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Segmental incorporation of [^{3}H]leucine in the rat spinal cord

Is the protein metabolism of nerve cells related to the size of their axons?

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INTRODUCTION

Experimental studies of the protein metabolism of nerve cells are concerned mainly with data relating to the functional activity of nerve cells (Gaitonde & Richter, 1956; Flanigan, Gabrieli & MacLean, 1957; Altmann, 1963), their state of development (Hydén, 1943; Gaitonde & Richter, 1956; Palladin, 1957; Lajtha, Furst & Waelsch, 1957; Adams & Lim, 1966; Schain, Carrer & Copenhaver, 1967; Gutmann, Jakoubek, Fischer & Babicky, 1967) or pathological alterations (Brattgård, Hydèn & Sjöstrand, 1958; Fischer, Lodin & Kolousek, 1958; Francoeur & Olszewski, 1968). However, a substantial portion of the proteins synthesized by the nerve cell perikaryon are carried into the axon by way of axonal flow (Droz & Leblond, 1962, 1963; Ochs, Johnson & Ng, 1967). The volume of the axon, therefore, may influence the basic rate of protein synthesis of the perikaryon. Experimental elucidation of the relations between axonal volume and the rate of protein synthesis in the neuronal perikaryon is beset with discouraging difficulties. It is not practical to determine the length and diameter of the axon for each of the cells of a given nucleus. Also, it is of little value to compare various nuclei that project over different distances since the function of these nuclei may not be comparable.

The present investigation of segmental variation in amino acid uptake in rat spinal cords avoids such difficulties in experimental design. The motor neurons of the anterior horn can be considered a functionally homologuous cell population; also, obvious differences in average axonal length exist between the segments that supply the extremities and those supplying the neck and trunk. Our report includes quantitative determinations of the uptake of tritiated leucine per segment of spinal cord, using scintillation counting, and autoradiographic studies of leucine uptake per cell as well as measurements of cell size.

MATERIALS AND METHODS

Scintillation counting. Three adult rats (Sprague–Dawley strain) received 15 μc/g body weight of tritiated leucine (specific activity, 54 c/mM) in three injections at hourly intervals; they were killed 2 h after the last injection. The animals were perfused from the heart with neutral formalin under deep chloroform anaesthesia. The spinal cords were removed and segments of exactly 3 mm in length were cut, starting at the cranial end of the cord, by inserting the cord into a U-shaped plexiglass holder with knife guard. The cords and segments were stored in a moist chamber, weighed

and extracted with 3:1 (v/v) ethanol–ether overnight, with occasional shaking. The samples were washed twice in ethanol–ether and then homogenized in the counting vial, using a glass piston. All particles were washed down with 1 ml of 1 N-NaOH, hydrolysed for 45 min at $75 \pm 1°$ C, and scintillation solution (Bray's solution) was added to the hydrolysate. Counts were made in a Unilux T. M. II liquid scintillation system. Counts were corrected for 100% efficiency with internal standard solution.

The 3 mm segments used in this study were identified in terms of anatomical segments by preparing the cord with attached roots of three additional rats. Incisions were made at 3 mm intervals and the relative positions of the cervical, thoracic and lumbosacral segments were determined by comparing the origin of the roots with the incisions. These calibrations are shown at the top of Figs. 1 and 3.

Autoradiographic methods. Four adult rats (Sprague–Dawley) were given a total of 30 μc/g body weight of tritiated leucine (specific activity, 45 c/mM) in three intraperitoneal injections at hourly intervals. The reasons for this distribution of the dosage are given in the discussion. Two hours after the last injection the rats were perfused under deep anaesthesia from the heart with isotonic formalin (Cammermeyer, 1960). The spines were then dissected free and postfixed in formalin for several days. The cords were removed, washed under running tap water to remove unbound leucine and cut into segments of exactly 3 mm length, numbered beginning with the cervical-medullary junction. All segments of a given cord were dehydrated at the same time and were embedded in one paraffin block in three tight columns of eight to nine segments each; the whole set of cross-sections measured approximately 10 by 35 mm. This method of embedding minimized variation due to the following factors: tissue shrinkage during dehydration and embedding; thickness of the section; deformation of the tissue in cutting; and emulsion coating. The sections were counterstained with fast green Kernechtrot.

