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Unbiased linear property estimation for spheres, from sections exhibiting overprojection and truncation

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SUMMARY

In the biological sciences, stereological techniques are frequently used to infer changes in structural parameters (volume fraction, for example) between samples from different populations or subject to differing treatment regimes. Non-homogeneity of these parameters is virtually guaranteed, both between experimental animals and within the organ under consideration. A two-stage strategy is then desirable, the first stage involving unbiased estimation of the required parameter, separately for each experimental unit, the latter being defined as a subset of the organ for which homogeneity can reasonably be assumed. In the second stage, these point estimates are used as data inputs to a hierarchical analysis of variance, to distinguish treatment effects from variability between animals, for example.

Techniques are therefore required for unbiased estimation of parameters from potentially small numbers of sample profiles. This paper derives unbiased estimates of linear properties in one special case—the sampling of spherical particles by transmission microscopy, when the section thickness is not negligible and the resulting circular profiles are subject to lower truncation. The derivation uses the general integral equation formulation of Nicholson (1970); the resulting formulae are simplified, algebraically, and their efficient computation discussed. Bias arising from variability in slice thickness is shown to be negligible in typical cases.

The strategy is illustrated for data examining the effects, on the secondary lysosomes in the digestive cells, of exposure of the common mussel to hydrocarbons. Prolonged exposure, at 30 $\mu g\ l^{-1}$ total oil-derived hydrocarbons, is seen to increase the average volume of a lysosome, and the volume fraction that lysosomes occupy, but to reduce their number.

DERIVATION OF UNBIASED LINEAR PROPERTY ESTIMATES

The mathematical problem addressed by this paper is the well-known 'Holmes effect' of stereology (e.g. Goldsmith, 1967), in which a medium containing spherical particles is viewed by transmission microscopy of a sample section. The sphere diameters are assumed to vary independently of each other and of the positions of the sphere centres. The latter are taken to be randomly and dilutely distributed, that is, they form a Poisson field of sufficiently low intensity for there to be negligible overlap when observing the profiles projected through a slice of thickness t. Alternatively, overlap effects become negligible if t is small in relation to average sphere diameter. However, the results of this paper are not restricted to such 'thin' sections; indeed, relatively large values of t lead to greater stability of estimation for some properties.

Let the number of circular profiles with centres in a fixed section area, A, be denoted by N

and the circle diameters, in increasing order, by $\{y_1, y_2, \ldots, y_N\}$. It is assumed that profiles of diameter less than a known limit (q) are not measured. The exact removal of bias resulting from such lower truncation is the central purpose of the mathematical treatment in this paper. Notice that no assumption is made about q being small in relation to a typical profile diameter; in fact it may sometimes be desirable to select q larger than the smallest observable circle diameter, in order to exclude all contributions from spheres below a certain size.

Relevant literature on the Holmes effect for spheres includes Bach (1959), Goldsmith (1967), Keiding *et al.* (1972), Piefke (1976) and Coleman (1979, 1980). The first two papers consider only the case $q=0$ and, with the exception of Piefke (1976), all concentrate on the relation between circle diameter and sphere diameter distributions rather than unbiased estimation of specific properties. Keiding *et al.* (1972) take a parametric approach and Coleman (1980) considers a more general definition of the lower resolution limit.

The distribution function (d.f.) of the diameters (x) of all spheres in the medium for which $x \geq q$ is denoted by $G(x|q)$, and the number of such spheres per unit volume by $N_V(q)$. No parametric assumptions are made about $G(.|q)$, so properties of the sphere diameter distribution for $x < q$ are clearly not identifiable. Unbiased estimation is therefore sought of linear properties of the form

$$\theta(q)=N_V(q)\int_q^\infty l(x)\,\mathrm{d}G(x|q) \tag{1}$$

where $l(x)=\pi x^3/6$, πx^2, x and 1 define $\theta(q)=V_V(q)$, $S_V(q)$, $J_V(q)$ and $N_V(q)$, respectively the total volume, surface area, diameter and number of particles of diameter $\geq q$ in a unit volume of the medium.

Appendix A discusses an unbiased estimation procedure for $\theta(q)$; the estimator takes the general form

$$\hat{\theta}(q)=A^{-1}\sum_{i=1}^{N} h(y_i) \tag{2}$$

where $h(y)$ is the solution of a particular integral equation, derived from the general formulation of Nicholson (1970). For each of the four linear properties mentioned above, the estimator is manipulated into a convenient computational form, involving the evaluation of a simple numerical integral over (at most) N narrow ranges. As an example, the following are the steps in the estimation of $N_V(q)$. First evaluate the N summations

$$a_j=\sum_{i=j}^{N}\exp\{\pi(y_i^2-q^2)/(4t^2)\} \qquad (j=1,\ldots,N) \tag{3}$$

and the N numerical integrals

$$I_j=t^{-2}\int\exp\{-\pi z^2/(4t^2)\}\,\mathrm{d}z \qquad (j=1,\ldots,N) \tag{4}$$

the integration being over the range $(y_{j-1}^2-q^2)^{1/2}$ to $(y_j^2-q^2)^{1/2}$, where $y_0\equiv q$. Then,

$$\hat{N}_V(q)=A^{-1}\left\{(a_1/t)-\sum_{j=1}^{N} a_j I_j\right\} \tag{5}$$

This is the simplest of the four estimators to describe, and corresponds to a stereological result already known (Bach, 1967). However, calculation of the other estimates involves an identical procedure, with only a little extra complication in execution by computer program; see Appendix A, equations (A17)–(A25).

