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A method to correct adequately for the change in neuronal size when estimating neuronal numbers after nerve growth factor treatment

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Summary

A method is described to compute the correct distribution of nuclear diameters in thick sections of superior cervical ganglia of rats from the observed distribution of nuclear profiles. This method is applicable to the problem of correcting for the differing diameters of cells observed after treatment with nerve growth factor (NGF). It overcomes the errors due to the failure to count small fragments of nuclei too thin to be seen and the multiple counting of nuclei in more than one section. It has been shown that the changes in number of neurons after NGF treatment were not as large as previously supposed. An estimate of the volume occupied by the cell bodies suggests that the numbers calculated here are correct.

Introduction

The qualitative morphological effects of nerve growth factor (NGF) on the rodent sympathetic nervous system have been well described by many workers (Levi-Montalcini and Booker, 1960a; Thoenen *et al.*, 1971; Zaimis *et al.*, 1972). Quantitative studies, however, have failed to correct for the effect of changing cell diameters on the final number of cell profiles counted. This has resulted in an overestimation of the number of adrenergic cells in NGF treated ganglia. It was the intention in this study to devise an accurate method to determine the total neuron number in sympathetic ganglia from normal and NGF treated animals. This method gives an accurate correction for the changes in cell size with treatment and is suitable for thick sections used in light microscopy.

Many of the problems of counting absolute numbers of neurons in a nerve centre have been reviewed by Konigsmark (1970). Sympathetic ganglia provide a defined volume in which to estimate total numbers thus eliminating one of the problems involved in counting cells in C.N.S. nuclei. The first major problem in cell counting is to settle on a satisfactory unit to count, either whole cells, nuclei or nucleoli. The

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most accurate results come from nucleolar counts if there is only one per cell. In the sympathetic system, however, there are commonly 3 or more nucleoli per cell thus rendering this method impracticable. Nuclear counts have two major drawbacks, the first is the overestimate of the number due to the inclusion of a nucleus in more than one section (the split cell problem), and the second is the underestimate of the numbers due to the inability to see small segments of nuclei because of underlying cytoplasm. Counts on whole neurons are rendered difficult by these same problems, and, in addition, irregular shape and branches lead to an overestimate of numbers and an underestimate of the diameters as a result of the counting of small pieces of cytoplasm, for example the enlargements at the base of dendrites and the axon.

The split cell problem which results in an overestimate of cell numbers has been examined by several workers, and a mathematical correction can be applied to sections of thickness T containing cells of average diameter D by the method of Abercrombie (1946), such that

$$N_V = N_A \left(\frac{T}{T + D}\right),$$

where N_v is the number of units that should be counted in the volume of the section and N_A is the actual number of units counted in the area of the section. This correction factor, however, makes no allowances for the failure to count small undetectable fragments of cells or nuclei. Floderus (1944) proposed the correction formula:

$$N_v = N_A \left(\frac{T}{T + D - 2b}\right)$$

where b is the thickness of fragments too small to be detected. Both of these correction formulae, however, assume one size of cell or nucleus and depend upon an accurate determination of the average diameter. If the sections are very thin compared to the diameter of the cell then an approximation is that the true average diameter = $4/\pi \times$ average diameter of the profiles. If, on the other hand, the section is very thick compared with cell diameters then the true average diameter approximates the average diameter of the profiles. When counting cells, however, there is a tendency to underestimate the diameters due to the inclusion of the smaller diameters of cytoplasmic processes, thus resulting in an overestimate in the number of cells present. This is partially compensated for by the failure to recognize thin fragments of cells which are included in the theoretical distribution.

The unit used in this study was the nucleus because it is approximately spherical and has no irregular protrusions. The very nearly circular profiles resulted in improved accuracy in the estimate of the diameters and the elimination of the risk of counting re-entrant pieces of the same unit as may occur with the more irregular shaped cells. Coupland (1968) analysed this problem when a range of sizes of units is present, in relation to counting adrenal granules in thin sections used for electron microscopy, and proposed a method to arrive at the true distribution of diameters

from the distribution of profiles observed. This method has been recently revised by Anker and Cragg (1974) and used by Cragg (1974) to estimate the number of synapses in cat cortex.