Sections were cut at 10 μm, mounted with their long axis across 50×75 mm slides, and coated with Kodak NTB-2 emulsion. The purpose of mounting crosswise was to minimize the gradient in emulsion thickness along the slide which results from the draining of the emulsion after dipping. A gradient in emulsion thickness along a 35 mm specimen was difficult to control; it was found to produce slight variation in the grain density along the specimen, evidently due to inadequate emulsion thickness in the portion that was up when the emulsion was drained. Mounting the sections crosswise made it easier to obtain a minimum layer of 3 μm emulsion, with only negligible variation along the 10 mm axis of the specimen. The emulsion was gelled immediately after dipping by chilling the slides on a cold plate (Rogers, 1967) to avoid stress artefacts. Slides of animals without label were exposed as controls for chemography. Trial slides were exposed for 2, 4 and 6 weeks to determine optimum grain density for counting. Grain counts were made with an oil-immersion lens, using a square ocular net micrometer; the term grain density refers to the number of grains per arbitrary unit area in a 10 μm thick section (see Discussion for further comments). The grain density over nerve cells was approximately 10–20 grains per field after 2 weeks exposure, which was optimal for counts. Average grain density of background was 0·1–0·5 grain per field and was considered negligible.

The cells were identified and numbered in drawings of each anterior horn so that grain counts and measurements of cell size could be made for the same cells. Cells

of the anterior horn were selected at random, but only cells with a visible nucleolus were studied. Counts of grain density were made at a magnification of × 1250, using an ocular net micrometer. The grain density over a source of radiation is uniform only for those portions that are farther away from the edge of the source of radiation than the track length of the highest energy particle (Rogers, 1967). Accordingly, care was taken to make all counts in fields at least 3 μm off the cell membrane. Counts were made for cytoplasm, avoiding the nucleus. Additional counts over the nucleus were analysed separately. Ten counts were made per cell if the size of the cell permitted; proportionately fewer counts were possible for smaller cells. Ten cells per segment were counted at the levels of the thoracic and lumbosacral enlargement. Only three to six cells satisfying all of our criteria could be found in the sections of the thoracic and sacral segments. No effort was made to collect a total of 10 cells for each segment from several sections, since the statistical error due to variation in cell number per segment was considered more acceptable than the error due to variations among slides. To avoid investigators bias, segments were counted at random. All counts were made by the same person.

Determination of cell size. The size of the cell, the nucleus and the nucleolus was measured of each cell in which grain counts were made. For measurements of cell and nuclear size, the cells were projected on transparent paper attached to the screen of a Zeiss Ultraphot I photomicroscope. The cell body and nucleus were outlined and measured planimetrically with an Ott disc planimeter. It will be understood then that the terms of cell size, nuclear size and area of cytoplasm (defined as area of cell minus area of nucleus) as used in the following text refer to the respective areas in a cross-section through the largest dimension of the cell, and not to an actual three-dimensional reconstruction of the volumes. In another series of measurements, the diameters of the nuclei and of the nucleoli were measured directly with an ocular micrometer. All measurements refer to fixed, paraffin-embedded tissue; no effort was made to correct for shrinkage during embedding.

Statistical methods. Measurements of cell and nucleus were subjected to statistical regression analysis; correlation coefficients, slopes and intercepts were calculated.

Statistical evalution of the grain counts involved analysis of variable numbers of up to 10 counts per cell, made in up to 10 cells per segment, in 27 to 28 segments. Separate analyses of variance were made for the cells of each given segment; with only one exception at the 0·05 level, there was no statistically significant difference in grain density among cells. Next, all grain counts per segment were pooled, and an analysis was made of the variance among segments. Measurements of cell, of nucleus and nucleolus per segment were also subjected to analysis of variance.

RESULTS

Only one set of data is shown for each of the experiments; these are representative of all others.

