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A New Computer Assisted Method for Morphological Assessment of Peripheral Nerve Regeneration: Statistical Analysis of Spatial Patterns of Axons*)

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ABSTRACT

Free grafts of the common peroneal nerve of rats were performed, and the state of regeneration was observed at four week intervals the 24th post-operative week. The specimens used consisted of whole transverse sections of the nerve. These sections were magnified 2,100X by electron microscope, and radom photogrphs were taken of 10 sites. The negatives were enlarged threefold to produce 6,300X prints.

Using the photos, the axon coordinates, diameters and peripheral lengths of the specimens were entered into a minicomputer (NOVA-01) that has an effective tablet area of 350×350 mm. with a Graf/Pen Model GP3 (Scientific Accessories Corporation). The computer programs were developed by the authors exclusively for this study.

The axon spatial patterns were classified into three groups, those with regular, random and clustered distributions, respectively. Each of the 10 photographs of each section was tested to determine the category into which they fell. Two methods for analysis of the spatial patterns are available, The 'distance' method and the 'quadrat' method. We used both, but the results obtained by the former are presented in this report.

It was noted that the sequence of spatial pattern changes in myelinated fibers during the regenerating process was clustered random, regular, thus gradually approaching the normal pattern.

INTRODUCTION

A new analytical method for a histological pattern of axons will be introduced. This new method was developed for the following reasons. Observation of the transverse section of peripheral nerves in various stages such as normal, degeneration and regeneration appeared to present different distribution patterns of myelinated and unmyelinated axons. It is felt that if this distribution pattern could by analyzed, classified and experessed quantitatively, it could be that we would have another index in addition to the heretofare used size and number of axons.

On the basis of this line of thinking, we sought to analyze with the aid of a computer, the spatial pattern of common peroneal nerve of rats which had been severed and repaired by nerve grafting. A brief description will be made of the problematic points and the future outlook.

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MATERIALS AND METHODS

The right peroneal nerve of 48 male rats weighing between 250 to 300 g were used. Nerve segments of four defferent lengths, 1.0, 1.5, 2.0 and 2.5 cm, were taken and then resutured orthotopically to the original site using 10-0 threaded nylon suture with the aid of operating microscope. The state of regeneration was observed at 4-week intervals up to the 24th week after surgery.

The nerve diameter in situ was approximately 1 mm. Transverse sections were taken as specimens from three sites, 5 mm distal to the proximal suture site, 5 mm proximal to the distal suture site and the distal cut end. These specimens were placed upon a singled hole grid, magnified 2,100X. using a transmission electron microscope and photos of the 10 sites were made. The area photographed represents approximately 40% of the inner trannsverse section of the nerve bundle (funiculus, fascle). The films were enlarged 3-fold to produce prints of 6,300X, which were used for analysis. This is the minimum size of enlargement needed to make identification of unmyelinated fibers.

The photos were used to input the axon coordinates, diameter and circumference of the myelinated axons and center coordinates of the unmylinated axons into a Graf/Pen Model GP3 (Scientific Accessories Corporation) which has an active tablet area of 350×350 mm and a minicomputer Nova-01. The computer program was developed specifically for this purpose. The data obtained were simulated using another computer and expessed using an X-Y plotter to ascertain as to whether the data had been entered correctively.

The center of gravity of the myelinated axon in calculated from these data, and using this center of gravity a perfect circle equal in area to the axon was simulated by calculation. This is the reason that the illustratiaon shows areas of overlapping with adjoining axons. The cut surface area and central coordinates of individual myelinated axons were reproduced so that they would be equal in area to those of the original photo. As was considered that at this low degree of magnification, the difference in cut surface area between the respective unmyelinated axons could be ignored, an average value was used. As parts of the axons along the periphery of the photo were lost, accurate input of data could not be made. Careful comparison of the original against the simulated photos was made and such axons were excluded from the study.

METHOD OF ANALYSIS

The axon spatial patterns were classified into three groups, namely regular distribution, random distribution and clustered distributaion (Fig. 1). In other words, the 10 shotos made of each section were classified into one of the three categories.

