories of cellular ageing [7–9].

However, it is unclear whether this antibiotic can enter normal mammalian cells, as it has been reported that there were no effects on growth, protein synthesis, and intracellular bacteria when mouse 3T3 and 3T6 cells in culture were treated with high concentrations of various aminoglycoside antibiotics [10,11]. On the other hand, Tulkens and Trouet [12], using a microbiological assay to measure antibiotic activity, have described the kinetics of uptake and intracellular localization of streptomycin, gentamycin, kanamycin, and amikacin in rat fibroblasts in culture.

In this report, the techniques of fluorescent microscopy, metrizamide gradient subcellular fractionation, and fluorimetry were used to demonstrate the presence and intracellular location of dansyl-paromomycin (DNS-Pm), a fluorescent derivative of the aminoglycoside antibiotic, paromomycin (Pm), in human diploid fibroblasts. We also report on the synthesis and properties of DNS-Pm and comment upon possible mechanisms of uptake and release of this compound by human fibroblasts.

MATERIALS AND METHODS

5-dimethylaminonaphthalene-1-sulphonyl chloride (dansylchloride or DNS-Cl) was obtained from Sigma (Poole, Dorset, UK); paromomycin sulphate was a gift from Parke Davis & Co. (Pontypool, Gwent, UK); cation-exchange resin AGI-x8 was from Bio-Rad (Richmond, California); while all other chemicals were analytical grade and were supplied by BDH (Poole, Dorset, UK).

Preparation of Dansylated Paromomycin

We dissolved 3.7 mmol of DNS-Cl in the minimum amount of acetone; 1.4 mmol of paromomycin sulphate in 0.2% NaHCO$_3$; and the solution brought to pH 7.5 with bicarbonate. The DNS-Cl solution was slowly added to the Pm solution and the mixture was incubated for 18 hr at 37°C with continuous agitation.

The reaction mixture was then extracted with amyl acetate to free the aqueous solution of unreacted DNS-Cl. After extraction, the aqueous layer was then treated with acetone and the precipitate that formed was removed by centrifugation. The aqueous acetone solution was passed through a cation-exchange resin column (Bio-Rad AGI-x8, 6 × 1.5 cm) to remove dansylsulphonic acid. Fractions (5 ml) were collected and the fluorescent ones combined and freeze-dried. The yield of purified DNS-Pm was 4%. The presence and purity of DNS-Pm was monitored by thin-layer chromatography (TLC) using silica gel G and a mixture of chloroform, methanol, and 35% ammonia (1:4:3 v/v/v). R$_f$ values from Pm and DNS-Pm were 0.42 and 0.90, respectively. (TLC plates were treated with ninhydrin solution (1 gm ninhydrin, 100 ml acetone, 2 ml glacial acetic acid) and the color was allowed to develop in an ammonia-free atmosphere).

Estimation of the Number of Dansyl Groups Present in Dansylated Paromomycin: Ultraviolet and Fluorescent Spectrophotometry

The OD at the absorption maximum of DNS-Cl (262 nm) and DNS-Pm (254 nm), in conjunction with the known concentration of each compound in 96% alcohol, was used to calculate the molar ratio of the dansyl group to Pm. The expected and actual values at different dansyl:Pm ratios suggest that a molar ratio of one best fits the data.

The fluorescent spectrum of DNS-Pm in butanol showed that the excitation maximum was 340 nm, while the emission maximum was 515 nm.