The present work outlines a method based on the principles described by Coupland (1968) which is applicable to the analysis of nuclear counts in thick sections. It overcomes the errors due to the failure to count small fragments of the nuclei, split nuclei, as well as allowing for a distribution of nuclei of differing sizes.

Principle of the calculation

The principle of this calculation is to reduce the observed distribution of nuclear diameters to the theoretical distribution of true nuclear diameters. This is achieved by an iterative subtraction of all fragments of larger cells, that appear in the distribution as smaller profiles, to the limit where the fragments are too thin to be seen. Only nuclei with their centres in the section are included in this distribution so that the accurate calculation of the mean nuclear diameter can be made and the

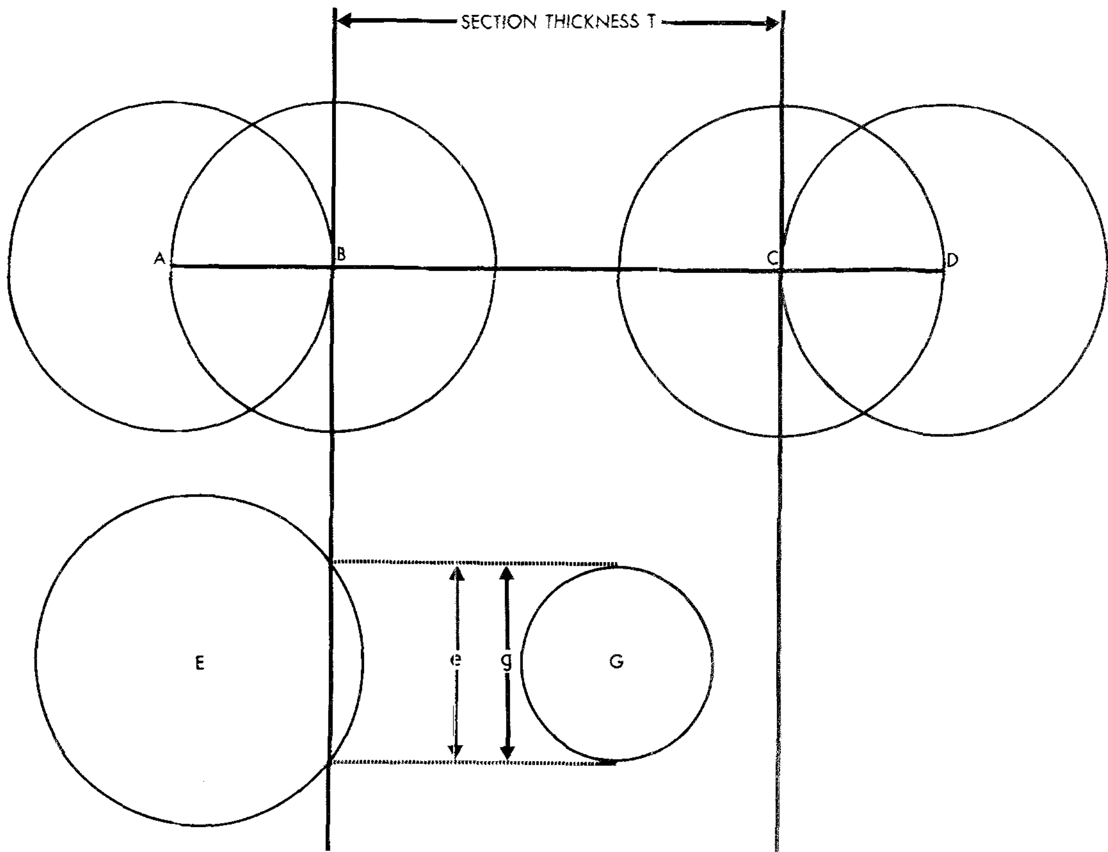

Fig. 1. Schematic representation of possible positions for nuclei in a tissue section of thickness *T*. For explanation see text.

correction factor to calculate total neuron numbers from the crude cell counts is determined directly.