The conventional methods for sapatial pattern analysis used in other fields of science[1-5,7-9] can be largely classified into two, that is, the 'distance' method and 'quadrat' method. The 'distance' method was introduced by Hopkins and Skellam (1954). Under this method, the classification is made by calculating the ratio

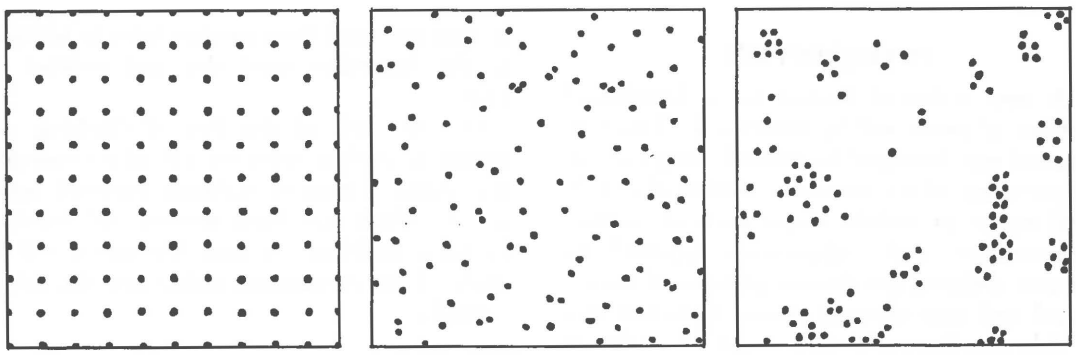

Fig. 1. The spatial pattern of axons was classified into regular, random and clustered. Using Hopkin's (1954) index, radom distribution is $A=1$, clustered $A>1$ and regular $A<1$.

of the squared distance from a random point to the nearest adjoining individuals, the 'quadrat' method involves the counting of the number of individuals within a specific area and classification is made on the basis of coefficient of variation. The latter method was first reported by Strand (1972). We carried out analysis employing both methods, but in this report analysis based on the 'distance' method will be described.

The application of the method of Hopkins and Skellam to the analysis of regenerated axons involves the measurement of the squared distance (U) from a random point to the nearest neighboring axon, and squared distance (V) from a randomly selected axon to its nearest neighbor, and

$$A=\sum U/\sum V$$

is calculated. If the pattern is a random distribution, as the mean value of $\sum U$, the sum of the squared distance from a random point of the nearest randomly distributed axon, and $\sum V$, the sum of the squred distance from a randomly selected axon to its nearest nwighbor, are the same, thus, $A=1$. On the other hand, if the pattern is a homogeneous distribution, $\sum U$ will be smaller than $\sum V$, thus $A<1$, and in clustered distribution, $\sum U$ will be larger than $\sum V$, thus $A>1$. The value of A is sought through such calculations, and on the basis of the results the pattern of distribution is determind.

However, the value of A is frequently found not to be exactly 1 even on random distribution, and shows a certain degree of variation depending on the number of axons in the measured area. Therefore, it is necessary to carry out tests to ascertain whether the difference is so great that a value of 1 can be considered significant. The following equation is used for this purpose.

$$X=A/(1+A)$$

We have the properties of the statistics X as follows the average mean of X

$$E(X)=1/2,$$

the variance of X

$$V(X)=1/4(2n+1).$$

For the large samples, X is approximately distributed as the normal distribution with the parameter of $E(X)$ and $V(X)$.

At the level of significance of $\alpha=0.05$,

if $1/2-1/\sqrt{2n+1}\leqq X\leqq 1/2+1/\sqrt{2n+1}$

then this is a random distribution, where n is the number of axons.

If $X<1/2-1/\sqrt{2n+1}$,

then it is a homogeneous distribution.

And if $X>1/2+1/\sqrt{2n+1}$,

then it is a clustered distribution.

RESULTS

First, a number of photos will be cited as examples, and their respective evaluations will be described.

Fig. 2 shows a normal common peroneal nerve. A total of 58 myelinated and 136 unmyelinated fibers can be seen. However, as some fibers along the periphery which had not been accurately simulated were excluded, those in the study area subjected to analysis numbered myelinated fibers 52 and unmyelinated fibers 136. Random coordinates were produced by the computer, and the ratio of $\sum U$ and $\sum V$ sought by calculation was $A=0.610$, $X=0.379$ and $1/\sqrt{2n+1}=0.098$. As the value of X at the 0.05 level of significance was less than 0.402, the spatial pattern was evaluated as a homogenous distribution.

The values for the unmyelinated fibers were $A=71.569$, $X=0.986$ and $1/\sqrt{2n+1}=0.061$. As X is greater than 0.561, this was judged as being in a clustered state of distribution. Examples thus evaluated are shown in Fig. 3 to 6. The readers are asked to compare the histological pattern with the results of analysis.

As there is a difference in state of regeneration by site even in the same section, it is felt the most appropriate approach would be to take samples from a number of areas and analyze each photo independently, then sum up the values and make an overall evaluation. In other words, summarize them as in Table 1, and evaluate the results. Using this method, The evaluation of the 10 photos made of the 4-week samples showed that 8 had clustered distribution while 2 had random. The results for the 12-week, 16-week and normal samples are also given.