In the current computing environment, such calculations are neither complex to implement nor do they involve substantial CPU time; storage requirements are minimal and the procedure can readily be programmed on a microcomputer. The estimators are well-behaved and numerical difficulties are not encountered unless t becomes small relative to a typical sphere

diameter; it is well known that the equivalent estimators, derived by Nicholson (1970) for the case $t=0$, $q\neq 0$, are unstable. The other special case, $q\rightarrow 0$, $t\neq 0$, causes fewer problems; the procedure described in Appendix A will converge for very small q, though the computation time will increase. For q exactly zero, the above equations should be used to calculate $\hat{N}_V$ and the remaining unbiased estimates obtained from the 'classical' formulae (e.g. Weibel, 1980):

$$\hat{J}_V = A^{-1}N - t\hat{N}_V \tag{6}$$

$$\hat{S}_V = 4A^{-1}\sum_{i=1}^{N} y_i - 4t\hat{J}_V \tag{7}$$

$$\hat{V}_V = A^{-1}\sum_{i=1}^{N}(\pi y_i^2/4) - (t/4)\,\hat{S}_V \tag{8}$$

A common practical problem is that the slice thickness, t, is not a fixed quantity but varies within known limits. Under some simple, heuristically stated conditions, it is straightforward to determine the likely biasing effect of such variation on the estimator $\hat{\theta}(q)$. Appendix B gives the relevant mathematical details; it can be seen that, for many practical purposes, smooth fluctuations in slice thickness contribute negligible bias to the estimation, a more likely source of bias being the failure to determine the mean slice thickness adequately.

STATISTICAL ANALYSIS OF LYSOSOMAL DATA

This section expounds a strategy for statistical analysis which may be appropriate to a number of stereological problems in the biological sciences. This strategy, and the concomitant estimation procedure of Appendix A, are illustrated by data extracted from a study of digestive cell lysosomes from the digestive diverticula of the common mussel *M. edulis* (Lowe *et al.*, 1981).

For the purposes of the present illustration, the data are restricted to two treatments: (a) control and (b) continuous exposure of the animals for 103 days to the water accommodated fraction of North Sea crude oil (30 μg l^{-1} total oil derived hydrocarbons). Secondary lysosomes were distinguished in cryostat sections, of thickness 10 μm, by their azo-dye reaction product for lysosomal β-*N*-acetylhexosaminidase (Fig. 1, A and B). Five randomly selected animals, of shell length in the range 50–60 mm, were studied for each treatment regime; three digestive tubules were randomly selected from each animal and a number of quadrats falling within each tubule epithelium were examined. These were selected, using random number tables, from the set of all quadrats, in a systematic grid, which fell entirely within the tubule's epithelial cells. The number of profiles and their diameters were recorded, proper attention being paid to the elimination of size-biased selection of profiles by the quadrats, using extended 'forbidden lines' (Gundersen, 1977). It was decided to ignore profile diameters of less than (approximately) 0·6 μm; this value for q excludes any contributions from the functionally different primary lysosomes. On the other hand, it is thought that most (if not all) of the sphere diameters of secondary lysosomes are larger than 0·6 μm; thus $\hat{\theta}(q)$ estimates the linear property θ for the full diameter distribution. (Setting a value for q is therefore seen to be an active part of the experimental design, in focusing attention on a restricted range of sphere diameters. For q set above the absolute resolution limit, it becomes increasingly irrelevant whether the observed diameter distribution is distorted, near that limit, by a simple truncation, or by the 'capping' mechanism first advocated by Keiding *et al.*, 1972.)

The purpose of the analysis is to assess the statistical significance and magnitude of any changes to the lysosomal structure with prolonged oil exposure. An important feature, in common with many applications of stereology in the biological sciences, is the lack of homogeneity of the linear properties across different animals from within the same treatment regime, and even across different tubules from within the same animal. Thus, the assumption of a Poisson field of constant intensity (or some other, weaker, stationarity assumption) may only be justified within a single digestive tubule epithelium. It is then potentially very misleading to pool all the data for a specific treatment, arising from several tubules and several animals, and