The aim in this method is to reduce all nuclei to unit points represented by the centre of the nucleus. In this way the split cell error is eliminated and only nuclei with centres within the plane of section are counted. These nuclei will all present full diameters within the plane of section whereas the fragments of nuclei to be eliminated from the count will present only fractions of the full diameter.

In Fig. 1 the possible extremes are shown. Nucleus A lies just outside the section and will not be seen. As its centre moves from position *A* to *B*, for example *E*, with apparent diameter (*e*), the fragment of the nucleus included in the section will become larger until it reaches position B when it will present a full diameter in the section and have its centre within the section. If its centre lies anywhere between *B* and *C*, for example *G*, it should be included in the count and presents a full diameter (*g*) to the section. As the centre goes from *C* to *D* outside the volume of the section it will present less than full diameter fragments until it can no longer be seen in the section. In a random series of sections through the ganglion, nuclei have an equal chance of having their centres anywhere between *A* and *D*. The proportion of nuclei

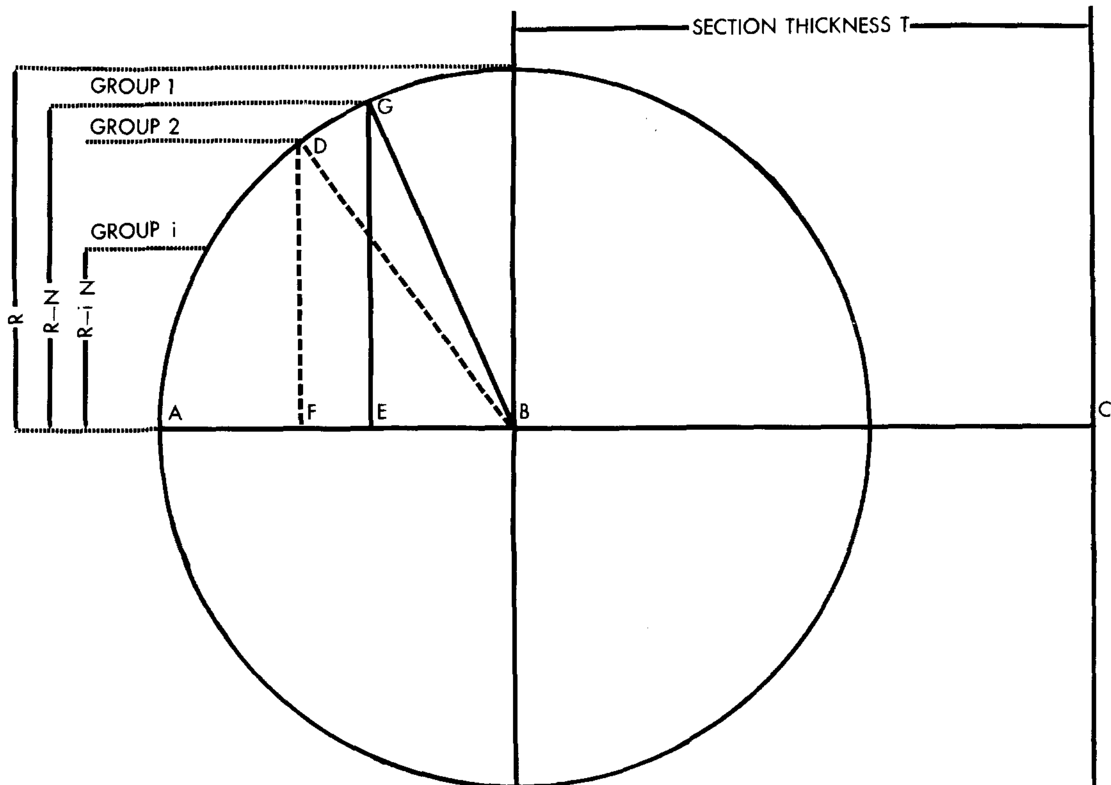

Fig. 2. Schematic representation of origins of fragments of nuclei in a population of cells of radius *A—B* which could be included in a tissue section of thickness *T*.

having their centres in the section which should be included in the count compared to the total number of profiles seen is BC/AD.