Proton Magnetic Resonance Spectrometry

It is possible to obtain nuclear magnetic resonance signals characteristic of groups of protons, ie, those that arise from the protons on the anomeric carbons of paromomycin and those from the aromatic nucleus of DNS-Cl (Fig. 1). Thus, there
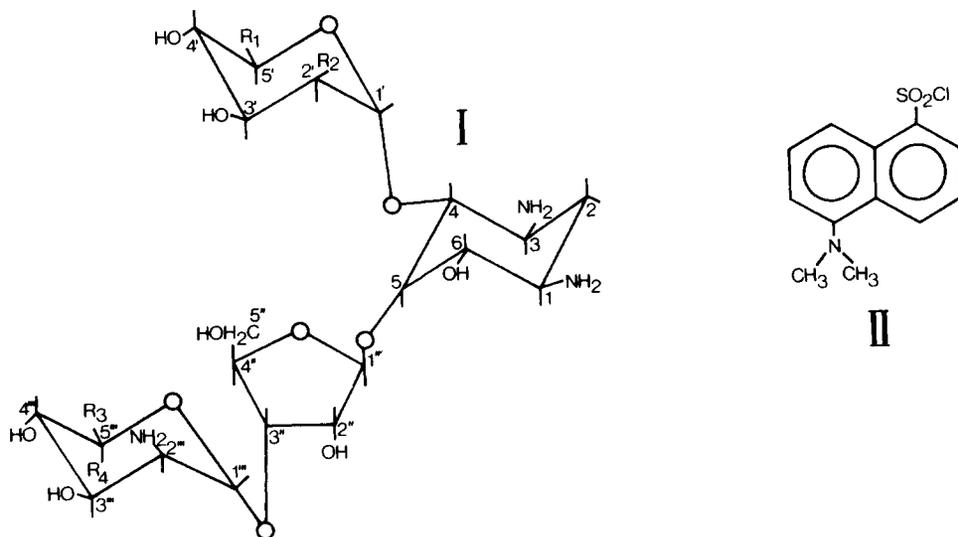
Fig. 1. Structures of paromomycin (I) and dansyl chloride (II). Paromomycin exists in two forms:

	R_1	R_2	R_3	R_4
1.	CH_2OH	NH_2	CH_2NH_2	H
2.	CH_2OH	NH_2	H	CH_2NH_2

are three protons bonded to anomeric carbons (1', 1'', 1''') of the paromomycin moeity. These protons give a characteristic chemical shift (δ) of between 5.2–5.8 ppm, while the aromatic protons in the DNS-Pm molecule have a δ of between 7.5–8.5 ppm. Spectra were obtained from solutions of each compound in deuterated water. The proton chemical shift of dioxan (δ = 3.7 ppm) was used to calculate the above chemical shifts.

The addition of a dansyl group to Pm is probably through a sulphonamide bond with either of the amino groups in the 3 or 2' position. It is known from inactivation studies that substitution of either of these amino groups can render the drug inactive [13]. Using the UV molar extinction of DNS-Cl and DNS-Pm, we have calculated that each paromomycin molecule is labeled with one dansyl group. In order to verify this conclusion, the proton magnetic resonance spectrum of Pm and DNS-Pm was examined and it was concluded that a number of compounds were contributing to the spectrum. We suggest that these compounds are probably positional isomers of Pm I and Pm II [13]. However, we have integrated the area of the peaks representing the six aromatic protons (δ = 7.4–8.5 ppm) of the dansyl group and compared this area with that of the three anomeric protons, 1', 1'', and 1''' of Pm, an expected ratio of 2. This integration indicated that the ratio of the peak areas (aromatic to anomeric) was 2.2:1, which agrees with a 1:1 ratio of DNS to Pm found by ultraviolet spectrophotometry. We have been unable to prepare our samples of DNS-Pm completely free of Pm. At present, we have determined that the purity of our samples is

about 97%. Therefore, any antibiotic activity we measure and attribute to DNS-Pm may be due to this level of contamination by Pm (results not shown). However, we now have evidence that DNS-Pm behaves in an analogous manner to other amino-glycoside antibiotics in respect of the cellular studies reported here, although DNS-Pm may have little or no antibiotic activity.

Cell Cultures

Human fetal lung diploid fibroblasts (MRC-5) were routinely subcultured at 37°C using Eagle's minimum essential medium F-15 (Gibco Biocult), supplemented with 10% fetal calf serum and cells were either grown in plastic tissue culture flasks (25 cm^2 and 81 cm^2 surface area) or on sterilized microscope coverslips in petri dishes.