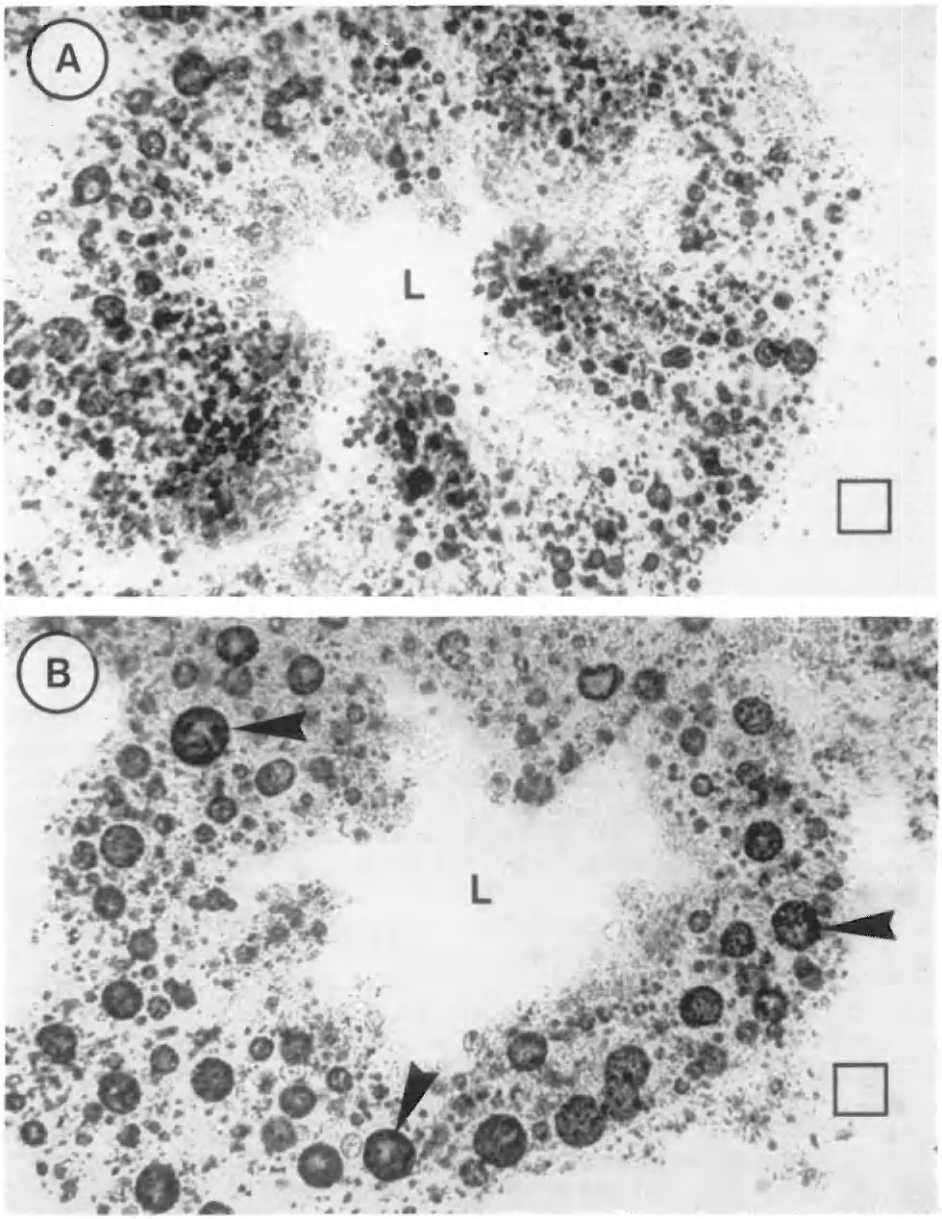

Fig. 1. (A) A cryostat section (10 μm) through a digestive tubule from a control mussel, sampled after 103 days, showing darkly stained lysosomes reacted for β-*N*-acetylhexosaminidase activity in the digestive cells of the tubule epithelium. (B) A section as in (A), from an experimental mussel exposed to the water accommodated fraction of North Sea crude oil for 103 days, showing greatly enlarged secondary lysosomes (arrowed) reacted for β-*N*-acetylhexosaminidase activity in the digestive cells. The number of lysosomes is reduced from the control condition. L = lumen of the digestive tubule. Sample quadrat = 100 μm^2.

'unfold' the resulting single histogram. The reason, of course, is that the readings composing such a histogram are not independently and identically distributed. A more correct course is to estimate a linear property of interest separately for each 'unit' of data, the 'unit' being defined as the level at which the structure can reasonably be regarded as homogeneous (a single tubule

here). These point estimates are then input to a standard heirarchical analysis of variance (ANOVA) which assesses the significance of the difference between treatments in the context of variability of the linear property between animals and tubules. A method of estimating linear properties from small numbers of profiles is therefore required; N is as small as 9 for one of the data units discussed below. The unbiased estimation procedure of the previous section satisfies this need here—though, naturally, the smaller the value of N the larger the variance of the estimator will be.

The relevant ANOVA design is a two-way mixed model (Scheffé, 1959) in which the treatment effects are regarded as 'fixed' and the animal effects as 'random', animals being nested within treatments. The three tubules for each animal are the replication level of the design. In order to use the estimates of (say) $V_V(q)$ from each tubule as 'raw data' input to such an ANOVA, it is desirable that they be approximately (a) unbiased, (b) normally distributed, and (c) of constant variance. The first of these conditions provides the rationale for the mathematical derivation in Appendix A. It is often argued by statisticians that unbiasedness is not an important criterion in estimation, the minimization of mean square error (a combination of bias and variance) being more relevant. This is certainly true for the usual one-stage type of analysis, the conclusion of which is a confidence interval for a particular parameter; however, for the type of two-stage analysis considered here, a large variance for the first-stage estimates can be less damaging than a moderate bias. The large variability simply makes the test of equality for the treatment regimes more conservative, whereas a bias which changes in magnitude for different treatments (as happens here if q is assumed to be zero) may invalidate the analysis—see, for example, the discussion in Cox & Hinkley (1974).

