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# Quantitation of secretion by rat basophilic leukemia cells by measurements of quinacrine uptake

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**A novel method for quantitating secretion is described based on measurements of the cellular uptake of the fluorescent aminoacridine dye quinacrine into low-pH secretory granules. The quinacrine fluorescence remaining in the medium was found to decrease after incubation with increasing numbers of the 2H3 rat basophilic leukemia line. This depletion of dye from the medium decreased after a secretory stimulus. Assuming that quinacrine partitions according to mass action, a quantitative model was derived to allow calculation of the percent secretion from dye uptake data. A good correlation was obtained when the values for the percent secretion determined by the quinacrine uptake method were compared with secretion measured by release of the granule enzyme β-glucuronidase.**

## Introduction

Many cells contain cytoplasmic granules, whose contents are released in response to an appropriate stimulus at the plasma membrane [1–3]. The ability to follow and quantitate secretion provides information regarding these interactive processes. One type of method for quantitating secretion is the analysis of secretory products into the medium from the secretory granule. However, not all secretory products of interest can be easily analyzed. Alternative techniques, such as electrical measurements of membrane area [4] or release of fluorescent dye loaded granules [5–7], have been used to follow secretion temporally [5,7] but have not been successful in quantitating secretion.

One class of fluorescent dyes, the aminoacridines, are weak bases which have been used to identify acidic compartments of various cells microscopically [8,9]. These dyes are membrane permeable in their neutral form and effectively partition into low pH compartments by mass action [10,11] where they are retained after acquiring a positive charge [10]. The aminoacridine dyes have been used to follow secretion by monitoring the loss of fluorescence from a preloaded secretory cell (see, for example, Ref. 6). Since the fluorescence of such dyes in intracellular compartments is greatly affected by the local conditions such as pH and dye concentration [12], quantitative measurements of secretion are difficult.

In this paper we investigate the possibility of quantitating secretion by the reduction in the uptake of an aminoacridine dye into secretory cells as their acidic granules undergo exocytosis. We have used the dibasic dye quinacrine, whose dual protonation at low pH permits more effective trapping compared with the monobasic aminoacridine dyes and thereby allows the use of lower

Abbreviations: DNP-, dinitrophenyl-; BSA, bovine serum albumin.

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loading concentrations. We show that uptake of quinacrine into the model secretory cell RBL-2H3, a rat basophilic leukemia cell line, allows quantitation of secretion induced by the calcium ionophore ionomycin. Secretion calculated by this technique shows an excellent correlation with secretion measured by release of the granule enzyme β-glucuronidase into the medium. This approach offers several advantages over currently used methods of quantitating secretion.

## Materials and Methods

*Materials.* Ionomycin was obtained from Calbiochem, San Diego, CA. Quinacrine dihydrochloride, *p*-nitrophenol-β-D-glucuronide, EGTA, and nigericin were purchased from Sigma Chemical Co., St. Louis, MO. The buffer we have utilized throughout this study was Hanks' balanced-salt solution (HBSS) containing 0.01 M Hepes (pH 7.4).

*Cells.* Rat basophilic leukemia (RBL,2H3 line) cells, a gift from Dr. S. Dreskin, were maintained in culture using Eagles Minimal Essential medium with 15% fetal calf serum along with 4 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were harvested at the point of confluence in the flask by incubating for 6 min at 37° C with a trypsin-EDTA mixture. Cell viability was > 95% by Trypan blue exclusion. Cells were subsequently washed twice with 0.01 M Hepes-BSS solution (pH 7.4) and placed on ice.

*Secretion.* A suspension of RBL cells ($2 \cdot 10^6$ cells/ml) in HBSS was divided into two aliquots of 2 ml each. Typically, at time $t = 0$, 100 μl of $10^{-5}$ M ionomycin in HBSS was added to one aliquot, mixed, and both aliquots incubated at 37° C for 25 min. In order to vary the degree of secretion both the time (10 min to 90 min) and ionomycin additions (from 50 μl to 200 μl) were occasionally varied. Secretion was then stopped by adding 0.4 ml of 12 mM EGTA/HBSS solution to both the control and ionomycin fractions. This EGTA concentration was found to block ionomycin-induced secretion from RBL cells. After EGTA addition cells were examined microscopically with trypan-blue and occasional experiments showing less than 90% viability in either incubation fraction were excluded. Both the control and ionomycin-treated RBL suspensions were subsequently assayed for granule enzyme secretion and quinacrine uptake (described below). To evaluate whether the quinacrine assay worked in a system in which secretion was induced via cross linking of surface receptors, we incubated RBL cells with 7.6 μg/ml α-DNP-IgE [13] for one hour at 37° C then washed with the buffered HBSS solution twice. Secretion was subsequently induced by adding DNP-BSA to a final concentration of 40 ng/ml to both the control cells and those with bound IgE. After 20 min the secretion was terminated by the addition of EGTA. The α-DNP-IgE and DNP-BSA were generous gifts from Dr. S. Dreskin.