(1) *Uptake of* [^3H]*leucine per segment of cord*

Scintillation counts of the segmental uptake of [^{3}H]leucine are shown in Fig. 1. There was no variation in the uptake of leucine per mg of tissue along the cord. Accordingly, the total uptake per segment of cord was proportional to the weight

of the segment, also shown in Fig. 1. The weight of cord segments, exactly 3 mm in length, showed increases with the cervical and lumbosacral enlargements of the cord, as well as a general increase from the caudal to the cranial end; the latter evidently reflects the caudo-cranial increase in the mass of the fibre tracts.

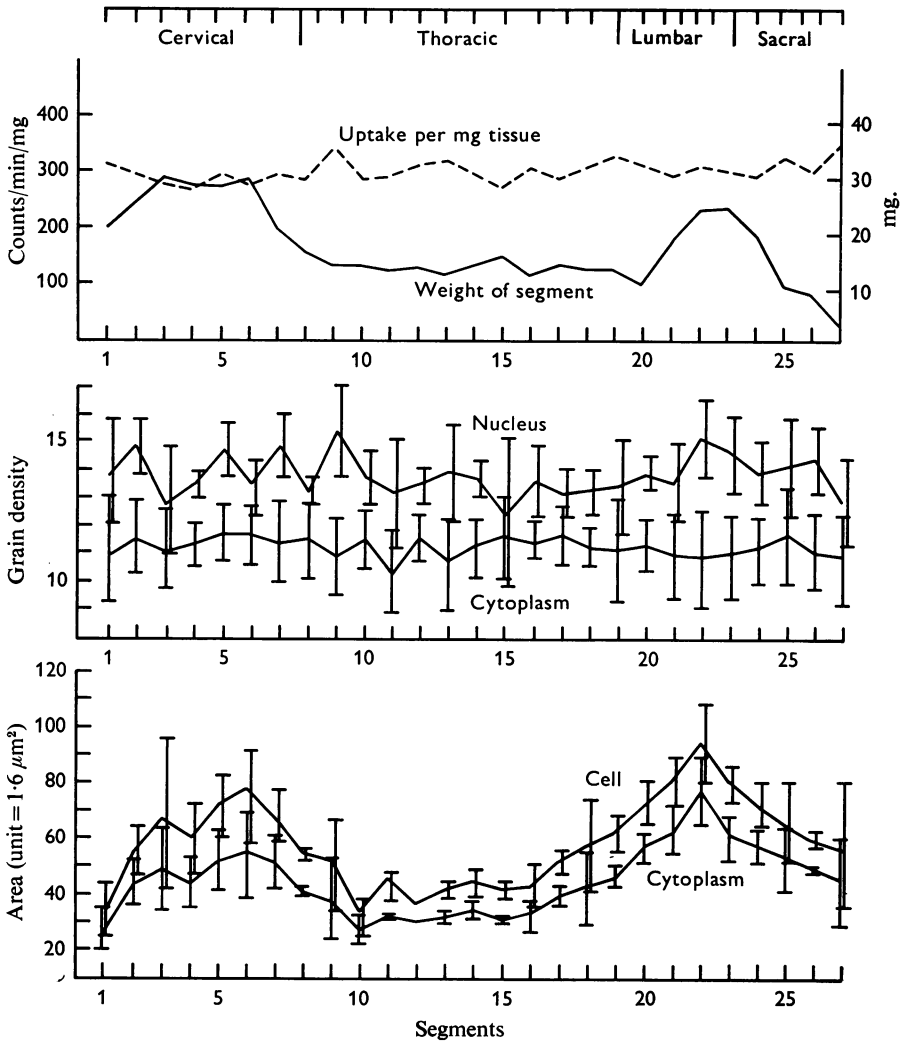

Fig. 1. Upper set of curves: uptake of [^{3}H]leucine per mg of cord tissue (broken line) determined by scintillation counts. The value for each segment is the average of four counts of the same sample corrected for 100% efficiency. The weight of the segments is shown by a solid line. Middle set of curves: grain densities in autoradiographs of [^{3}H]leucine uptake by anterior horn motor neurons tabulated separately for nuclei and cytoplasm. See 'Material and Methods' for details. The brackets indicate standard deviations. Lower set of curves: planimetric measurements of the areas of the whole cell and the cytoplasm (calculated as area of cell minus area of nucleus) in cross-sections through the largest extension of the cells. Brackets indicate standard deviations. This set of measurements represents the same cells for which the grain counts are shown. 'Segments' refers to cord segments of exactly 3 mm in length; the correlation with anatomical segments is shown at the top of the figure.