The requirement for normality of errors is partly met by noting the simple additive structure of the estimator (2). An appeal to the central limit theorem, slightly modified to allow for the stochastic sample size (Clarke, 1975), establishes its asymptotic normality. However, for small samples, the right-skewed form of the profile diameter distribution suggests that convergence to normality will be slow, particularly since (8) indicates that, for volume density estimation at least, the distribution of $h(y)$ will be even more right-skewed than that of y. It is therefore desirable to transform the point estimates $\hat{\theta}(q)$, logarithmically, before calculating the ANOVA. This has the additional advantage of inducing approximate constancy of variance, for the following reason. It can readily be shown, using the conditional variance formula, that

$$\text{var}\{\hat{\theta}(q)\} = \{\theta(q)^2/E(N)\}\{1+C_h^2\} \tag{9}$$

where

$$C_h^2 = \text{var}\{h(y)\}/E^2\{h(y)\} \tag{10}$$

For some estimates—$\hat{N}_V(q)$ for example—the coefficient of variation, C_h^2, is negligibly small. For others—notably $\hat{V}_V(q)$—it can be large, but the evidence from the current data indicates that C_h^2 remains approximately constant as $\theta(q)$ varies. The correct variance-stabilizing transformation is therefore logarithmic, the usual Taylor series expansion showing that

$$\text{var}\{\log \hat{\theta}(q)\} \simeq (1+C_h^2)/E(N) \tag{11}$$

It follows that the number of quadrats examined per tubule should be determined separately for each treatment, to achieve approximate equality in the expected number of profiles seen. Here, a small pilot sample indicated that the numbers of quadrats to be examined for oil-exposed and control animals should be in the ratio 8:3.

Note that the use of the logarithmic transformation introduces a small element of bias into the estimation, since $E\{\log \hat{\theta}(q)\} \neq \log \theta(q)$; however, the attainment of *exact* unbiasedness is irrelevant—more important is whether the degree of bias introduced is consistent for different $\theta(q)$. The consistency follows from the second order approximation

$$E\{\log \hat{\theta}(q)\} \simeq \log \theta(q) - (1+C_h^2)/\{2E(N)\} \tag{12}$$

in which the correction term is seen to be approximately constant—in fact usually quite negligible.

Table 1 sets out the $N_V(q)$ and $V_V(q)$ estimates, calculated from equation (A24) of Appendix A, for the thirty (treatment, animal, tubule) combinations. Tables 2 and 3 give the details of the first of these thirty calculations; these have been included to facilitate the checking of any computer software that the reader may produce from the formulae of Appendix A. Tables 4 and 5 give the two-way ANOVA tables for $N_V(q)$ and $V_V(q)$ estimation, respectively, the natural logarithms having been taken of the first-stage estimates in Table 1. The increase in volume fraction for the oil-exposed group is seen to be clearly significant, with 95% confidence intervals for mean $V_V(q)$ of $(1209, 3131) \times 10^{-5}$ for the control group and $(3441, 8911) \times 10^{-5}$ for the

Table 1. Number density $N_V(q)$ ($\mu m^{-3} \times 10^5$) and volume density $V_V(q)$ ($\times 10^5$) estimates of digestive cell lysosomes, for each of three tubules from five control and five oil-exposed mussels. Also given in each case is N, the observed number of profiles in section area A ($=300$ μm^2 for control and 800 μm^2 for exposed animals); $q = 0{\cdot}6$ μm and $t = 10$ μm. The 'subtotal' rows give the means of the three estimates for each animal; these are geometric averages since the later analyses are on logarithmically transformed data.

		Control			Oil exposed		
Animal	Tubule	N	$N_V(q)$	$V_V(q)$	N	$N_V(q)$	$V_V(q)$
1	1	34	1,043	1,604	33	358	9,414
	2	35	1,077	2,471	37	399	6,091
	3	38	1,173	1,605	21	209	7,684
			1,096	1,853		310	7,609
2	1	31	918	4,855	29	300	10,445
	2	35	1,067	3,124	55	599	5,064
	3	38	1,197	2,083	40	430	9,263
			1,054	3,161		426	7,883
3	1	22	696	653	30	325	4,376
	2	19	576	1,050	29	302	5,048
	3	24	726	2,466	35	395	1,635
			663	1,191		338	3,306
4	1	20	598	2,955	16	154	9,186
	2	20	570	5,634	9	84	4,351
	3	25	736	2,898	12	133	3,289
			631	3,640		120	5,085
5	1	17	517	898	29	324	3,624
	2	24	737	1,609	22	235	4,314
	3	17	515	916	21	212	8,799
			581	1,098		253	5,162

Table 2. Frequency (f_i) of lysosome profile diameter midpoints (y_i), for a section area $A = 300$ μm^2 from a single digestive tubule epithelium (control animal); resolution limit $q = 0{\cdot}6$ μm and slice thickness $t = 10$ μm.

y_i (μm)	0·6	0·9	1·2	1·5	1·8	2·1	3·0	N
f_i	12	3	5	7	5	1	1	34

Table 3. Linear property estimates, and their standard deviation estimates, for the data of Table 2.