*β-Glucuronidase assay.* In this assay the granule enzyme β-glucuronidase [14] was determined in both medium and cells, and the number of enzymatic units in the medium were expressed as a percentage of the total. Using microtiter plates, 100 μl aliquots of the above RBL cell suspension were centrifuged for 5 min, at $700 \times g$. Aliquots of 70 μl of the supernatants were assayed for enzymatic activity as described below. The remaining cell associated enzyme was determined by adding 150 μl of 1% Triton X-100 in HBSS to each well and carrying out the enzyme assay on 70 μl of this extract. The number of enzyme units associated with the residual media was subtracted out to give the cell associated enzyme and the percentage of total β-glucuronidase in the medium calculated.

The β-glucuronidase assay was modified from that of Hall et al. [15]. Briefly, 70 μl of the sample to be assayed was incubated with 10 μl of 0.5 M sodium acetate, 0.4% Triton X-100 (pH 5.0) and 20 μl of 0.1 M *p*-nitrophenol-β-D-glucuronide for 90 min at 37° C. The reaction was stopped by addition of 150 μl of 50 mM NaOH. *p*-Nitrophenol release was read in an ELISA reader using a 405 nm filter.

*Quinacrine uptake assay.* After incubating RBL cells with and without the secretogogue, quinacrine uptake as a function of cell number was measured as follows: 6–12 aliquots of each cell suspension containing multiples of $2 \cdot 10^5$ cells were placed in 15 ml tubes and the volumes adjusted to 0.2 ml with HBSS. The first tube contained no cells and was used to determine the maximum fluorescence, $Fl_T$. An identical set of tubes was set up for the

control cells without secretagogue. To each tube, 1.8 ml of 3 mM EGTA and 5 $\mu$M quinacrine in HBSS was added. After incubation for 45 min at 37 °C, the tubes were centrifuged at 900 × $g$ for 5 min and one ml of supernatant from each tube added to 1-cm$^2$ (4.5 ml) polystyrene cuvettes (Fischer Sci., Silver Springs, MD) containing 1 ml of HBSS. The fluorescence of these samples was read on a Perkin-Elmer Spectrofluorimeter with an excitation of 400 nm and an emission of 500 nm. The cuvette sample chamber was maintained at 26 °C. Although quinacrine fluorescence is pH sensitive in the neutral range, we found that the HBSS provides adequate buffering for this system. We have found that addition of a strong pH 10 buffer to the supernatant gives a 2.5-fold increase in sensitivity of quinacrine fluorescence, but this was not done in the experiments reported here. At the concentrations of quinacrine and EGTA used, the viability (Trypan-blue exclusion) of the stimulated cells was not different from those of untreated controls. Coulter volumes of quinacrine treated and untreated cells as determined on a FACS analyzer (Becton-Dickenson, Sunnyvale, CA) varied by less than a 3%. The quinacrine fluorescence was a linear function of the quinacrine concentrations.

## Results

*Quinacrine uptake by RBL-2H3 cells*

We have performed a number of experiments to investigate whether RBL cells decrease quinacrine uptake as a result of secretion induced by ionomycin. A typical experiment is shown in Fig. 1A. The ratio of quinacrine fluorescence in the medium of samples with ($Fl_1$) and without cells ($Fl_T$) is plotted as a function of the cell number. Curve I is an example of a control incubation in which there was no induced secretion. As the incubation cell number is increased there is a monotonic decrease in supernatant fluorescence, reflecting greater quinacrine uptake as the cell number increases. Curve II is an example of a quinacrine uptake experiment using the same RBL cells in which secretion has been induced with ionomycin. Less quinacrine uptake was observed (cf. curve II and curve I, Fig. 1A). The nonlinearity of curves I and II indicates that the dye taken