(2) *Autoradiographic and quantitative cytological data*

Grain counts of the uptake of [^{3}H]leucine by nerve cells (Fig. 1) showed no evident variation in grain density for segments of the spinal cord. Variation statistical analysis confirmed this impression. With one exception among 55 segments, there was no significant variation ($P > 0{\cdot}05$) among the cells of each segment. Analysis of the variation for pooled grain densities from each segment showed no significant variation ($P > 0{\cdot}05$) among the segments. Next, the data for all cells, regardless of segmental localization, were arranged in order of cell size or area of cytoplasm, respectively, and the grain density per cell was plotted (Fig. 2). The grain density was found not to depend on cell size. Since the location of every cell was recorded in drawings, additional comparison could be made of the medial and lateral cell

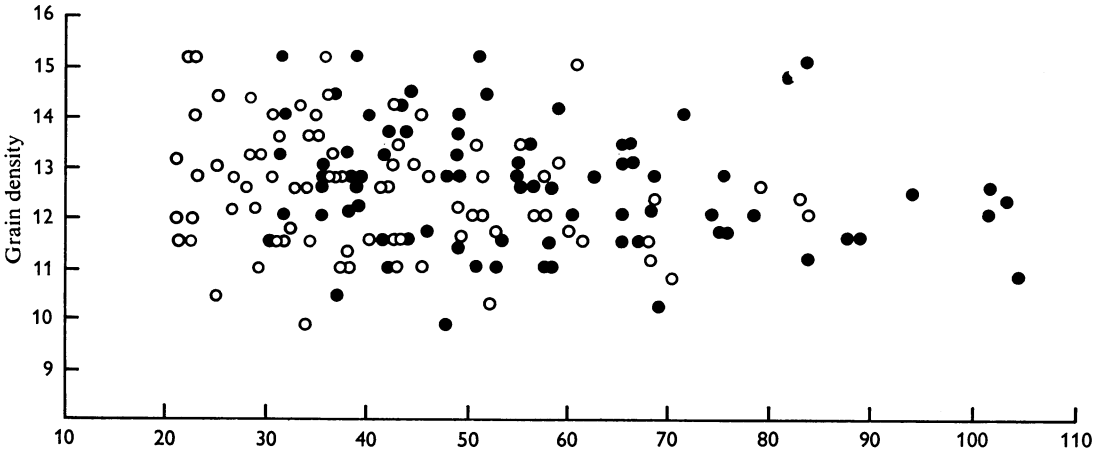

Fig. 2. Average grain density per cell plotted as a function of the area of the cell body, or the area of cytoplasm, respectively, regardless of the segmental location of the cell. The matrix of data is the same as that analysed in Fig. 1. ●, Area of cell body; ○, area of cytoplasm (unit = 1·6 μm^2).

groups of the anterior horn; the results of this comparison (not shown here) were also negative. The above statements pertain to grain densities over cytoplasm. The grain density over nuclei was generally higher (Fig. 1), but showed the same behaviour as grain density over cytoplasm.

The above data indicated that leucine uptake per volume of cytoplasm was independent of cell size. Accordingly, the size of the nerve cell was an approximate measure of the amount of leucine taken up by the cell. Data from Fig. 1 (bottom) compared with Fig. 3 show marked segmental variation in cell size, paralleled by similar variation in size of the nuclei. Analysis of these data showed a linear relation between nuclear size and cytoplasm (Fig. 4), with correlation coefficients of 0·68–0·70. There was also a linear relationship between the diameter of the nucleus and that of the nucleolus, with correlation coefficients of 0·88–0·89 (Fig. 5).

It appears, therefore, that the size of the perikaryon of a motor nerve cell or that of its nucleus, respectively, can be considered a fairly accurate measure of its total uptake of leucine and, hence, of the cell's rate of protein synthesis (see Discussion).