Fluorimetric Measurements of DNS-Pm Extracted From MRC-5 Cells

Time-dependent uptake of DNS-Pm by MRC-5 Cells. A confluent layer of MRC-5 cells (2–3 × 10^6) was treated with 660 μM DNS-Pm for 1–7 days. After the completion of the treatment, cells were removed by scraping with a rubber policeman and collected by centrifugation (275 g, 10 min). Cell pellets were washed twice with PBS, resuspended in 0.5 ml butanol saturated PBS, and sonicated for 8 sec. The cell sonicate was extracted with two volumes of butanol (1 ml) saturated with PBS until no fluorescence was visible in the sonicate under UV. The fluorescence thus extracted was measured on a Turner 110 fluorimeter using an excitation wavelength of 340 nm and emission wavelength of 515 nm. The concentration of DNS-Pm was determined from a standard curve generated from known concentrations of DNS-Pm dissolved in PBS and extracted in butanol. This relationship was linear, the slope representing 8.28 units of fluorescence per μM with an intercept of -0.05 and an $r^2 = 1.00$ (linear regression data). Cells treated with dansylsulphonamide, dansylsulphonic acid, and dansylchloride did not show accumulation of any of these compounds.

Dose-dependent uptake of DNS-Pm by MRC-5 cells. Confluent layers of cells were treated with varying concentrations of DNS-Pm (from 50 to 660 μM) for 2 days. Cells were processed as described above and their extracted fluorescence was measured.

Release of DNS-Pm from MRC-5 cells. Cells treated with 660 μM DNS-Pm for 2 days were given fresh medium without DNS-Pm. The medium was changed twice a day up to 4 days. DNS-Pm was extracted from the cells as described above and the fluorescence measured. Examination of the absorption and emission spectra of the extracted fluorescence and the fluorescence in the medium showed no change in these spectra, indicating no intracellular degradation had occurred. For fluorescent micro-scopic studies cells were examined every day for a 3-week chase period.

Localization of DNS-Pm in MRC-5 Cells

Metrizamide gradient cell fractionation. The method of cell fractionation used was described previously [14]. Cells (5 × 10^7) in tissue culture flasks were treated with 660 μM DNS-Pm for 2 days. After the treatment, cells were collected by scraping and were washed twice with PBS as described previously. Cell pellets were resuspended in 2 ml of 10 mM Tris-Cl (pH 7.5) containing 15% metrizamide.

Cells were homogenized (15–20 strokes) in a tight-fitting Dounce homogenizer and breakage was checked by acridine orange staining. The homogenate was centrifuged at 270 g for 10 min to remove nuclei and the nuclear pellet was washed twice in 1 ml of 10 mM Tris-Cl buffer. The supernatant and washings from nuclei were pooled and EDTA was added to a final concentration of 10 mM. This solution was placed on a discontinuous gradient of metrizamide in 10 mM Tris-Cl and centrifuged for 2 hr at 4°C (40,000 g). The interfaces from the gradient were collected and samples were taken to measure the protein content [15]. Acid phosphatase and succinic dehydrogenase activities of the various fractions were also measured [16,17]. The remainder of each fraction was extracted in butanol saturated with PBS, as described earlier, and the fluorescence measured.

RESULTS

Uptake and Release of DNS-Pm by MRC-5 Cells

Fluorescent microscopy. DNS-Pm was rapidly taken up by MRC-5 cells in culture (Fig. 2) and within 2 hrs of treatment with DNS-Pm, highly fluorescent granules (FG) were seen distributed throughout the cytoplasm. These FG performed saltatory motion, were phase-dense and after 18 hr could be seen crowded in the perinuclear region of the cells. There were some phase-dense granules that were not fluorescent (Fig. 2). No fluorescence was ever seen inside nuclei. Longer periods of treatment with DNS-Pm (> 18 hr) caused enlargement of the FG, which were still to be seen in the perinuclear region.