Property	(units)	Estimate	Standard deviation
Number density $N_V(q)$	(μm^{-3})	0·010435	0·001794
Height density $J_V(q)$	(μm^{-2})	0·012250	0·002277
Surface density $S_V(q)$	(μm^{-1})	0·055923	0·012998
Volume density $V_V(q)$	(1)	0·016043	0·004977

Table 4. Two-way, mixed model ANOVA, performed on the logarithms of the $N_V(q)$ estimates of Table 1. The animal factor is nested within the treatment factor and is regarded as a 'random' effect; the relevant denominator mean square in the test for equality of treatment effects is taken from the 'between animals' row and not the residual ('between tubules') row. * = significant at 5%, ** = significant at 1%.

Effect	Sum of squares	d.f.	Mean square	F ratio
Treatments	8·5365	1	8·5365	17·42 (1, 8 d.f.)**
Animals	3·9204	8	0·4900	9·70 (8, 20 d.f.)**
Residual	1·0103	20	0·0505	
Total	13·4671	29		

'Control' mean (SE) = 6·654 (0·181)
'Exposed' mean (SE) = 5·587 (0·181)

Table 5. ANOVA, as in Table 4, for the logarithms of the $V_V(q)$ estimates of Table 1.

Effect	Sum of squares	d.f.	Mean square	F ratio
Treatments	8·2027	1	8·2027	12·85 (1, 8 d.f.)**
Animals	5·1081	8	0·6385	3·15 (8, 20 d.f.)*
Residual	4·0500	20	0·2025	
Total	17·3607	29		

'Control' mean (SE) = 7·573 (0·206)
'Exposed' mean (SE) = 8·619 (0·206)

oil-exposed group (these are back-transformed from the end-points of symmetric confidence intervals on the logarithmic scale). Also, the significance of the animal effect implies that little useful purpose would be served by examining more tubules per animal (or more quadrats per tubule). The precision of the mean $V_V(q)$ estimates for the two treatments is largely determined by the between-animal variability; confidence interval widths would only be significantly reduced by an increase in the number of animals examined. (This is a familiar feature of statistical analyses throughout experimental biology, though it is only relatively recently that it has been brought to the attention of stereologists, e.g. Gundersen & Østerby, 1981). Similarly, the number density analysis shows both a treatment and an animal effect, the 95% confidence intervals for mean $N_V(q)$ being (511, 1177) for control and (176, 405) for soil exposure, in units of $\mu m^{-3} \times 10^5$.

It is worth noting that the local 'reference areas' (the epithelial section areas for each tubule) are available here, and can be used to estimate the total volume and number of lysosomes per unit length of tubule. In this case, the reference areas do not differ significantly between control and exposed animals, so it comes as no surprise to find that the above conclusions hold (increased total volume and decreased number of lysosomes, with oil exposure) per unit length as well as per unit volume of tubule. In fact, the most suitable parameter for use as a 'stress indicator' is, perhaps, the average lysosomal volume, $v(q)$, for lysosomes of diameter $\geq q$. This is not a linear property but the ratio of two linear properties,

$$v(q) = V_V(q)/N_V(q) \tag{13}$$

Its estimate is computed as the ratio of $\hat{V}_V(q)$ to $\hat{N}_V(q)$ and it is again appropriate to input logarithms of these estimates into the second-stage ANOVA. (The discussion leading up to equation (12) carries over to this case, bearing in mind that the logarithm of a ratio is the difference of the logarithms.) The ANOVA is presented in Table 6 and, naturally, indicates a marked increase in the average lysosomal volume, from a 95% confidence interval of (1·5, 4·3) μm^3 for the control to (12·1, 35·5) μm^3 for the exposed animals. It must be appreciated that the results of the three ANOVAs are not unrelated, in the sense of independently reinforcing the same conclusion, since the point estimates on which they are based are all functions of the same data set.

Table 6. ANOVA, as in Table 4, for the logarithms of the average lysosomal volume estimates, $\hat{V}_V(q)/\hat{N}_V(q)$, calculated from Table 1.