A few additional comments are in order as to the segmental variation in cell size. The curves in Fig. 1 show large motor neurons at the origin of the cervical and lumbo-sacral plexus, but these segments also contained small nerve cells. Accordingly, there was a greater standard deviation for cervical and lumbar segments than for the rest of the cord. Statistical analysis of variance gave no significant differences ($P > 0{\cdot}05$) for cell size, perikarya, nuclei and nucleoli among segments. These data do not contradict the generally accepted concept that larger nerve cells are found in those

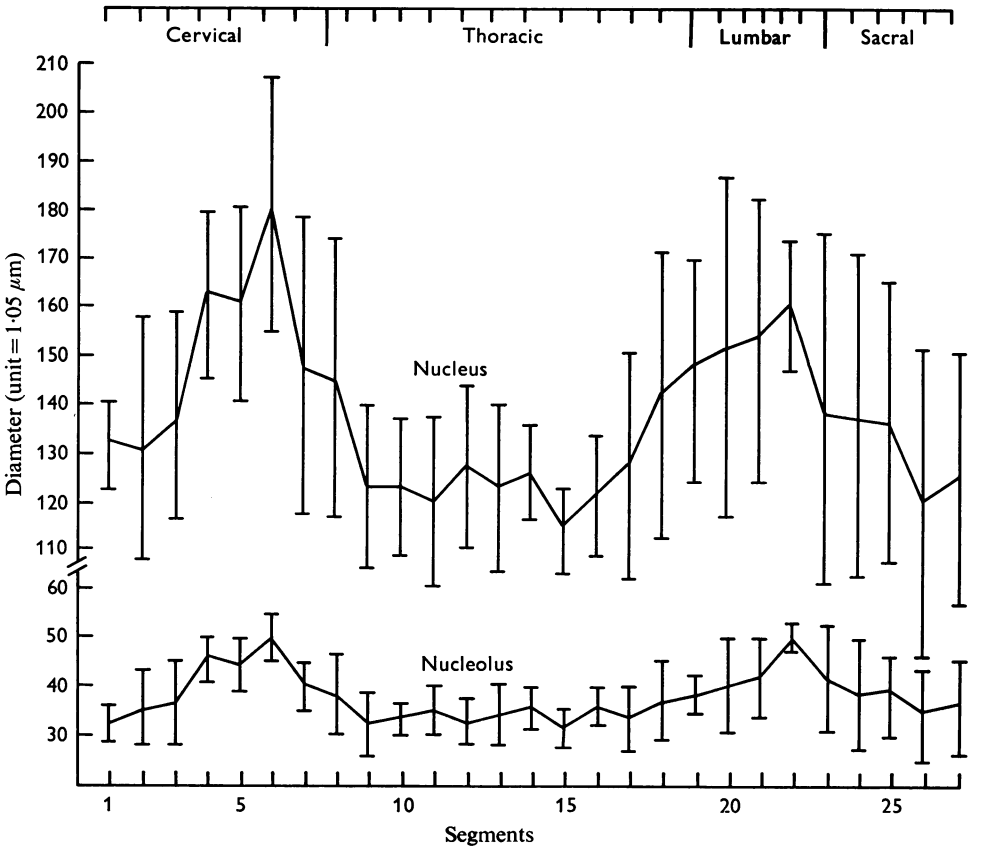

Fig. 3. Segmental variation of the diameters of the nuclei and nucleoli of nerve cells. The cells measured were the same as in Fig. 1. Each value was the mean of two measurements of the longest and shortest axis of the nucleus or nucleolus, respectively. The brackets indicate standard deviations.

segments that give origin to the fibres supplying the extremities; rather, they show that these segments do not contain large neurons only. Analysis of variance, therefore, was not an adequate test for segmental variation in maximum neuronal size. Highly significant differences ($P > 0{\cdot}01$) were found between neurons at the origin of the extremity plexus (segments 3–7 and 19–25 in Figs. 1 and 3) and those in the rest of the cord by using the rank sum test for comparison of mean cell size, nuclear size and nucleolar size. A measure of the largest neurons in each segment was obtained