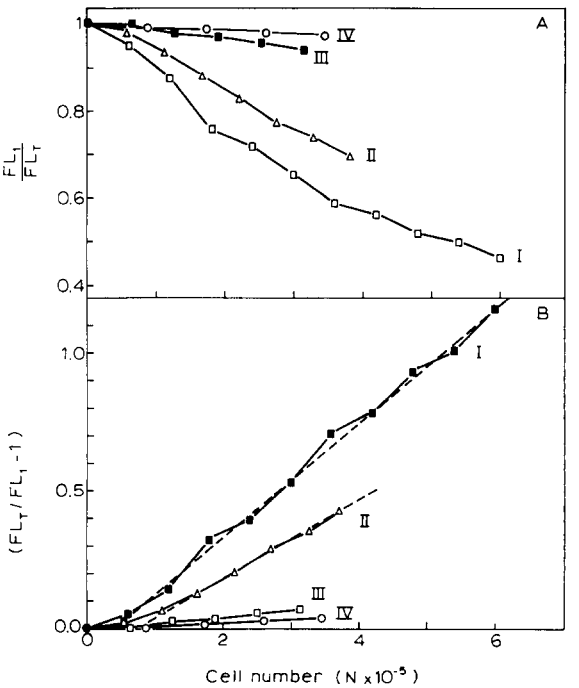


Fig. 1. Quinacrine uptake by RBL (rat basophilic leukemia) cells. (A) The quinacrine fluorescence of the supernatant ($Fl_1$), shown normalized to the undepleted supernatant fluorescence ($Fl_T$) in which there was no uptake (i.e. zero cells), plotted against the number of RBL cells in the incubation ($N$). Curves I and II represent RBL cells previously incubated for 20 min in normal medium and medium containing 0.5 $\mu$M ionomycin, respectively. Curves III and IV show quinacrine uptake by unstimulated RBL cells (as in curve I) in the presence of 10 mM ammonium chloride or 2 $\mu$M nigericin, respectively. (B) Data of Fig. 1A are replotted according to Eqn. 1 of the text. The dotted lines have been fit to curves I and II for $N > 2 \cdot 10^5$ by linear-regression analysis. For the line fit to curve I $r = 0.996$, the intercept is $-0.085$, and the slope is 0.207. For the line fit to curve II $r = 0.998$, the intercept is $-0.108$, and the slope is 0.143.

up per cell is less at higher cell numbers. When lower amounts of quinacrine were used the curvature of these plots was enhanced, whereas higher amounts of quinacrine resulted in less curvature (data not shown).

The two upper curves (III and IV) are examples of quinacrine uptake by unstimulated RBL cells in the presence of ammonium chloride and nigericin, respectively. These agents are known to raise the pH of acidic intracellular compartments by different mechanisms [16,17]. It is clear that both of these agents dramatically diminish quinacrine up-

take into RBL cells. These curves indicate that under these conditions the majority of the quinacrine uptake is not due to binding to cellular components, but is dependent on the low pH of intracellular compartments. Using β-glucuronidase release as a measure of RBL cell degranulation [14], neither nigericin nor ammonium chloride, at the concentrations used in Fig. 1, caused a detectable increase in secretion over the background (5% of the total over a 20-min incubation). This indicates that differences between the control curve (I) and the upper two curves (III and IV) is not due to a stimulation of secretion by ammonia or nigericin.

*Quantitation of quinacrine uptake as a means of assessing secretion*

The appendix presents a model for quinacrine uptake into a cell containing an acidic intracellular compartment (e.g., secretory granules). The determinants of quinacrine uptake into the cells are the low pH and volume of the acidic compartment. Since the latter decreases when secretion occurs, measurements of changes in quinacrine uptake in cells can be used to calculate the degree of secretion. The appendix describes the formulation for the quantitation of secretion using this model by measuring the depletion of quinacrine fluorescence from the medium. One method of calculating secretion directly utilizes the equation

$$(Fl_T/Fl_1) - 1 = N\alpha \qquad (1)$$

where $\alpha$ is the slope of the curve determined by $((Fl_T/Fl_1) - 1)$ and $N$ (Eqn. A-9). $\alpha$ is a function of both the intragranular pH and the supernatant pH (which is buffered at 7.4), and is proportional to the average granule compartment volume per cell ($\bar{V}_G$).