Uptake of DNS-Pm by fibroblasts could be inhibited when cells were kept at 4°C. Most of the fluorescence at that stage was seen on the cell membrane only. However, with the rise in temperature, FG started appearing inside the cells. A cyclic peptide antibiotic, bacitracin (0.5–2 mg/ml), which is known to block the receptor-mediated uptake of some ligands [18], had no visible effect on the uptake of DNS-Pm by MRC-5 cells at 37°C. Replacement of Pm with DNS-Pm in cultures treated for several passages with Pm did not inhibit the uptake of DNS-Pm.

Fluorimetric studies. A direct correlation was found between the extracellular concentrations of DNS-Pm and the butanol-extracted fluorescence (intracellular concentration) from MRC-5 cells (Fig. 3). At an extracellular concentration of 660 μM of DNS-Pm, there was a sharp increase in the extractable intracellular fluorescence after 2 days of treatment. The amount of DNS-Pm continued to increase for up to 7 days (Fig. 4). Almost 80% of the intracellular DNS-Pm was lost in 1 day from cells when DNS-Pm was removed from the medium (Fig. 5). After this initial rapid loss of intracellular DNS-Pm there was no further release of DNS-Pm from the cells and the intracellular concentration of between 1–1.2 μg/mg of protein was maintained for at least 4 days.

Cell fractionation studies. After centrifugation, metrizamide gradients were examined under UV illumination and it was observed that most of the fluorescence was present at interface 1 (10% → 20% metrizamide). There was some fluorescence seen at interface 2 (20% → 25%), the intensity of which varied from one experiment to the other. However, little or no fluorescence was seen in interfaces 3 (25% → 30%) or 4 (30% → 35%). The fluorescence contained in these metrizamide fractions was extracted and measured. A typical result of the distribution of DNS-Pm on the
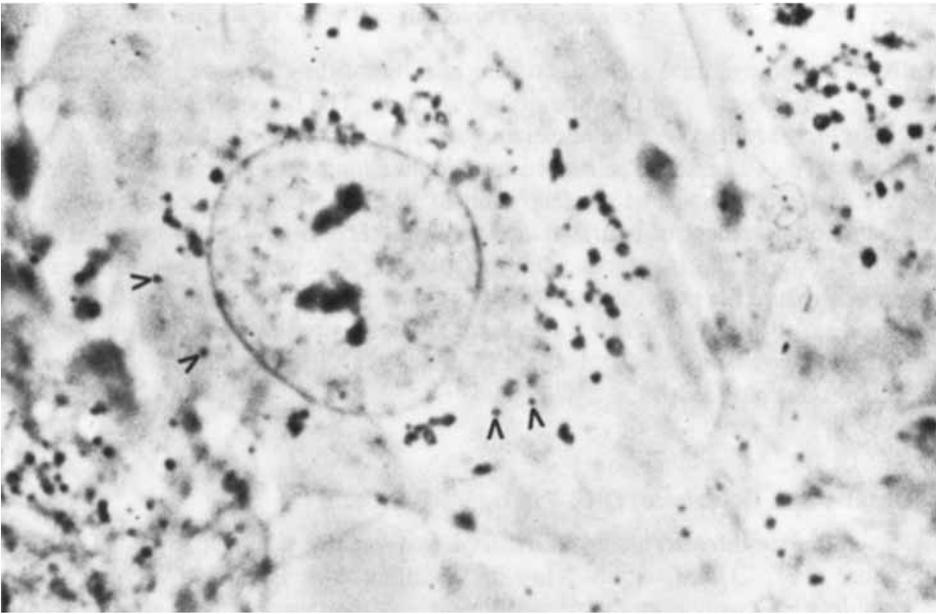
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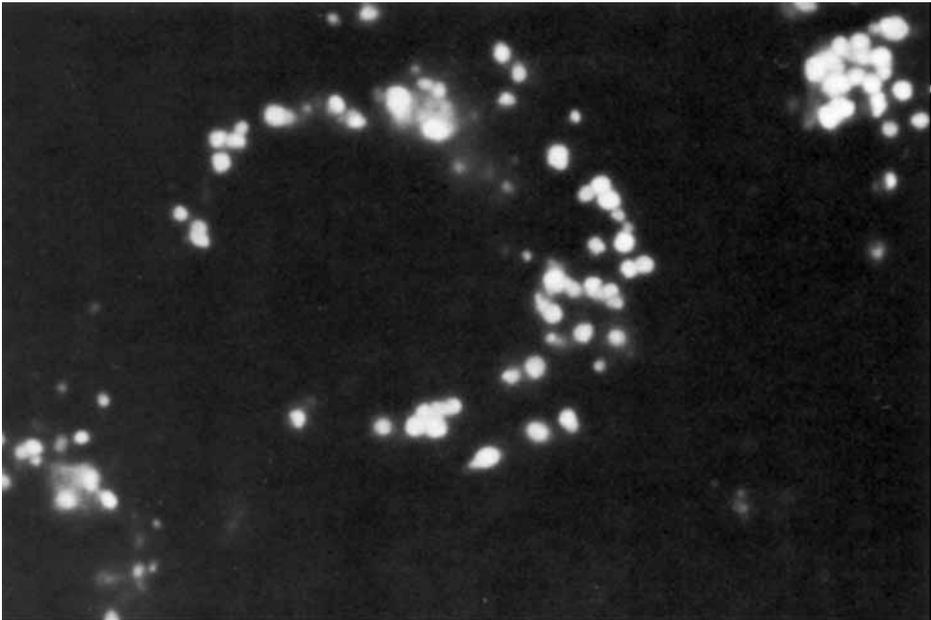
b