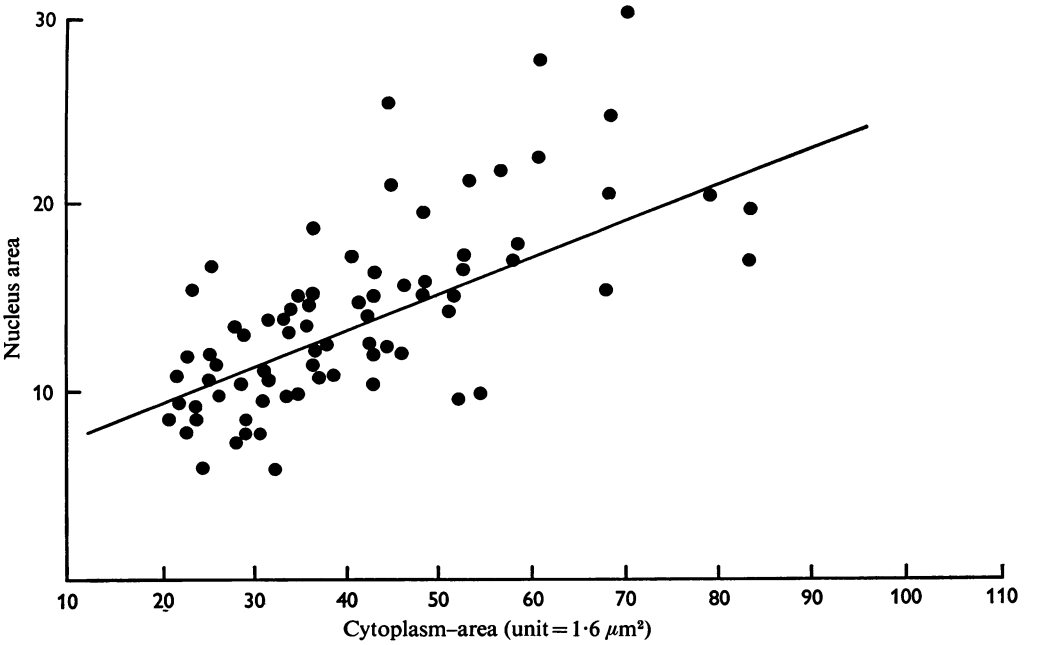

Fig. 4. Regression curve calculated by the least square method and correlation coefficient for planimetric measurements of the areas of the nucleus and the cytoplasm. Correl. coeff. $r = 0{\cdot}688$; slope $b_{yx} = 0{\cdot}22$; intercept $a_{yx} = 5{\cdot}16$.