Effect	Sum of squares	d.f.	Mean square	F ratio
Treatments	33·4696	1	33·4696	40·77 (1, 8 d.f.)**
Animals	6·5679	8	0·8210	2·73 (8, 20 d.f.)*
Residual	6·0243	20	0·3012	
Total	46·0618	29		

'Control' mean (SE) = 0·919 (0·234)
'Exposed' mean (SE) = 3·031 (0·234)

A more comprehensive study would employ greater replication and additional design levels and factors; however, the resulting higher-way ANOVA would present no new difficulties and the general strategy would be unchanged. Though small in scale, the current data set confirms previous stereological findings of increased lysosomal volume density and decreased lysosomal number density in the digestive cells of experimentally treated mussels (Lowe *et al.*, 1981). These greatly enlarged lysosomes are known to be less stable than those of the controls as they show significantly reduced latency for β-*N*-acetylhexosaminidase (Widdows *et al.*, 1982). It is also known that the physiological scope for growth in these experimentally treated mussels is less than that of the controls, indicating a tendency towards catabolic processes (Widdows *et al.*, 1982). These factors taken together are indicative of an autophagic role for these enlarged lysosomes. The alteration in secondary lysosomal configuration for these cells represents a considerable disturbance of the structure and function of the lysosomal-vacuolar system, which is normally active in the heterophagic digestion of food.

APPENDIX A. MATHEMATICAL AND COMPUTATIONAL DETAILS

1. *Solution of the integral equation*

The assumptions described at the start of the paper place the problem within the general framework discussed by Nicholson (1970). He shows, firstly, that N has a Poisson distribution with mean

$$E(N) = N_V(q)\, E\{r(x) \mid q\} \tag{A1}$$

where $r(x)$ is the volume of the 'catchment region' around the slice, within which a sphere of diameter x must have its centre for it to become an observable profile; $E(.\mid q)$ denotes expectation over the distribution $G(x \mid q)$. Secondly, there exists an unbiased estimator for $\theta(q)$ of the form

$$\hat{\theta}(q) = A^{-1} \sum_{i=1}^{N} h(y_i) \tag{A2}$$

if a function $h(y)$ can be found to satisfy (for all x) the integral equation

$$\int h(y)\, \mathrm{d}G(y \mid x, q) = Al(x)/r(x) \tag{A3}$$

where $G(y \mid x, q)$ is the conditional d.f. of circle diameter y, given that a sphere of diameter x produces an observable profile. The estimator (A2) is uniquely unbiased, within a certain class of linear estimators, if $h(y)$ uniquely satisfies (A3). Here,

$$r(x) = A\{t + (x^2 - q^2)^{1/2}\} \qquad (q \leq x < \infty) \tag{A4}$$

$$G(y \mid x, q) = \begin{cases} \{(x^2 - q^2)^{1/2} - (x^2 - y^2)^{1/2}\} / \{t + (x^2 - q^2)^{1/2}\} & (q \leq y < x) \\ 1 & (x \leq y < \infty) \end{cases} \tag{A5}$$

and the relation between circle diameter probability density function (p.d.f.), $f(y|q)$, and sphere diameter p.d.f., $g(x|q)$, is

$$f(y|q)=\left\{tg(y|q)+y\int_y^\infty g(x|q)/(x^2-y^2)^{1/2}\,\mathrm{d}x\right\}/\{t+\mu(q)\} \tag{A6}$$

where

$$\mu(q)=\int_q^\infty (x^2-q^2)^{1/2}\,\mathrm{d}G(x|q) \tag{A7}$$

see Coleman (1979), for example. Substitution of (A4) and (A5) in (A1) and (A3) gives

$$E(N)=N_V(q)\{t+\mu(q)\} \tag{A8}$$

$$\int_q^x yh(y)/(x^2-y^2)^{1/2}\,\mathrm{d}y+th(x)=l(x) \tag{A9}$$

The Volterra integral equation (A9), to be solved for $h(y)$, is similar to equation (A6), when solved for $g(.|q)$. The latter is accomplished by Bach (1959) and Goldsmith (1967); they take $q=0$ but the extension to $q\neq 0$ is straightforward. Coleman (1979) and Clarke (1975) give solutions involving only elementary mathematics. Defining $\psi(x)$ as the normal survivor function,

$$\psi(x)=\int_x^\infty (2\pi)^{-1/2}\exp(-t^2/2)\,\mathrm{d}t \tag{A10}$$

(A9) can similarly be shown to have unique solution

$$h(y)=t^{-1}\left[l(y)+\int_q^y (x/t)\{(\pi/t)\exp\{c(y^2-x^2)\}\psi(\{2c(y^2-x^2)\}^{1/2})-(y^2-x^2)^{-1/2}\}l(x)\,\mathrm{d}x\right] \tag{A11}$$

where $c=\pi/(4t^2)$. Evaluation of $\hat{\theta}(q)$ seems to involve a double numerical integration for each measured diameter—an impractical procedure. However, an integration by parts (integrating $x\exp\{c(y^2-x^2)\}$ and differentiating the $\psi(.)l(.)$ term) gives

$$h(y)=(2/t)\left[\exp\{c(y^2-q^2)\}\psi(\{2c(y^2-q^2)\}^{1/2})l(q)+\int_q^y \exp\{c(y^2-x^2)\}\psi(\{2c(y^2-x^2)\}^{1/2})l'(x)\,\mathrm{d}x\right] \tag{A12}$$

where $l'(x)$ denotes the derivative of $l(x)$—this result may be found in Piefke (1975, 1976) and Clarke (1975). The estimator for $N_V(q)$ follows immediately, as the second term disappears when $l(x)=1$. It agrees with that quoted by Bach (1967), derived in a different way. Similarly straightforward is $S_V(q)$ estimation, for which $l'(x)=2\pi x$, since the same integration by parts can be repeated. However, for $J_V(q)$, (A12) involves the double integral