Fig. 1B shows the data of Fig. 1A replotted according to the function shown in Eqn. 1. It can be seen that for $N > 2 \cdot 10^5$ cells, curves I and II can be fit by straight lines. Fig. 1B shows that there is a value of the cell number above which $\alpha$ is a constant allowing the curves to be fit by straight lines. Neither of these straight lines go through the origin, which would be predicted from Eqn. 1 if $\alpha$ were a constant independent of cell number. It is possible that this curvature is due to a systematic error in the measurement of the fluorescence, but may also be due to effects of the dye concentration on intragranular pH [10,18]. Since all cells used to determine a curve are taken from the same suspension prior to quinacrine uptake they will have the same average granule volume, and thus in the region of constant $\alpha$ the intragranular pH will be constant. In the region where $\alpha$ (slope of unstimulated control cells) and $\alpha'$ (the slope of the stimulated cell suspension) are constant their ratio will be equal to the ratio of their respective average granule volumes and

$$f_S = 1 - (\alpha'/\alpha) \qquad (2)$$

where $100 \times f_S$ is the percent secretion (Eqn. A-13).

Using Eqn. 2 and the values for $\alpha$ and $\alpha'$ obtained in Fig. 1B, the percent secretion ($100 \times f_S$) is calculated to be 30.9%. In this experiment, the ionomycin-induced secretion was measured by release of the granule enzyme β-glucuronidase into the supernatant. The fraction of the total released for the stimulated cells was 34%, while the control fraction spontaneously released was 5%, giving a net secretion due to the secretogogue of 29%. This value is in good agreement with the above esti-

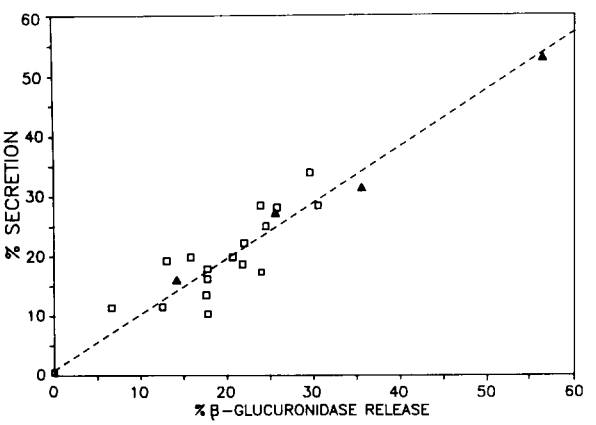


Fig. 2. The percent secretion calculated from quinacrine uptake is plotted against the secretion of β-glucuronidase for parallel experiments. Experiments were performed on different days with ionomycin concentrations from 0.25 μM to 1 μM, and cell numbers ranging from $1 \cdot 10^6$ cells/ml to $5 \cdot 10^6$ cells/ml to induce variations in the amount of secretion prior to the quinacrine assay. The triangles (▲) represent experiments in which RBL were induced to secrete using α-DNP-IgE and DNP-BSA (Methods). The dashed line represents the linear fit to the data for the ionomycin-induced secretion.

mate determined by quinacrine uptake.

Fig. 2 shows the combined results of 19 quinacrine uptake experiments with RBL cells stimulated to secrete with ionomycin. The percent secretion as determined by the quinacrine uptake technique is compared with the β-glucuronidase release measured in parallel. Ideally one would expect an intercept of zero with a slope and regression coefficient both equal to one. A linear fit to the curve yields a slope of 0.93, an intercept of 1.0% and a regression coefficient of 0.88, all in good agreement with the ideal case. When secretion was induced by crosslinking α-DNP-IgE bound to the IgE receptor (triangles, Fig. 2), we obtain a linear fit with a slope of 0.86, an intercept of 4% and a regression coefficient of 0.99. This line is not significantly different than the linear fit to the ionomycin-induced secretion data.

## Discussion

In this paper we have measured secretion in the RBL-2H3 mast cell line by monitoring the uptake of the aminoacridine dye quinacrine. We have developed a quantitative model to describe quinacrine uptake into cells containing low-pH compartments such as secretory granules. Our results show that quinacrine uptake into RBL cells conforms to predictions of the model. They further indicate that such measurements can provide quantitative estimates of secretion in these cells. This is demonstrated by the good quantitative correlation of secretion by quinacrine uptake and measurement of supernatant release of the intragranular enzyme β-glucuronidase.

The use of fluorescent dyes to follow secretion allows for the possibility of a sensitive and minimally perturbing method which can be simply and quantitatively carried out. Several different approaches have been used to exploit fluorescent dyes in the studies of secretion. Rabbit kidney collecting tubule cells preloaded with endocytosed fluorescein-dextran show a reduction in fluorescence intensity with increasing $CO_2$ levels consistent with vesicle exocytosis [5]. The fluorescence of a suspension of mast cells preloaded with the aminoacridine dye acridine orange increased when induced to secrete, presumably due to release of the self-quenched dye from granules [6]. Bronner et al. [19] have detected an increase in plasma membrane area due to the fusion of granules during mast cell secretion by using a lipophilic dye which fluoresces when it partitions into the plasma membrane from the medium.