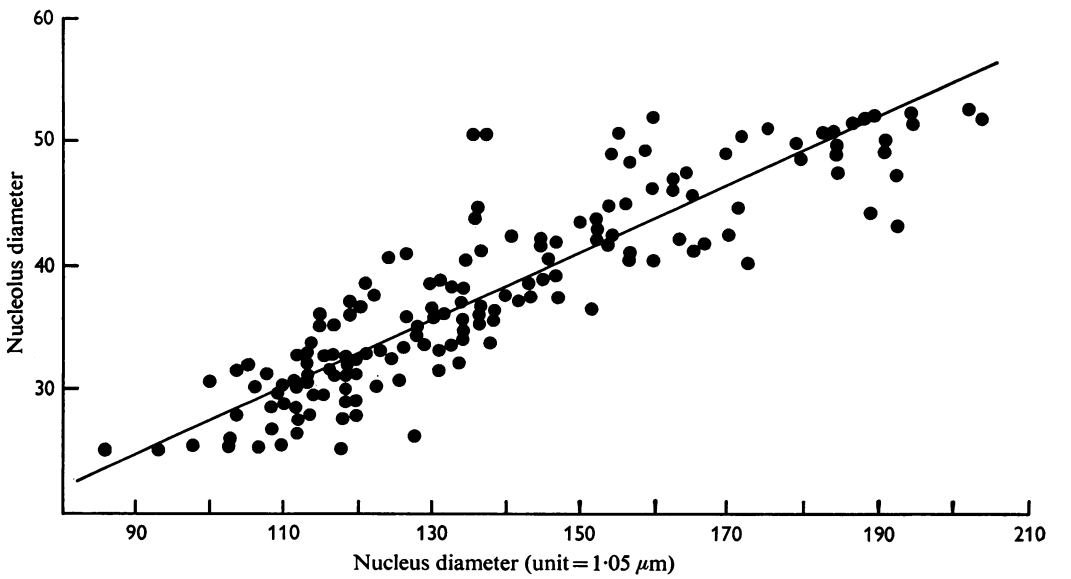

Fig. 5. Regression curve calculated by the least square method and correlation coefficient for measurements of the diameter of the nucleus and the nucleolus of nerve cells. The measurements are the same as those shown in Fig. 3. Correl. coeff. $r = 0{\cdot}89$; slope $b_{yx} = 0{\cdot}26$; intercept $a_{yx} = 2{\cdot}03$.

by plotting the mean plus two standard deviations (not shown here). Such a plotting indicated that the largest neurons at the origin of the extremity plexus were approximately twice the size of those in the thoracic segments.

DISCUSSION

Before considering the biological significance of our data, we shall discuss several technical aspects that pertain to the limitations of the methods and sources of error.

Leucine uptake as a parameter of protein synthesis. The uptake of leucine by nerve tissue, as shown by scintillation counting or autoradiography, is generally accepted as a parameter of the rate of protein synthesis. There is biochemical evidence for the rapid incorporation of amino acids into proteins of nerve tissue. Our extraction and dehydration methods for scintillation counting and for autoradiography enabled us to extract the free amino acids and most of the lipid material. The label remaining after extraction can be considered incorporated into proteins (Ochs, Johnson & Ng, 1967); non-specific binding of amino acids was found to account for only 4% of the label bound in formalin-fixed tissue (Peters & Ashley, 1967). With these procedural limitations, the following discussion considers leucine uptake to be indicative of protein synthesis by the nerve cell.

Limitations of autoradiography. The 'Material and Methods' section lists precautions taken to assure uniformity of the autoradiographs and to eliminate investigator's bias in the counts. Beyond such technical considerations autoradiography is subject to serious limitations when used as a quantitative tool. Since our sections were cut 10 μm thick, the efficiency of the autoradiograms was only about 2%, assuming uniform distribution of the label (Rogers, 1967). Our efforts to standardize tissue shrinkage and emulsion coating were thus merely aimed at obtaining consistent aliquots of the label present in the tissue section, providing the distribution of label in the section is uniform. This assumption seems justified in regard to cytoplasmic proteins, as discussed in the next paragraph. Error may arise if the distribution of label is not uniform, e.g. by concentration in specific organelles. For example, if tritium is distributed homogeneously, the relative number of particles reaching the emulsion is 0·25; if the tritium occurs in the upper 0·25 μm of the section, the yield is 1·52 (Pelc & Welton, 1967). Hence, the differences in grain density between cytoplasm and nuclei (or nuclear membranes) shown in Fig. 1 should be considered as being of qualitative rather than quantitative significance.

Cellular redistribution of label. The next consideration concerns the possible effect of turnover and/or export of label during the period of observation. It has been shown that labelled amino acids are incorporated into nerve cell protein as early as 5–7 min after injection (Droz & Leblond, 1963; Droz, 1967). The label bound in nerve cells is subject to subsequent redistribution in the cell, with export into dendrites and axon; these account for a redistribution of label into the axon hillock and the myelinated axon in 2–6 h (Droz, 1967). On the basis of this information it was assumed that a reasonable equivalent of the rate of uptake could be shown by injecting the label in three equal doses, 1 h apart, and by killing the animals 2 h after the last injection. This mode of application minimized the possible effects in the intracellular redistribution of label during the period of observation.