Fig. 2. Fluorescence microscopy of DNS-Pm treated cells. Cells grown on coverslips were treated for 18 hr with DNS-Pm 165 μM. The coverslips were mounted on microscope slides and the cells photographed using phase contrast (a) and fluorescence (b). For phase and fluorescence photomicrography, High Speed Ektachrome 35 mm film was used. Exposure was for 5 sec (phase) and for 2 min (fluorescence) at a magnification of ×3700 approximately. Filters for fluorescence microscopy were BG12 excitation filter and No. 47 barrier filter (Carl Zeiss). Granules are visible in phase contrast (a), while those granules containing DNS-Pm can be seen in (b). A few phase-dense granules, which do not fluoresce, are marked with an arrow.

3 DNS–Pm Concentration (μM)

4 Days
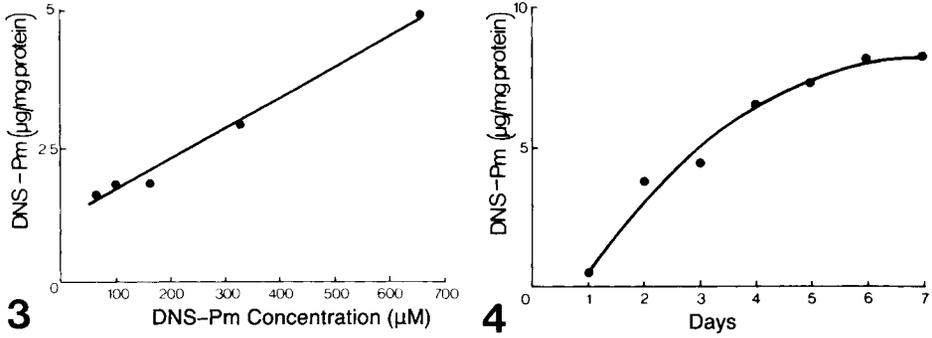
Fig. 3. Dose-dependent uptake of DNS-Pm by MRC-5 cells. Cells were treated for 2 days and fluorescence was extracted in butanol. Fluorescence units were converted to μg from a standard curve of DNS-Pm.

Fig. 4. Time-dependent uptake of 660 μM DNS-Pm by MRC-5 cells in culture, after extracting with butanol.