The aminoacridine dyes are weak bases permeable to membranes in their unionized form [10], and will accumulate in low pH granules in the protonated form by mass action [10,11]. These properties make them appealing because of simplicity of use as well as potential for quantitation of partitioning. The most frequently used aminoacridine dyes are the monobasic compounds 9-aminoacridine and acridine orange, and the dibasic dye quinacrine. A quantitative model for the accumulation of the dibasic dyes into cells containing low-pH granules, using the principles of mass action, is presented in the appendix. Because the dibasic dyes have an increased tendency to accumulate in granules compared with the monobasic dyes, their cytoplasmic concentrations can be neglected. This allowed us to develop an expression from which the percent secretion could be calculated using the experimentally determined quantities of supernatant fluorescence and incubation cell number.

In the present study we have used the fact that cell suspensions measurably deplete quinacrine from the medium. This depletion is dependent on both the granule volume and the intragranular pH. If one assumes that the average intragranular pH does not change during a secretory process then a diminution in quinacrine uptake reflects a decrease in granule volume. In our system such an assumption is borne out by Fig. 2. However, if there is a heterogeneity of granule pH and a selective exocytosis of more acidic or basic granules the average intragranular pH may shift during secretion. A clear advantage of the quinacrine uptake approach is that the dye fluorescence measurements are carried out in a controlled environment of the cell suspension media. It does not entail measurements of the dye in cells where the pH and other environmental influences are unknown and can affect the fluorescence efficiency. The quantitation of quinacrine uptake is simple to measure using a fluorimeter, and behaves in accordance with the model derived in the Appendix.

The measurement of secretion by quinacrine

uptake in RBL cells showed good correlation with release of the granule enzyme β-glucuronidase [14]. The RBL cells were chosen because of their well studied secretory physiology and also because they do not have the large internal volume of secretory granules (as seen in the EM [20]) characteristic of cells such as mast cells [9]. We thus expect that the quinacrine uptake method is applicable to a wide range of cell types. One potential limitation of this technique is pointed out by recent studies indicating that the mature secretory granules of exocrine cells do not have a low pH [21], and in such cases the quinacrine uptake method would not be applicable. However, this is not a problem in most instances and, when applicable, the quinacrine uptake method has the advantage that fluorimetric measurements are simple and rapid to perform. This would be useful in cells in which the secretory products are difficult to assay.

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## Appendix

*Quantitation of secretion by changes in quinacrine uptake by secretory cells*

In order to model the uptake of quinacrine or other lysosomotropic dyes into secretory cells, we have assumed that such cells have three compartments into which quinacrine can partition: (1) the extracellular space; (2) the intracellular space; and (3) the low-pH granules. For each compartment the protonation of the weak base lysosomotropic agents can be depicted by the following equations where all variables are in molar concentrations

$$Q_i + H_i \rightleftharpoons (QH)_i \tag{A-1a}$$

$$(QH)_i + H_i \rightleftharpoons (QH_2)_i \tag{A-1b}$$

where $i$ designates the compartment number, Q the unprotonated form of the quinacrine, H the hydrogen ion, $(QH)_i$ the singly protonated form of quinacrine and $(QH_2)_i$ is the doubly protonated form. Since Q is uncharged it is assumed to be the major membrane permeant form [10] and therefore, $[Q] = [Q]_i$ for all $i$.

From Eqns. A-1a and A-1b the respective dissociation constants, which are independent of compartment, can be defined by

$$K_1 = [Q][H]_i/[QH]_i \tag{A-2a}$$

$$K_2 = [QH]_i[H]_i/[QH_2]_i = [Q][H]_i^2/K_1[QH_2]_i \tag{A-2b}$$

It follows that the total dye concentration in the system $[Q]_T$ is given by

$$[Q]_T = \{(V_T - V_C)([Q] + [QH]_1 + [QH_2]_1) + (V_C - V_G) \times ([Q] + [QH]_2 + [QH_2]_2) + (V_G)([Q] + [QH]_3 + [QH_2]_3)\}/(V_T) \tag{A-3}$$

where $[Q] = [Q]_i$ for all $i$ (since the neutral form is membrane permeant), $V_T$ is the total system volume, $V_G$ the granule volume, and $V_C$ the intracellular volume (which includes $V_G$).