Biological significance of the data. Our autoradiographs, including controls from the cerebrum of brain stem, were in every way comparable to previous observations showing marked incorporation of amino acids in the bodies of nerve cells, especially into large nerve cells (MacLean, 1955; Fischer, Kolousek & Lodin, 1956; Flanigan *et al.* 1957; Oehlert, Schultze & Mauer, 1958; Schultze & Oehlert, 1958; Altmann, 1963; Merei & Gallyas, 1964). The similarity of such autoradiographs with Nissl-stained preparations was stressed by Schulze & Oehlert (1958).

Scintillation counts of total leucine uptake in spinal cord showed variation in uptake per segment, but there was no variation in uptake per mass. Such counts of whole cord segments are difficult to interpret because of the multiplicity of factors that enter the analysis. For example, the ratio of grey to white matter changes caudo-cranially, as well as in the cervical and lumbar enlargements of the cord. Changes in volume of grey matter, in turn, correspond to changes in the number of cells, and in the size of the cells. Separation of these factors is not feasible without quantitative cytological studies. The autoradiographic data presented here pertain only to the motor neuron population; yet, the data show a surprising similarity to the data for whole cord in that the variation in uptake was proportional to the size of the structures involved, but no variation occurred in the concentration per volume unit. The uptake of [^{3}H]leucine per volume of cytoplasm of motor neurons was fairly constant and independent of cell size. Accordingly, the size of a nerve cell was proportional to the total leucine uptake (or protein synthesis) per perikaryon. If autoradiographs are studied under the microscope, they give the impression that the grain densities are higher in the larger neurons; this impression is evidently misleading as one tends to estimate cell size and total grain number rather than grain density.

Our findings are only in partial agreement with Peterson (1966) who reported no variation in grain density for mouse spinal motor neurons larger than 200 μm^2; more than twice the grain density was found for the smaller neurons. Our data do not support his latter observation (Fig. 2). It appears possible that high uptake in small neurons reported by Peterson is due to higher grain densities over the nucleus (Fig. 1) which occupies a relatively larger portion of the cross-section of a small cell; if no effort is made to separate counts for nucleus and for cytoplasm, relatively higher counts for small cells can be obtained.

Is there a relation between axon size and protein metabolism of nerve cells?

Nerve cells that send their axons into the extremities evidently have longer axons than cells supplying the neck and trunk; fibre spectra indicate that the axons supplying the extremities are also thicker (Duncan, 1934). The segmental differences among nerve cells of the cord shown in the present study imply a relationship between the amount of protein synthesized by a nerve cell and the size of its axon. The segments from which the limb plexuses arise contain larger neurons which synthesize more protein per cell. However, they do so merely by having larger nucleoli, nuclei and perikarya, and not because their rate of protein synthesis per volume of cytoplasm is higher. A more exact determination of the relationship between protein synthesis in nerve cells and axon size would require exact determinations of volumes of perikaryon and axon; of the rates of protein synthesis in both (if indeed axonal protein synthesis occurs as recently indicated by Singer & Salpeter, 1966); and of

the rate of protein decay in cell body and axon. Such a comprehensive picture of the 'economy of a neuron' is beyond the scope of the present investigation.

SUMMARY

The uptake of [^{3}H]leucine in segments of rat spinal cord was studied in order to test the hypothesis that the protein metabolism of nerve cells may be related to the length of their axons. The basis for this study was the assumption that the spinal motor neurons supplying the extremities have longer and thicker axons than those supplying the neck and trunk.

Scintillation counts of leucine uptake in cord segments of equal length showed variation in total uptake per segment corresponding to the variation in weight of the cervical and thoracic enlargements, but showed no variation in uptake per weight of cord tissue.

Autoradiographic studies of the uptake of [^{3}H]leucine by motor neurons showed no variation in uptake per volume of cytoplasm among segments of the cord or among nerve cells of different sizes. The total amount of [^{3}H]leucine taken up by a nerve cell appeared to be a function of cell size. There was a linear correlation between the area of cytoplasm in nerve cells and that of their nuclei; nuclear and nucleolar sizes also were in a linear correlation.

The data support the concept that nerve cells with larger and longer axons do indeed synthesize more protein per cell. However, they do so merely because they have larger nucleoli, nuclei and perikarya, and not because the rate of protein synthesis per volume of cytoplasm is higher.

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