Fig. 2 looks more closely at the origin of the profiles in a section of thickness T. The radius of the fragment that arises from a nucleus with its centre outside the section will be given by the perpendicular from its centre to the circle centred on B. For example, a nucleus with its centre at E will create a fragment in the section with maximum radius EG (e in Fig. 1). If we arrange all these possible fragments into groups of interval N we can construct a histogram of the total number of profiles that will be in each group. In the group of largest radii from R to $(R-N)$ the smallest radii will come from nuclei with centres at E so that $EG = (R-N)$. In this group there will be both complete nuclei and fragments. The fragments will come from nuclei on both sides of the section so that the proportion (P_1) of nuclei with centres within the section in this group is:

$$P_1 = BC/(BC + 2BE)$$

or, since $BC = T$ and $BE = \sqrt{[R^2 - (R-N)^2]}$,

$$P_1 = \frac{T}{T + 2\sqrt{[R^2 - (R-N)^2]}}\,. \tag{1}$$

Thus, if S_1 profiles are counted in the size group R to $(R-N)$, there will be $P_1 S_1$ nuclei with centres within the section, and only this number $P_1 S_1$ will be counted into the density of nuclei within the section.

The nuclei with the same size R will occur with centres further from the section than E, and these will give rise to smaller profiles in the section. It is necessary to calculate how many of these smaller profiles are to be expected, for if the observed number of smaller profiles exceeds this expectation, it means that nuclei of a smaller size than R must also be present.

A group of profiles between $(R-N)$ and $(R-2N)$ will be generated by nuclei of size R, the centres of these nuclei lie between E and F, and the number Z_2 of these profiles will be:

$$Z_2 = \frac{2EF}{BC + 2BE} \times S_1$$

or

$$Z_2 = \frac{2\{\sqrt{[R^2 - (R-2N)^2]} - \sqrt{[R^2 - (R-N)^2]}\}}{T + 2\sqrt{[R^2 - (R-N)^2]}} \times S_1\,. \tag{2}$$

In the general case in the group i with nuclei between $(R-(i-1)N)$ and $(R-iN)$ there will be

$$Z_i = \frac{2\{\sqrt{[R^2 - (R-iN)^2]} - \sqrt{[R^2 - (R-(i-1)N)^2]}\}}{T + 2\sqrt{[R^2 - (R-N)^2]}} \times S_1 \tag{3}$$

of the profiles in the largest group. Thus a series can be calculated giving the number

of fragments of nuclei in the smaller groups as a fraction of the number in the largest group.

However, there is more than one nuclear size in the sympathetic ganglion and this theoretical distribution of fragments can be used to derive the actual distribution of nuclear radii in the ganglion. This can be done by taking the largest group (S_1) and calculating the number of nuclei in this group whose centres are contained within the section ($P_1 S_1$). The number of fragments (Z_i) that this number of nuclei will contribute to each of the subsequent groups is subtracted from that group. Thus all the fragments attributable to the number of nuclei in the largest group are eliminated. The remaining number of profiles in the next smaller group (S_2) is treated in the same way as this process is repeated until all the profiles originally present can be accounted for in the final distribution of nuclear radii.

This procedure results in negative values being found in several of the smaller groups (i.e. $Z_i > S_i$). This is the result of several factors culminating in the failure to observe the entire theoretical distribution outlined above. The major factor is the difficulty in distinguishing the thin fragments cut from the pole of nuclei from the underlying cytoplasm. It is also possible that the knife may not cut through the pole but displaces it so that again the theoretical small fragment is not seen. The diameter of the thinnest section that can be seen varies for the size of the nucleus. In Fig. 1 that fragment of the nucleus centred on E has the same diameter as the nucleus centred on G, but is very much thinner. In the smallest diameter group the thickest fragments, not having the full nuclear diameter, arise from cells in the next largest group. As no profiles can be observed in the next smallest group, the diameter of which is within the resolution of the microscope, it must be assumed that these fragments are the thinnest that can still be seen. Thus the thickness of the thinnest profile can be calculated from the radius r of the second smallest group:

$$t = r - \sqrt{(2Nr - N^2)}. \qquad (4)$$

Thus for any nucleus of radius R the radius of the thinnest fragment will be:

$$\sqrt{(2Rt - t^2)}. \qquad (5)$$

The series of subtractions should, therefore, be continued until this limit is reached for any nuclear size.

