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Method for quantitating the molecular content of a subcellular organelle: Hormone and neurophysin content of newly formed and aged neurosecretory granules

(vasopressin/oxytocin/posterior pituitary/stoichiometry)

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ABSTRACT **A method is described for the quantitative determination of the content of subcellular organelles such as secretory granules. Purified subcellular fractions of the organelle are prepared and aliquots are assayed for hormones, for example. To determine the number of organelles per fraction, known numbers of latex particles of a size similar to the organelle are added to other aliquots of the subcellular fractions. Latex particles and organelles are then pelleted together by centrifugation. The ratio between latex particles and organelles can be determined by morphometric analysis of ultrathin sections taken through the full thickness of the pellet. The number of organelles and hence their content of the substance assayed can then be calculated. We have applied this technique to posterior pituitary neurosecretory granules, the content of which has already been estimated by a different method. Newly formed neurosecretory granules from oxen and rats were found to have a content of ≈85,000 molecules of hormone and neurophysin. Aged neurosecretory granules from the same neural lobes appeared to contain less hormone and neurophysin, but this was shown to be the result of loss of material from the granules during isolation in media of 360 mosM. Such loss could be prevented by isolation in hypertonic (660 mosM) media.**

Quantitative studies on subcellular organelles of any type frequently demand a knowledge of the average content of an organelle. To calculate the average content, the number of organelles in a given sample must be determined. If the number of organelles is not known, then their contents cannot be expressed absolutely but only relatively (for example, in terms of protein or DNA). For many organelles, there may not be a stable parameter to which the other measurements can be related.

An assessment of the number of organelles in a tissue sample can be made by morphometry (1). This has been used to determine the number of neurosecretory granules (NSG) in the neural lobe of the pituitary gland of the rat. The granules are formed in the perikarya of magnocellular neurones in the hypothalamus and carried by rapid axonal transport to the neural lobe, where they are stored in the nerve endings and nerve swellings. No synthesis of neurosecretory material occurs in the neural lobe. From the hormone content of the gland and the number of granules determined by morphometry, the hormone content of an average NSG was calculated to be 84,000 molecules (2). However, calculation of the hormone content in this manner makes the assumption that all hormone is intragranular within the neural lobe.

It has been argued by a number of authors that significant amounts of hormone are located extragranularly—particularly as a "cytoplasmic pool" (see ref. 3 for discussion). Therefore, a direct method of assessing the hormone content of isolated NSG is needed to avoid the assumptions inherent when whole tissue is used. This, in turn, requires the determination of the number of granules isolated in a fraction. The hormone and neurophysin content of the granules can be determined by radioimmunoassay or bioassay or both. Neural lobe NSG are not a homogeneous population but can be separated into newly formed NSG (NF-NSG) and aged NSG (A-NSG) by fractionation on appropriate gradients (4).

We have devised a method for assessing the number of granules in such fractions by adding to the fractions known numbers of latex particles of similar size to the granules, pelleting this mixture, and measuring the ratio of latex particles to granules in the pellet. In this way, the total number of granules in the fraction was determined and the average hormone content of a granule calculated. By this method, we calculate that NSG contain ≈85,000 molecules of hormone. The agreement of this value with that derived by morphometry of the entire tissue not only validates the data but also indicates that essentially all hormone is intragranular. We suggest that this method may be widely applicable in the quantitative studies of other particulate organelles.

EXPERIMENTAL PROCEDURES

Isolation of Granule Fractions. Neurosecretory granules were isolated by the method described by Nordmann *et al.* (4). Briefly, neural lobes from rats and oxen were homogenized in 0.3 M sucrose buffered with 10 mM Hepes (pH 6.8). The homogenate was centrifuged at 800 × *g* for 10 min, then centrifuged at 3,000 × *g* for 10 min, and finally centrifuged at 9,000 × *g* for 10 min. The resultant supernatant was centrifuged for 20 min at 27,000 × *g*. This produced a crude granule pellet that was resuspended in sucrose and layered onto an iso-osmotic continuous gradient made of 0.3 M sucrose and 33% AG 6227 (Hexabrix; Laboratoires Guerbet, Aulnay-sous-Bois, France; ref. 5), which had an average osmolarity of 360 mosM. This was then centrifuged at 129,000 × g_{av} for 60 min to isopycnic equilibrium. By this means, the A-NSG were separated from the NF-NSG as two fractions. The volumes of the fractions were measured and a number of identical aliquots were taken for assay of hormone, neurophysin, and protein content. In a second group of experiments, all of the procedures for the preparation of the granule fractions were carried out by using media containing 0.52 M sucrose. In this case, the gradient had an average osmolarity of 660 mosM.