$$\int_q^y\int_x^y u\exp\{c(u^2-x^2)\}/(y^2-u^2)^{1/2}\,\mathrm{d}u\,\mathrm{d}x \tag{A13}$$

and $\psi(.)$ remains in the integrand of the outer integral, even if the order of integration is reversed. Fortunately, the joint substitution

$$v=(u^2-x^2)^{1/2},\qquad z=qx^{-1}(y^2-u^2)^{1/2} \tag{A14}$$

gives a simpler double integral of $v\exp(cv^2)/(q^2+z^2)$. After inner integration over v, the most complex term left contains only the single numerical integral

$$\int_0^Y \exp(-cz^2)/(q^2+z^2)\,\mathrm{d}z \tag{A15}$$

where $Y=(y^2-q^2)^{1/2}$. Also, y appears only in the upper limit of this integral and not in the

integrand, so that by ordering the $\{y_i\}$ the N integrals in (A2) can be evaluated cumulatively. Substituting $l(x)=\pi x^3/6$ in (A12), an analogous derivation exists for $V_V(q)$, though the manipulation is rather more tedious.

All four estimates can be written in the following consistent format, allowing easy programming of their computation. In general,

$$h(y)=h_0(y)-\exp(cY^2)\int_0^Y h_1(z)\exp(-cz^2)\,\mathrm{d}z \tag{A16}$$

where for

$$N_V(q):\quad h_0(y)=t^{-1}E,\qquad h_1(z)=t^{-2} \tag{A17}$$

$$J_V(q):\quad h_0(y)=1+qt^{-1}E-2\pi^{-1}\sin^{-1}(D)$$
$$h_1(z)=2q\pi^{-1}F+qt^{-2}-zt^{-1}F^{1/2} \tag{A18}$$

$$S_V(q):\quad h_0(y)=4Y-4t+4t(1+cq^2)E$$
$$h_1(z)=4(1+cq^2) \tag{A19}$$

$$V_V(q):\quad h_0(y)=(\tfrac{1}{4}\pi y^2-ty+t^2)+qt(1+2cq^2/3)E$$
$$+\tfrac{1}{2}(y^2+c^{-1})\{D(1-D^2)^{1/2}(1+2D^2/3)-\sin^{-1}(D)\},$$
$$h_1(z)=\tfrac{2}{3}q^5(cF+2F^2+2c^{-1}F^3)-tzF^{1/2} \tag{A20}$$

and $D=q/y$, $E=\exp\{c(y^2-q^2)\}$, $F=(q^2+z^2)^{-1}$, $Y=(y^2-q^2)^{1/2}$, $c=\pi/(4t^2)$.

2. *Computation of the estimates*

Re-arrange the data, if necessary, to ensure that $q(=y_0)\leq y_1\leq y_2\leq\ldots\leq y_N$; then for $j=1,\ldots,N$ define

$$I_j=\int h_1(z)\exp(-cz^2)\,\mathrm{d}z,\qquad a_j=\sum_{i=j}^{N}\exp\{c(y_i{}^2-q^2)\} \tag{A21}$$

where the integration for z is over $(y_{j-1}{}^2-q^2)^{1/2}$ to $(y_j{}^2-q^2)^{1/2}$. Substituting (A16) into (A2),

$$\hat{\theta}(q)=A^{-1}\left(\sum_{i=1}^{N}h_0(y_i)-\sum_{i=1}^{N}\left[\exp\{c(y_i{}^2-q^2)\}\sum_{j=1}^{i}I_j\right]\right) \tag{A22}$$

and, by a summation interchange, the most convenient computational form is obtained as

$$\hat{\theta}(q)=A^{-1}\left(\sum_{i=1}^{N}h_0(y_i)-\sum_{j=1}^{N}a_jI_j\right) \tag{A23}$$

For grouped frequency data, the ith of n groups having mid-point y_i and frequency f_i (so that $\sum_{i=1}^{n}f_i=N$), equation (A23) is replaced by

$$\hat{\theta}(q)=A^{-1}\left(\sum_{i=1}^{n}f_ih_0(y_i)-\sum_{j=1}^{n}a_j{}^{\star}I_j\right) \tag{A24}$$

where

$$a_j{}^{\star}=\sum_{i=j}^{n}f_i\exp\{c(y_i{}^2-q^2)\} \tag{A25}$$

The integrand for I_j is always bounded (when $q>0$, $t>0$) and, typically, is very flat over the narrow range of the limits; the simplest of numerical integration routines will usually be adequate for evaluation of I_j. The data analysis discussed in the text uses the following 'Simpson rule' algorithm.

(a) Calculate the scalar $H_0=\sum f_ih_0(y_i)$ and the vector $\{a_j{}^{\star};\ j=n,n-1,\ldots,1\}$.