5 Days

6 Metrizamide Gradient Interface
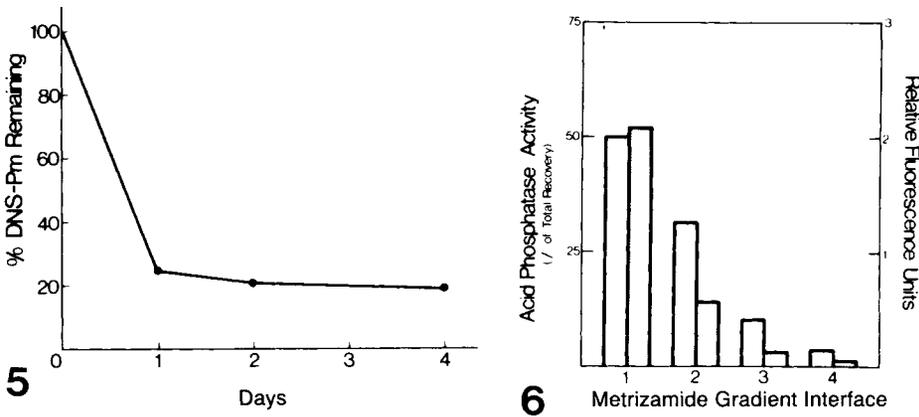
Fig. 5. Release of DNS-Pm by MRC-5 cells in culture. Cells treated with 660 μM DNS-Pm for 2 days yielded 4.5 μg/mg protein (= 100%). After 1 day of chase period only 1 μg/mg protein DNS-Pm was recovered. There was no further significant release up to 4 days.

Fig. 6. Distribution of fluorescence (DNS-Pm) and acid phosphatase activity in interfaces after metrizamide gradient cell fractionation. Recovery of acid phosphatase was about 60% of the activity in the homogenate. (1) Interface < 20% (lysosomes); (2) interface 20% → 25%; (3) interface 25% → 30% (mitochondria); (4) interface 30% → 35%.

gradient is shown in Figure 6. Marker enzyme assays showed that most of the fluorescence was associated with the fraction that had maximum acid phosphatase activity (Fig. 6). Most of the succinic dehydrogenase activity (a mitochondrial marker enzyme) was found to be in the interface 3 and 4 (results not shown). This association of fluorescence with acid phosphatase was found to be reproducible.

DISCUSSION

We have shown that a fluorescent derivative, dansyl-paromomycin, prepared from an aminoglycoside antibiotic, paromomycin, is taken up by human diploid fibroblasts in culture. Examination of treated cells by phase-contrast and fluorescent microscopy revealed the formation of bright FG after only 1 hr of treatment with DNS-Pm. Because of the intensity of fluorescence in these FG, we suggest that the DNS-Pm is concentrated within the vesicles at these early times. At later times (> 18 hr), these vesicles appeared to increase in size by swelling, by repeated vesicle fusion or by repeated fusion of vesicles with lysosomes, and continued to accumulate DNS-Pm. Marker enzyme assays of subcellular fractions obtained from metrizamide gradients showed that DNS-Pm accumulates in fractions containing acid phosphatase. These observations agree well with other reports that show that streptomycin, genta-mycin, kanamycin, and other aminoglycoside antibiotics are concentrated in the lysosomes of rat fibroblasts [12,19,20].

The uptake of DNS-Pm as a function of its concentration and the duration of treatment shows that at an extracellular concentration of 660 μM, DNS-Pm reaches a stable intracellular concentration of about 6-10 mM after 7 days incubation. On removal of DNS-Pm from the culture medium, about 75–80% of this intracellular concentration is lost from the cells within 1 day. However, the remaining fluorescence is retained by the cells and does not appear to be released.

From the uptake data we have estimated the intravesicular concentration of DNS-Pm to be between 15 and 250 mM. This estimate is based on the lysosomal volume being 4% of the cell volume, while treatment of cells with aminoglycosides, which caused lysosomal swelling, increased the volume 10-fold [20]. These results represent a 40–400-fold concentration of DNS-Pm over the extracellular concentration.

During this period of 7 days, we have calculated that about 0.02% of the extracellular DNS-Pm is present in the cells and in this time no degradation of DNS-Pm occurred. It is therefore unlikely that the extracellular concentration of DNS-Pm was depleted and could have affected the results.