Determination of the Number of Granules in a Fraction. Known numbers of latex particles (176 nm in diameter; Serva, Heidelberg, FRG) were added to aliquots of the granule fractions, and the mixtures were diluted with phosphate

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Abbreviations: NSG, neurosecretory granule(s); NF-NSG and A-NSG, newly formed and aged NSG, respectively; AVP, arginine vasopressin.

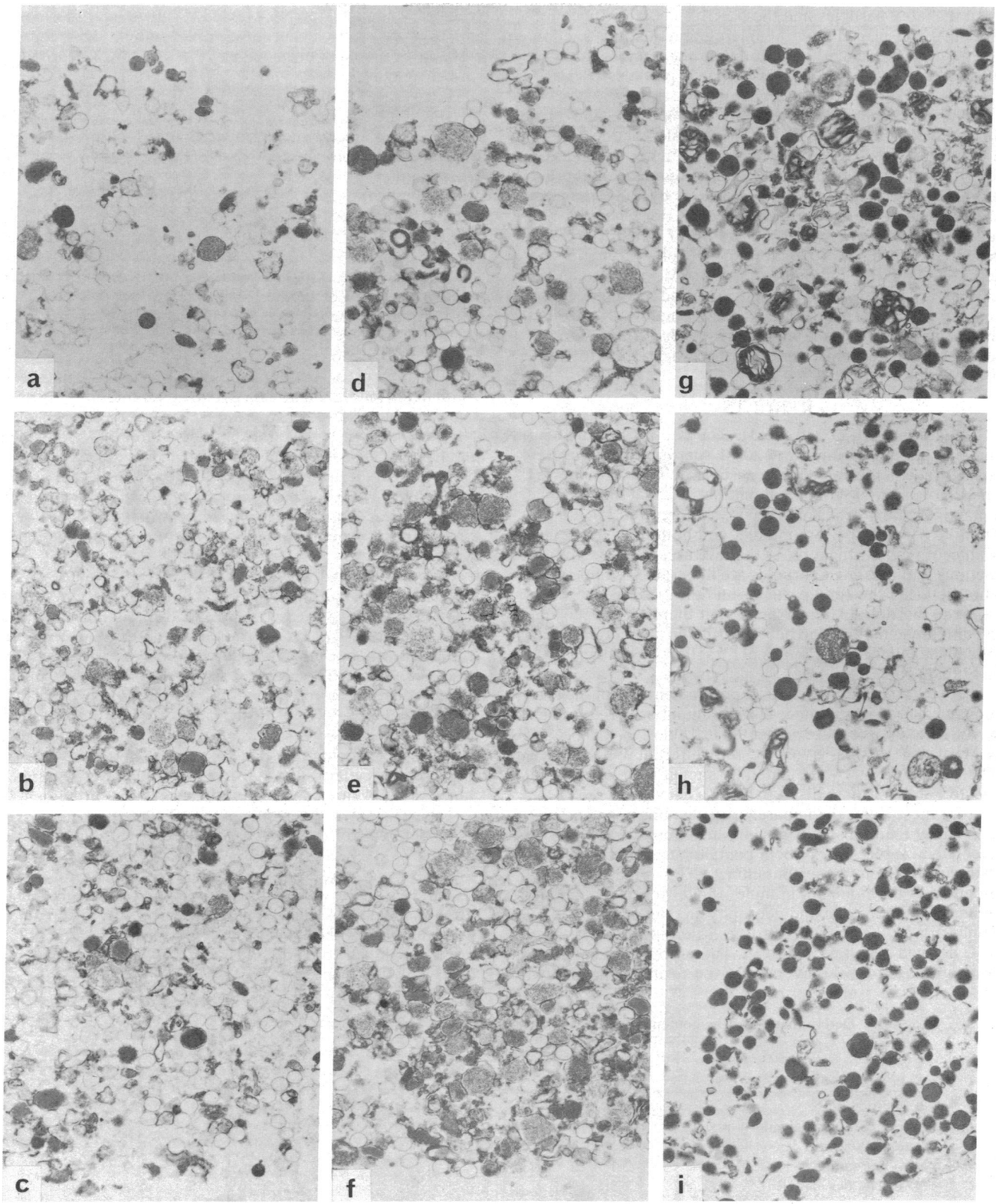

FIG. 1. Electron micrographs of pellets of mixtures of latex particles and NF-NSG (*a–c*) and A-NSG (*d–f*) isolated in 360 mosM media containing 0.3 M sucrose and NSG isolated in 660 mosM media containing 0.52 M sucrose (*g–i*). The top of the pellet is shown in *a*, *d*, and *g*; the middle part in *b*, *e*, and *h*; and the bottom in *c*, *f*, and *i*. (×19,000.)