The fraction (P_i) of the number of nuclei whose centres lie within the section to the total number of profiles counted gives the correction factor to correct nuclear profile density per unit area on the slide to the density of neurons per unit volume. The true distribution of diameters results in the ready calculation of the average diameter and its standard deviation.

Example calculation

Table 1 shows the individual steps in the calculation from the crude profile distribution or line 1 to the final true nuclear diameter distribution on line 15. The first step is to calculate the proportion P_1 of the profiles in the largest group

Table 1. Example of calculation for nuclei of normal 5 day old superior cervical ganglion.

Diameter 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0	0	2	14	46	96	104	102	80	39	25	24	4	4	1	1
2			2	14	46	96	104	102	80	38.9	24.9	23.9	3.91	3.89	0.84	0.60
3			2	14	46	96	104	102	79.9	38.9	24.9	23.9	3.82	3.76	0.51	
4			2	14	45.9	95.8	104	102	79.7	38.6	24.6	23.5	3.26	2.31		
5			2	14	45.8	95.7	104	102	79.5	38.4	24.2	23.0	2.04			
6			2	14	45.2	94.9	103	100	77.8	36.2	21.1	14.6				
7			2	14	44.6	94.1	101	98.8	75.9	33.4	13.6					
8			2	14	43.4	92.5	99.4	95.9	71.6	22.0						
9			2	14	40.4	88.3	93.6	87.3	48.3							
10			2	11.1	36.1	82.0	83.9	60.2								
11			2	7.70	30.7	73.4	59.3									
12			2	3.81	24.0	53.4										
13			2	1.96	18.1											
14			2	1.53												
15		0	1.64	1.53	18.1	53.4	59.3	60.2	48.3	22.0	13.6	14.6	2.04	2.31	0.51	0.60

Number of profiles = 542; number of diameters = 298; correction factor = 0.550; average nuclear diameter = 6.965 ± 2.002.

15–16 μm that have centres within the section. From Equation 1 substituting $T = 8$, $N = 0.5$, $R = 8$, we derive $P_1 = 0.60$. This represents the fraction of the distribution of cells with diameters 15–16 μm to be included in the final distribution. Nuclei in this size range will also have fragments in the next smaller group and the proportion can be calculated from Equation 2 as 0.16 which when subtracted from the profiles in this group leaves 0.84.

The subtraction continues to the limit of the thickness that cannot be seen. The smallest diameter fragments seen are in the group 2–3 μm and if these come from cells in the group 3–4 μm then the thinnest fragments seen are 0.67 μm thick substituting $r = 2$ in Equation 4. A nucleus of diameter 16 μm will have a fragment of diameter 6.4 μm as the smallest fragment that can be seen substituting $R = 8$ and $t = 0.67$ in Equation 5. Thus the subtraction continues until the group 6–7 μm is reached, or as i goes from 1–8 in Equation 3.

These values form a new distribution of profiles (line 2) and the correction can now be made for the nuclei in the group 14–15 μm and these corrected values are on line 3. This process continues until all the original profile distribution has been accounted for by complete nuclei. This final distribution appears on line 15 and is the true distribution of nuclear diameters in the ganglion. The total number of nuclei is line 15, 298, divided by the total number of profiles in line 1, 542, gives the correction factor of 0.55. Thus only 0.55 of the total number of profiles counted in the sections should be counted as nuclei and the remainder represent fragments.

Methods

NGF was purified by the method of Schenker *et al.*, (1976) and administered to one half of a litter of 8 rats daily from birth to 5 days. The other 4 litter-mate controls received saline.