Since these results showed that DNS-Pm accumulated within fibroblasts, reached saturation, and was stable for at least 7 days, the possible pathways of uptake and accumulation can be discussed. It is known that the uptake of soluble compounds by fluid-phase pinocytosis is directly related to the extracellular concentration of the solute, while by adsorptive pinocytosis cells can concentrate large amounts of solute without the intake of correspondingly large volumes of fluid [21]. It has been proposed that the uptake of aminoglycoside antibiotics by rat fibroblasts is either by fluid-phase or nonselective endocytosis, resulting in high concentrations of antibiotic in the lysosomes [12]. Our kinetic data and the lack of inhibition of endocytosis by bacitracin support fluid-phase pinocytosis as the main mechanism of uptake for DNS-Pm. A value (endocytic index) of about 1.4 μl/mg/day for the uptake of DNS-Pm represents a rate of pinocytosis comparable to that found for sucrose (0.08–0.13 μl/mg/hr), a fluid-phase pinocytic marker [22], and also close to the value (3.5 μl/mg/day) for other aminoglycosides [12].

These antibiotics and DNS-Pm are polycationic compounds, a characteristic that may influence their rates of uptake. If DNS-Pm accumulates in lysosomes, because it is polycationic and because of the low pH in these organelles, then the

rapid discharge of DNS-Pm in the absence of extracellular DNS-Pm cannot be due to simple diffusion of the trapped molecules. Neither can the discharge be due to the breakdown of DNS-Pm, since it has been shown that the compound is stable for at least 7 days. These facts suggest a rapid but incomplete exocytosis of FG over a 24-hr period. Further support for exocytosis of pinocytosed DNS-Pm comes from our fluorescent microscopy observation that when Pm was removed from cultures pre-treated for several cell generations with Pm and replaced by DNS-Pm, the latter was endocytosed. A similar conclusion that exocytosis was responsible for the release of endocytosed sucrose has been reached in a study using alveolar macrophages and human fibroblasts [23]. To explain the kinetics of sucrose uptake and release by such cells, these authors proposed a cellular model comprising of two to three compart-ments and in some respects our data may be accommodated in this model. However, one prediction arising from this model is that the long-term accumulation (days rather than hours) of solutes by fluid--phase pinocytosis should be linear. The results of present studies and others [12] do not show a linear rate of accumulation after 6 and 3 days, respectively. These results suggest that a steady state may have been estab-lished between endocytosis and exocytosis as the kinetics of uptake reached saturation.

The rapid release of DNS-Pm (80%) may also be explained in terms of the above model with two intracellular compartments [23]. However, the remaining DNS-Pm (20%) does not appear to be released. It is possible that this amount of DNS-Pm might be retained in the third (lysosomal) compartment but our kinetics of accumulation do not support this idea. However, there is evidence that a small amount of the aminoglycoside antibiotic, gentamycin, can be seen bound to the rough endo-plasmic reticulum of rat kidney cells [24]. In addition, recent evidence suggests that aminoglycosides may enter the cytoplasm [25]. It seems that a probable explanation for retention of DNS-Pm is that a proportion of DNS-Pm may escape into the cytoplasm during vesicular/lysosome membrane fusion and bind to ribosomes and rough endoplasmic reticulum.

Clearly, further studies of lysosomal traffic are necessary to substantiate the proposed mechanism of release of DNS-Pm from the lysosomes into the cytoplasm. Such studies could be extended to other aminoglycoside antibiotics, in particular Pm [26], since human fibroblasts cultured continuously in Pm showed a significant reduction in lifespan (Holliday and Rattan, manuscript in preparation). These results were not due to toxicity as cells treated with Pm showed no apparent effects on short-term growth and cell division. These authors also found that morphological changes, characteristic of senescence and increased amounts of heat-labile glucose-6-phosphate dehydrogenase (a nonlysosomal enzyme), accompanied the reduction in lifespan by Pm. These observations, taken together with the fact that Pm increases errors in translation in cell-free systems [1–6], provide the basis for the study of error propa-gation in mammalian cells [27] and for further tests of the error theory of cellular ageing [7–9].

ACKNOWLEDGMENTS

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