Using Eqns. A-2a and A-2b, we can rewrite Eqn. A-3 in terms of the variables in compartment 1

$$[Q]_T = ([Q] + [QH]_1 + [QH_2]_1)(((V_T - V_C)/V_T) + \beta + \gamma) \tag{A-4}$$

where

$$\beta = ((V_C - V_G)/V_T)\left(1 + [H]_2/K_1 + [H]_2^2/(K_1K_2)\right) / \left(1 + [H]_1/K_1 + [H]_1^2/(K_1K_2)\right)$$

and

$$\gamma = (V_G/V_T)\left(1 + [H]_3/K_1 + [H]_3^2/(K_1K_2)\right) / \left(1 + [H]_1/K_1 + [H]_1^2/(K_1K_2)\right)$$

If one works in the range in which fluorescence is proportional to the dye concentration (as mentioned in Materials and Methods) then

$$Fl_T/Fl_1 = [Q]_T/([Q] + [QH]_1 + [QH_2]_1) \tag{A-5}$$

where $Fl_T$ is the total fluorescence of the medium without any cells and $Fl_1$ is the fluorescence of compartment one, i.e. the supernatant fluores-

cence after centrifugation of the cells.

If we use the literature values of $pK_1$ and $pK_2$ as 10.28 and 7.7, respectively, for quinacrine [22], reasonable simplifying approximations can be made in Eqn. A-4. Assuming the pH of compartment three is 5.5 [16] and that of compartment two is about 7.1 [16] then the ratio $\beta/\gamma$ is approximately $((V_C - V_G)/V_G) \cdot 10^{-3}$. If $V_G > 0.001 \cdot V_C$ (e.g. for mast cells $V_G/V_C$ is about 0.25 [9]) we can neglect the second (cytoplasmic) compartment (the $\beta$ term) compared to the granular compartment (the $\gamma$ term), to a first approximation. Furthermore, following a similar argument as above, one can show that $V_C/V_T$ is small compared with the granule term (where $\gamma$ in Eqn. A-4 is approximately $(V_G/V_T) \cdot 10^4$). Within the framework of the above approximations Eqns. A-4 and A-5 can be combined to yield

$$Fl_T/Fl_1 = 1 + (V_G/V_T)[(1 + [H]_3/K_1 + [H]_3^2/(K_1K_2)) / (1 + [H]_1/K_1 + [H]_1^2/(K_1K_2))] \quad \text{(A-6)}$$

Since the number of cells is large we can define an average granule volume per cell, $V_G$, defined by

$$\bar{V}_G = V_G/N \quad \text{(A-7)}$$

where $N$ is the total number of cells.

Defining

$$\alpha = [(1 + [H]_3/K_1 + [H]_3^2/(K_1K_2)) / (1 + [H]_1/K_1 + [H]_1^2/(K_1K_2))](\bar{V}_G/V_T) \quad \text{(A-8)}$$

we can simplify and rearrange Eqn. A-6 to read

$$(Fl_T/Fl_1) - 1 = N\alpha \quad \text{(A-9)}$$

Eqn. A-9 allows experimental determination of $\alpha$ when $((Fl_T/Fl_1) - 1)$ is plotted as a function of $N$ (Fig. 1B).

The fractional secretion, $f_S$, given by the loss in granular volume of the cells, is calculated by first rewriting it in terms of the average granular volume

$$f_S = (V_G - V_G')/V_G = (\bar{V}_G - \bar{V}_G')/\bar{V}_G = (1 - (\bar{V}_G'/\bar{V}_G)) \quad \text{(A-10)}$$

where the unprimed and primed quantities represent those prior to secretion and those after secretion respectively.

From Eqn. A-9 it is easy to see that the fluorescence in the supernatant will be equal for both the control and secreting system when

$$N\alpha = N'\alpha' \quad \text{(A-11)}$$

Since the changes in granular pH that occur between the primed and unprimed system is small over the range of secretion we are looking (see the Results section of the text) it follows from Eqns. A-7 and A-8 that

$$\alpha'/\alpha = \bar{V}_G'/\bar{V}_G \quad \text{(A-12)}$$

and it follows from Eqns. A-10, A-11 and A-12 that

$$f_S = 1 - (N/N') = 1 - (\alpha'/\alpha) \quad \text{(A-13)}$$

and the percent secretion is simply $f_S \times 100$.

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