Superior cervical ganglia were removed and fixed overnight in 10% neutral formaldehyde, then dehydrated in a series of increasing strength ethanols, cleared in chloroform for 1 h and embedded in paraffin. Serial 8 μm sections were cut in the longitudinal plane of the ganglion and stained with 1% cresyl violet.

In this method the correction factor must be calculated from the same sample as the nuclear density because of the allowance for fragments too thin to be seen. It was decided to take 5 photographs from each ganglion as this sample with each photograph representing a volume of 4.8×10^{-4} mm^3. These were projected onto white paper at a final magnification of 500 and all nuclear sections that could be seen were outlined and a radius estimated using a Zeiss particle size analyser. For the determination of nuclear diameter sufficient photographs to give more than 500 profiles were taken and the other photographs used to obtain a larger sample for the cell density. If there were less than 500 profiles in the 5 photographs then all were used in the determination of the nuclear diameter. The value for the nuclear density obtained from the photographs was checked for the first control ganglion by counting 100 frames at a magnification of 400 directly through the microscope. This represented 5% of the volume of the ganglion. The values were 180.66 ± 6.26 (S.E.M.) for the photographs and 194.37 ∓ 1.31 (S.E.M.) for the direct measurements corrected to the volume in the photographs. The values for the direct count are higher due probably to the better resolution of small fragments through the microscope. This discrepancy is overcome by using the same source in obtaining the correction factor and nuclear density.

The volume of the ganglia was obtained by measuring the total area of the serial sections and multiplying by the thickness of the sections. The total number of neurons in the ganglia was

calculated from this volume multiplied by the average nuclear density and divided by the volume of the photograph.

The whole cell diameter was calculated by outlining all basophilic staining cytoplasmic areas and then using the same procedures as for the nuclear diameter. These measurements are subject to large errors due to the irregular shape of the cell. The cell volume was calculated from the average cell diameter assuming it to be spherical.

Results

This method of morphometric analysis was used to examine the effect of NGF on the neonatal rat superior cervical ganglion. Two groups of animals were examined, normal 5 day old rats and rats treated from birth daily for 5 days with 10 $\mu g\ g^{-1}$ NGF subcutaneously. A comparison of the nuclear diameters is shown in Fig. 3. The profile distribution for the crude nuclear profiles is shown in Figs. 3A and B for representative ganglia from each group. These crude measurements are corrected to form the distribution of the true nuclear diameters which are shown in Figs. 3C and

Table 2. Morphometry of neurons of the superior cervical ganglion of 5 day old rats with and without NGF treatment from birth.

	Ganglion volume (mm^3)	*Neuronal nuclear diameter (μm)*	*Neuronal cell diameter (μm)*	*Neuronal nuclear numbers*	*Total cell volume (mm^3)*	*Neurons % volume*
Control	0.086	6.97	11.2	17815	0.013	15
5 days	0.108	7.05	11.3	20013	0.015	14
	0.133	8.50	12.9	18835	0.021	16
	0.145	7.13	11.5	20082	0.016	11
mean	0.118	7.41	11.73	19186	0.016	14.0
SEM	0.013	0.36	0.38	539	0.002	1.1
NGF	0.447	10.25	15.3	24710	0.046	10
treated	0.371	10.63	14.6	26227	0.043	12
	0.469	11.45	16.1	25708	0.056	12
	0.386	11.00	14.9	22236	0.038	10
mean	0.418	10.83	15.23	24720	0.046	11.00
SEM	0.024	0.26	0.33	885	0.004	0.58
P<	.001	.001	.001	.001	.001	0.05

Animals were treated from birth for 5 days with 10 $\mu g\ g^{-1}$ NGF by subcutaneous injection daily; control animals received saline. Ganglia were processes as described in the text and morphometric parameters estimated as described in the section on the principle of the calculations. Total cell volume was calculated from the volume of the average cell times the number of cells present.