buffer (0.1 M; pH 6.8) containing glutaraldehyde at a final concentration of 2.5%. The granules were fixed for 75 min and then the fractions were centrifuged for 30 min at 60,000 × *g*. The resulting pellet was washed with phosphate buffer, postfixed with 1% osmium tetroxide in phosphate buffer, dehydrated, and embedded in Epon. Up to the dehydration step, the preparation was maintained at 4°C. Vertical ultrathin sections were obtained so that all levels of the pellet could be visualized and systematically sampled by micrographs. Point counting was used to calculate the ratio be-

tween the latex particles and the NSG. A square lattice of P test points was placed over the micrograph, and the fraction P_L/P_{NSG} was calculated after counting the number of those points superimposed on profiles of latex particles (P_L) and NSG (P_{NSG}).

Assays of Vasopressin, Oxytocin, Neurophysin, and Protein. Identical aliquots from all of the granule fractions isolated were assayed for vasopressin [arginine vasopressin (AVP)], oxytocin, and neurophysin in a number of different laboratories. Vasopressin was measured by radioimmunoassay using different antisera (M. Cazalis, Bordeaux; M. Delaage, Marseille; J. J. Legros and F. Louis, Liège; R. Sheaves, Oxford; B. T. Pickering, Bristol). Oxytocin was measured both by radioimmunoassay (M. Delaage, Marseille; J. J. Legros and F. Louis, Liège) and by milk-ejection bioassay (R. E. J. Dyball and F. Shaw, London). Neurophysin was measured by radioimmunoassay (J. J. Legros and F. Louis, Liège). Proteins were measured according to Bradford (6).

RESULTS

Granules Isolated at 360 mosM. The two most prominent bands of granules from the 0.30 M sucrose-containing 360 mosM gradients were collected separately to provide the NF-NSG- and A-NSG-containing fractions that had minimal contamination by other organelles, as described (4). Representative parts of the top, middle, and bottom of the pellets produced by centrifugation of the mixtures of these granule fractions with the latex particles are illustrated in Fig. 1. The ratios between the latex particles and the NSG in the pellets are given in Table 1. Fig. 2 shows that there was no systematic difference in this ratio between the top and bottom of the pellet. The number of granules per fraction was calculated as described (Table 1).

The AVP, oxytocin, and neurophysin contents of the fractions, measured in a number of different laboratories and using different antisera, are presented in Table 1 as the mean ± SEM of the results given by the different laboratories. Whereas the results were generally similar from one laboratory to another, some discrepancies existed.

From these data, the apparent average hormone and neurophysin content of single NSG in the different fractions was calculated (Table 1). Oxytocin-containing granules were not distinguished from those containing AVP, so that the results are given as hormone per granule. In view of the confidence limits for the hormone and neurophysin content and granule number data, there appears to be little difference between NSG derived from rats and oxen. Therefore, further calculations are based on the average of the results from the two species, which are, for NF-NSG, 15×10^{-8} ng of hormone and 16×10^{-7} ng of neurophysin per granule, and, for A-NSG, 8×10^{-8} ng of hormone and 7×10^{-7} ng of neurophysin per granule.

The average number of molecules of hormone and neurophysin in one NSG derived from these data is given in Table 2. Also given are the molar concentrations within the granules based on volumes for NF-NSG and A-NSG given by Nordmann *et al.* (4).

In a duplicate series of experiments in which only oxytocin and AVP were assayed, the average content of rat NF-NSG was 74,000 molecules of hormone per granule and of rat A-NSG was 33,000 molecules of hormone per granule. These values are a little lower than, but in essential agreement with, those of the first experiment.

From measurement of the total amount of protein in the fractions, we also calculated that one NF-NSG contains 2.2 fg of protein and one A-NSG contains 1.9 fg of protein. The amount of protein in the membrane of a granule has been estimated from the difference between the total protein content and that of the neurophysin. Data for the more stable NF-NSG have been used for this calculation. It is a maximal estimate because it assumes that other breakdown products of the precursor are too small to be detected by the protein assay that was used. The membrane of one granule thus estimated appears to contain ≈0.6 fg of protein, a figure very similar to the protein content of a chromaffin granule membrane (7).