(b) An initial evaluation of $\hat{\theta}(q)$ is

$$\hat{\theta}_0(q)=A^{-1}\left(H_0-\sum_{j=1}^{n} a_j^{\star}I_{j,0}\right) \tag{A26}$$

where

$$I_{j,0}=(Y_j-Y_{j-1})\,h_1(z_j)\exp(-cz_j^2),$$
$$z_j=(Y_{j-1}+Y_j)/2, \qquad Y_j=(y_j^2-q^2)^{1/2} \tag{A27}$$

(For some purposes, $\hat{\theta}_0(q)$ may already be quite accurate and further numerical integration unnecessary.)

(c) A sequence $I_{j,1}, I_{j,2}, \ldots$, of improved approximations to I_j, is then calculated separately for each j. $I_{j,1}$ uses a five-point Simpson evaluation, $I_{j,2}$ uses ten points, and this doubling is continued until convergence is reached. Notice that, to achieve a desired accuracy in $\hat{\theta}(q)$, the I_j need not be calculated to the same accuracy for each j; unnecessary evaluations can therefore be avoided by a convergence criterion of the form

$$A^{-1}a_j^{\star}|I_{j,r+1}-I_{j,r}|<k\hat{\theta}_0(q) \tag{A28}$$

where $k=0{\cdot}00001$ say. Often $\sum a_j^{\star}I_j$ is dominated by its first term and I_1 is the only integral requiring more than 10-point evaluation.

The general framework of Nicholson (1970) provides for variance estimation; here, an unbiased estimator of the variance of $\hat{\theta}(q)$ is

$$\widehat{\mathrm{var}}\{\hat{\theta}(q)\}=A^{-2}\sum_{i=1}^{N} h^2(y_i) \tag{A29}$$

Notice that it cannot be evaluated by a similar construction to (A23); if such variance estimates are needed, calculation of the N integrals in (A2) has to be performed directly, using (A16). Typically, this increases the computation time by an order of magnitude. In fact, it is argued in the text that the results of this paper are most usefully applied in a situation where routine calculation of variance estimates is not required.

It is not clear as to exactly how much of the Nicholson (1970) framework carries over to the assumption of a more general, stationary, marked stochastic process for the particles. The variance formula (A29) is firmly tied to the assumption of a Poisson distribution for N but the results of Mecke & Stoyan (1980) would suggest that the estimator $\hat{\theta}(q)$ remains unbiased under wider model assumptions than a Poisson process.

APPENDIX B. VARIABLE SLICE THICKNESS

It is assumed that the variation in slice thickness is gradual, such that, locally, the section planes are parallel and of fixed distance apart, t. The value of t is then assumed to vary from one local area to the next, independently of the size or number of spheres that the slice contains; the mean slice thickness is denoted by t_0 and the coefficient of variation by $C_t^2(=\mathrm{var}(t)/t_0^2)$. In practice, it is usually not difficult to supply an approximate value for C_t^2.

Letting $E_t(.)$ denote expectation with respect to the distribution of t, equations (A8) and (A6) become

$$E(N)=E_t\{E(N|t)\}=N_V(q)\{t_0+\mu(q)\} \tag{B1}$$

$$f(y|q)=E_t[t/\{t+\mu(q)\}]\,g(y|q)+E_t[1/\{t+\mu(q)\}]\,y\int_y^{\infty} g(x|q)/(x^2-y^2)^{1/2}\,\mathrm{d}x \tag{B2}$$

A Taylor series expansion shows that, approximately,

$$E_t[1/\{t+\mu(q)\}]\simeq[1/\{t_0+\mu(q)\}][1+C_t^2/\{1+\mu(q)/t_0\}^2] \tag{B3}$$

so that

$$f(y|q)\simeq(1-c_1C_t^2)[t_0/\{t_0+\mu(q)\}]\,g(y|q)+(1+c_2C_t^2)[1/\{t_0+\mu(q)\}]\,y\int_y^{\infty} g(x|q)/(x^2-y^2)^{1/2}\,\mathrm{d}x \tag{B4}$$

where $c_2 = 1/\{1+\mu(q)/t_0\}^2$ and $c_1 = \{\mu(q)/t_0\}c_2$. Regarding c_1 and c_2 as known constants, the integral equation (A9) becomes

$$(1+c_2C_t^2)\int_q^x yh(y)/(x^2-y^2)^{1/2}\,dy+(1-c_1C_t^2)\,t_0h(x)=l(x) \qquad \text{(B5)}$$

and the solution will be given by equations (A16)–(A25), exactly as before, except that t is replaced by $t' = t_0(1-c_1C_t^2)/(1+c_2C_t^2)$ and A is replaced by $A' = A(1+c_2C_t^2)$.

These corrections are usually easy to make and will very often be negligible. For example, $\mu(q)$ is approximately the mean sphere diameter, so that if this is roughly equal to t, $c_1 = c_2 = 0{\cdot}25$; in fact, the maximum values of c_1 and c_2 are 0·25 and 1 respectively. Typically, C_t^2 is very much less than unity; for example if $t_0 = 10$ μm, and the standard deviation of slice thickness is as large as 2 μm, C_t^2 is still only 0·04. Using these values with the data discussed in the text, the adjustments are negligible, of the order of 0·1–0·5% in $\hat{V}_V(q)$ and $\hat{N}_V(q)$.

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