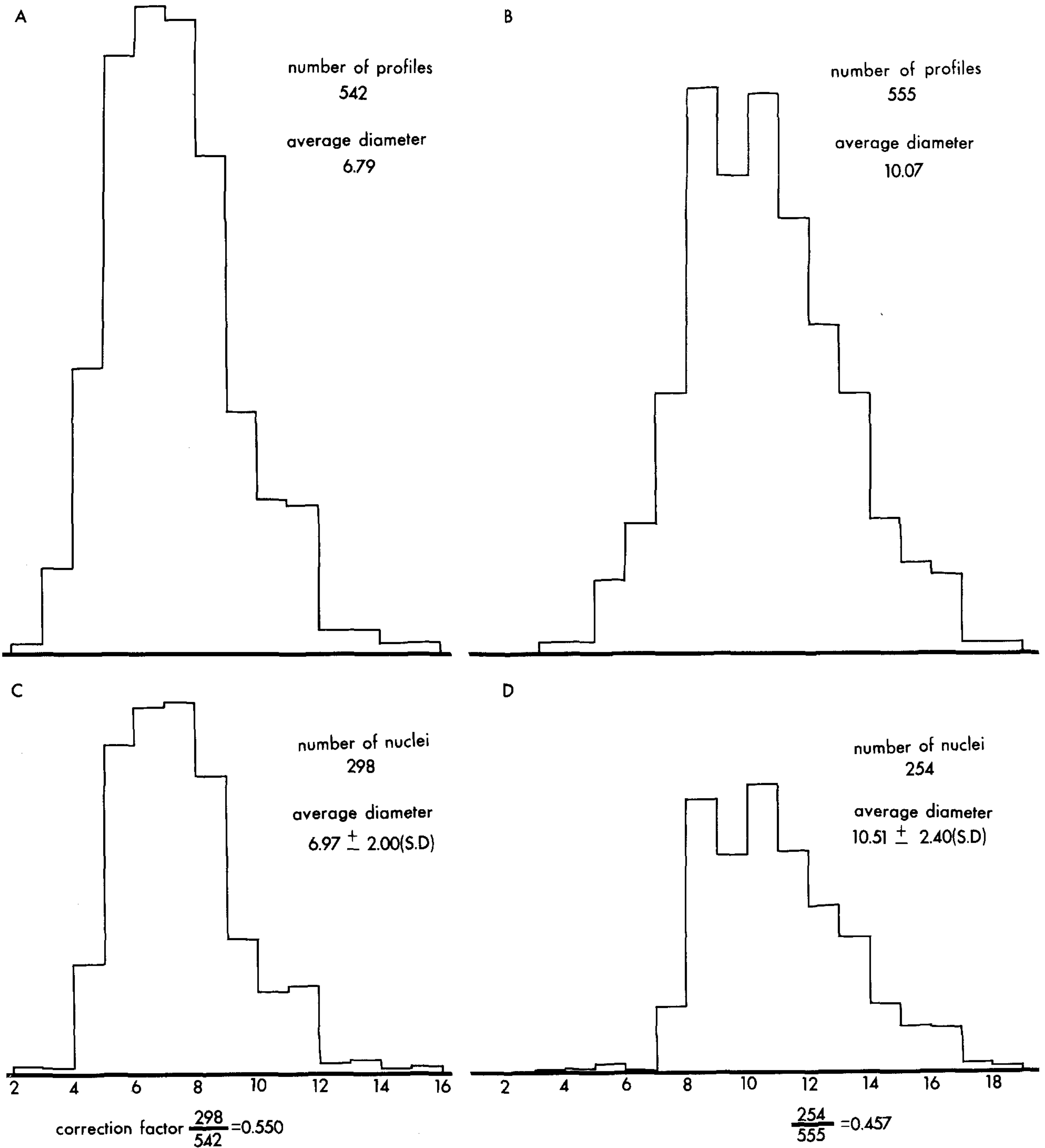

Fig. 3. Distribution of diameters for crude profiles for (A) control 5 day old ganglion and (B) NGF treated ganglion from birth to 5 days and true nuclear diameters derived in (C) from (A) and in (D) from (B). Three photographs were used to derive the values in the control ganglia and nine were used to obtain equivalent numbers in the NGF treated ganglion.