Granules Isolated at 660 mosM. When the granules were isolated using 0.52 M, 660 mosM media, only one major granule-containing band was obtained. This equilibrated at $\eta = 1.382$ (Table 3), and it should be noted that NF-NSG equilibrate at about this density when isolated in 360 mosM media (Table 1). The granules isolated in 660 mosM media appeared to be more electron dense and less swollen than those isolated in 360 mosM media (Fig. 1). Also, the bottom part of the pellet consisted almost entirely of granules, with latex particles being restricted essentially to the upper two-thirds of the pellet. Such a distribution illustrates the importance of analyzing complete vertical sections of the pellets.

Table 3 shows the results of duplicate experiments on NSG derived from rats and a single experiment on NSG derived from oxen, all isolated at 660 mosM. Again, only AVP and oxytocin were assayed in this experiment. As seen in Table 3, granules isolated at 660 mosM contained 80,000–100,000 molecules of hormone, a figure entirely in agreement with that for NF-NSG isolated at 360 mosM. No fraction of lighter granules containing less hormone could be detected after isolation using 660 mosM media.

Table 1. Determination of the content of NF-NSG and A-NSG isolated at 360 mosM

Parameter	Rat		Bovine	
	NF-NSG	A-NSG	NF-NSG	A-NSG
Refractive index	1.3790	1.3706	1.3802	1.3712
Latex, particles per ml	3.2×10^{12}	3.2×10^{12}	3.2×10^{12}	3.2×10^{12}
Latex/NSG ratio	1.4	1.3	2.3	2.8
NSG per sample	2.3×10^{11}	2.6×10^{11}	1.4×10^{12}	1.2×10^{12}
AVP,* ng per sample	2.8×10^{4}	1.5×10^{4}	12.5×10^{4}	4.5×10^{4}†
	$\pm 0.2 \times 10^{4}$	$\pm 0.1 \times 10^{4}$	$\pm 1.2 \times 10^{4}$	
Oxytocin,* ng per sample	1.1×10^{4}	0.3×10^{4}	5.8×10^{4}	3.8×10^{4}†
	$\pm 0.3 \times 10^{4}$	$\pm 0.1 \times 10^{4}$	$\pm 1.5 \times 10^{4}$	
Hormone,‡ ng per sample	3.9×10^{4}	1.8×10^{4}	18.3×10^{4}	8.3×10^{4}
Neurophysin, ng per sample	4.0×10^{5}	2.1×10^{5}	2.1×10^{6}	6.8×10^{5}
Hormone, ng per NSG	17.3×10^{-8}	7.1×10^{-8}	11.8×10^{-8}	8.2×10^{-8}
Neurophysin, ng per NSG	17.5×10^{-7}	8.1×10^{-7}	14.6×10^{-7}	5.8×10^{-7}

*Values are presented as mean ± SEM, except where indicated.
†Mean of two determinations.
‡AVP and oxytocin.

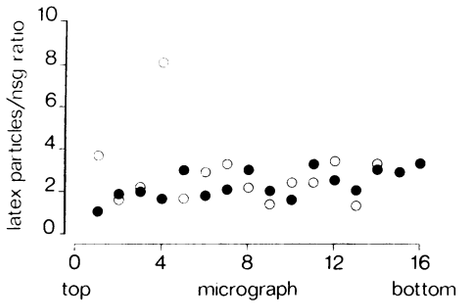

FIG. 2. Ratio of latex particles to NSG in individual micrographs sampled from the top to bottom of pellets of mixed latex particles and NF-NSG (●) and A-NSG (○).

DISCUSSION

The correlation of assay and ultrastructural data on a gland secreting material packaged in granules demands that the quantity of material stored in an individual granule be known. This argument can be extended to many subcellular organelles, so that a method for determining the amount of their content is necessary.

The previous calculation of the content of a neurohypophysial granule (≈84,000 molecules; ref. 7) made the assumption that all hormone is intragranular. The number of granules lost from the neural lobe after acute stimulation corresponds well with the hormone released if the calculations are based on a granule content of 84,000 molecules of hormone (8, 9). Evidence that oxytocin and vasopressin are packaged similarly comes from the finding that the granule and oxytocin content of homozygous Brattleboro rats, which fail to produce vasopressin as a result of a genetic defect (10), is again consistent with a granule content of ≈84,000 molecules of hormone (11).

Proponents of an extragranular pool of hormone cite as evidence the demonstration of hormone in the supernatant of subcellular fractions, the presence of granules with electron-lucent interiors, an allegedly disproportionate lack of granules in Brattleboro homozygotes, and the presence of immunocytochemically detectable hormone and neurophysin in the cytosol. We have discussed elsewhere the reasons for believing that such demonstrations reflect artefact rather than physiology (3), and to this can be added the observation that when granules are isolated at 660 mosM ambient osmotic pressure, 93% of the hormone is found in the granule fraction (12).

Clearly, it can never be proved that *no* hormone exists in the cytosol. However, the demonstration by a totally different method, which we present here, that NSG contain 85,000 ± 5,000 (mean ± SEM, n = 7, for NF-NSG and granules isolated at 660 mosM) molecules of hormone provides very strong confirmation for the effectively exclusive intragranular storage of releasable hormone. The recent demonstration of the partial sequence of the vasopressin precursor (13) substantiates the long-held opinion from circumstantial evidence that hormone and neurophysin originate together and are present in a 1:1 ratio in the granule. The data presented here again demonstrate this 1:1 ratio.

Table 2. Number of molecules of hormone and neurophysin and their concentration in NF-NSG and A-NSG isolated at 360 mosM

Parameter	NF-NSG	A-NSG
Hormone, molecules per NSG	84,000	44,000
Neurophysin, molecules per NSG	95,000	42,000
Hormone, mM in NSG	60.0	21.5
Neurophysin, mM in NSG	70.0	20.5

The data are averages for NSG from rats and oxen.

Table 3. Determination of the content of neurosecretory granules isolated in 0.52 M sucrose-containing 660 mosM media.

Parameter	Rat		Bovine
	Exp. 1	Exp. 2	
Refractive index	1.382	1.381	1.380
Latex, particles per ml	1.6×10^{12}	3.1×10^{11}	7.8×10^{11}
Latex/NSG ratio	0.71	0.69	0.71
NSG per sample	2.2×10^{12}	4.5×10^{11}	1.1×10^{12}
Hormone, ng per sample	3.7×10^{5}	6.1×10^{4}	1.5×10^{5}
Hormone, molecules per NSG	102,000	81,000	83,000

A-NSG isolated at 360 mosM contained less hormone and neurophysin than NF-NSG. Molecules could have been lost from A-NSG either *in vivo* or during production of the granule fraction. Gel electrophoresis demonstrates that only the A-NSG fraction contains detectable protein species of molecular weight smaller than neurophysins. These appear to result from continued intragranular proteolysis (14), and some are breakdown products of neurophysin because they react with antineurophysin sera (unpublished data). However, proteolysis would be expected to cause greater loss of neurophysin than of vasopressin, but both appear to be lost equally.

Some loss of granule content would also be expected to occur during preparation of the A-NSG fraction in 360 mosM medium (4, 15). Leakage through a damaged granule membrane would, at first sight, be expected to produce greater loss of hormone than of neurophysin on the basis of molecular size. However, consideration of the Stokes radius of neurophysin molecules indicates that monomeric, but not dimeric (16), neurophysin could diffuse out of smooth membrane-limited vesicles (17). Therefore, aging of NSG could include a change from dimeric to monomeric neurophysin.

Isolation of the NSG fractions at an ambient osmolarity of 660 mosM, at which 93% of the hormone and neurophysin remain intragranular (12), clearly demonstrates that the lower content of A-NSG isolated at 360 mosM is the result of loss during isolation and fractionation. Also, it demonstrates that media of what might appear to be grossly unphysiological osmolarity may be needed to preserve subcellular organelles with concentrated contents.

Because all neurohypophysial NSG appear to contain ≈85,000 molecules of neurophysin and hormones, the diffusion of neurophysin and hormones from the granules must be prevented *in vivo*. The mechanism that ensures this is not clear, but dimeric neurophysin (16, 17), neurophysin-hormone binding, and the low intragranular pH (18, 19) all appear to play a part.

The maximal amount of M_r 23,000 precursor (20) that could be contained in a 172-nm newly formed granule is 94,000 molecules because 1 g of protein occupies 0.74 cm^3 (21, 22). This is little more than the measured neurophysin-hormone content of NSG. This suggests that the magnocellular NSG can contain relatively few large molecules other than the precursor and the enzyme(s) that convert it to neurophysin and hormone. Certainly, the NSG could not accommodate 1 molecule of the M_r 80,000 precursor (23) for each of the 85,000 molecules of hormone.

In a parallel study, we have used the latex particle method described here to produce values for the contents of chromaffin granules that are very close to those reviewed by Winkler and Westhead (7). If other organelles of different sizes are to be studied by this method, it might be convenient to match organelle and latex particle sizes, and a range of particle sizes is available from Serva. However, differences could be corrected for using well-established stereological formulae (1).

In conclusion, we present a technique that permits the rapid determination of the *number* and the *concentration* of the assayable molecules contained in a subcellular organelle. We suggest that the method may have wide application in quantitative biological investigation.

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