D. It should be noted that different numbers of photographs were analysed, three for the control and nine for NGF treated to obtain nearly equal numbers of profiles. From these distributions a mean nuclear diameter can be calculated and an estimate of the standard deviation made. It can be clearly seen that there is a significant difference between these distributions ($P < 0.001$). Complete details for the number of neuronal nuclei, nuclear diameter and cell diameter, total ganglion volume and a calculation of the volume of the ganglion occupied by cell bodies for the two groups of ganglia are shown in Table 2. Overall there is a small increase in the number of neuronal nuclei in NGF treated ganglia ($P < 0.001$).

Discussion

The advantage of this procedure is in the calculation of the nuclear diameter. Previous techniques for neuron counting have assumed a constant cell size for the calculation of a correction factor (Floderus, 1944; Abercrombie, 1946). The cell diameter was derived from the average profile diameter, but there is no constant relationship between these two values which are approximately equal for very small cells and differ by a factor approaching $\pi/4$ for very large cells. The present technique results in the direct calculation of the average cell diameter over the entire range of diameters that occurs after treatment with factors such as NGF, but the correction factor is independent of it. Three different factors can be calculated using the Floderus technique, one assuming the profile diameter equals the nuclear diameter, the second using the nuclear diameter calculated by the above method and thirdly assuming the profile diameter equals $\pi/4$ times the nuclear diameter. These factors may also be altered by differing assumptions for the thickness of fragments that cannot be seen. If we assume that this thickness is the same as calculated in the present study then a comparison of the correction factors can be made. For the control ganglion the present method gives a factor of 0.55 and the Floderus method yields the three values 0.511, 0.572, 0.580 while the NGF treated ganglion has correction factors using the present method of 0.457 and using the Floderus method 0.400, 0.467, 0.480. It can be seen that the values calculated by the present method lie within the range of the Floderus values and hence differ only slightly from this method of correcting for changes in nuclear diameter.

The method of calculation of the number of neurons in a sympathetic ganglion presented here, enables correction for two of the major errors in estimating the number of cells in a neuronal population from thick sections – firstly the split cell error where one cell is counted more than once and secondly the failure to count thin fragments of the cell or nuclei. The use of the nucleus instead of cell bodies as the unit for counting results in a more accurate estimation of the numbers, as the nucleus is closer to being spherical and the numbers of small processes present on neurons are not counted. In addition, the estimation of the diameter of the profiles is more accurate in the more nearly circular profiles.

Previous reports have given higher estimates for the number of cells in neonatal

rat superior cervical ganglia. Levi-Montalcini and Booker (1960b) found 32 000 neurons in 7 day old rat superior cervical ganglia and Thoenen *et al.* (1971) found 29 800 neurons in normal 5 day old rat superior cervical ganglion and 74 000 in ganglia of treated rats. In neither of these reports was any description of correction for changing cell diameter given. There are several factors responsible for obtaining high neuronal counts in this system:

(1) Failure to correct for changes in cell diameter.

(2) Overestimation of cell numbers by counting nucleoli.

(3) Overestimation of cell numbers by counting small irregular processes of cell bodies.

(4) Underestimation of cell diameter due to counting small fragments leading to an overestimation of cell numbers.

(5) In addition there may be a significant difference in the total numbers of adrenergic neurons in the ganglion of different strains of rats.

The lower counts obtained in the present experiments are mainly due to the correction made for the changing cell diameter.

In the present series of experiments the diameter of the cell bodies is probably an underestimate due to problem 3 above. This will result in an underestimate in the volume occupied by neurons in the ganglion. The volume estimated is nevertheless in the range expected from the area occupied by the profiles which is around 30% in control ganglia and 25% in NGF treated ganglia. The calculation of the expected neuronal volume from the area of profiles is also subject to many errors but the % volume will be less than the % area measurement.

The moderate increase in the number of neurons in the ganglia after NGF treatment may be explained either by an enhanced survival of neurons or an increased rate of cell division. Preliminary results using [^{3}H] thymidine autoradiography indicate that NGF does not cause cell division during this period, suggesting that enhanced survival may be the mechanism (Hendry, 1975).

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