DISCUSSION

As computers have become more readily available cost-wise, coupled with the progress in data input units, more attention is being focussed on image analysis. However, it is not an over statement to say that their intro-

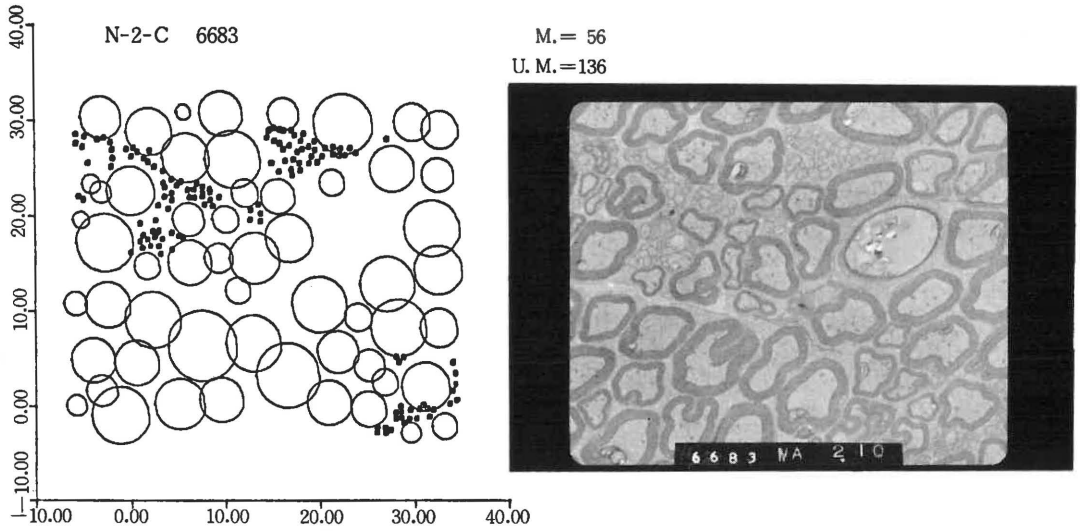

Fig. 2. Left photo taken normal nerve shows transverse section magnified 2,100 *X*. Right simulated description of photo.
Myelinated fibers; $A=0.616$, $X=0.379$, $1/\sqrt{2n+1}=0.098$, regular distribution.: Unmylinated fibesr; $A=71.569$, $X=0.986$, $1/\sqrt{2n+1}=0.051$, clustered distribution.

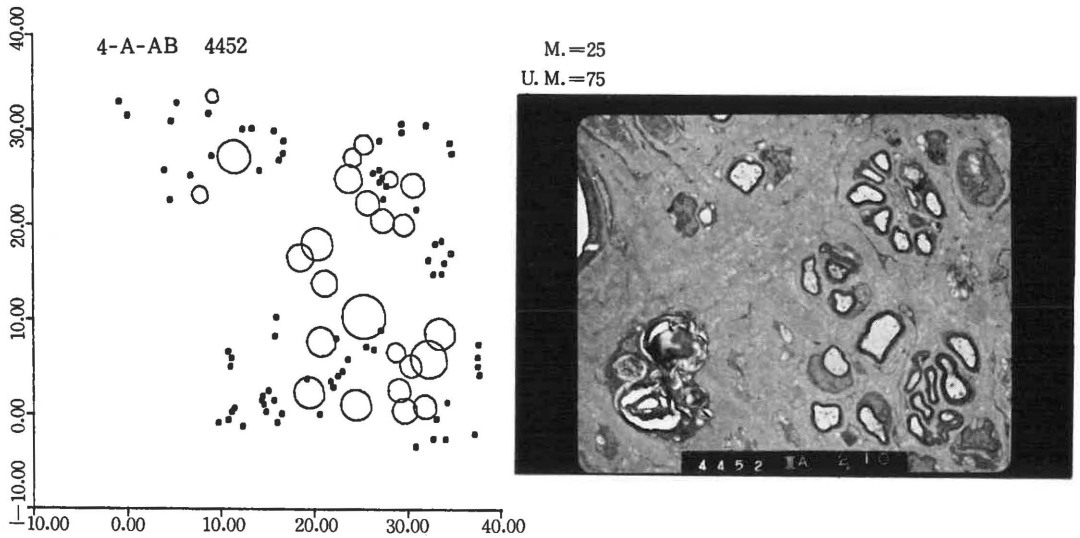

Fig. 3. (4 weeks after graft) Myelinated fiber; $A=2.412$, $X=0.707$ and $1/\sqrt{2n+1}=0.143$, clustered distribution.: Unmylinated fibesr; $A=8.986$, $X=0.900$ and $1/\sqrt{2n+1}=0.087$, clustered distrbution.

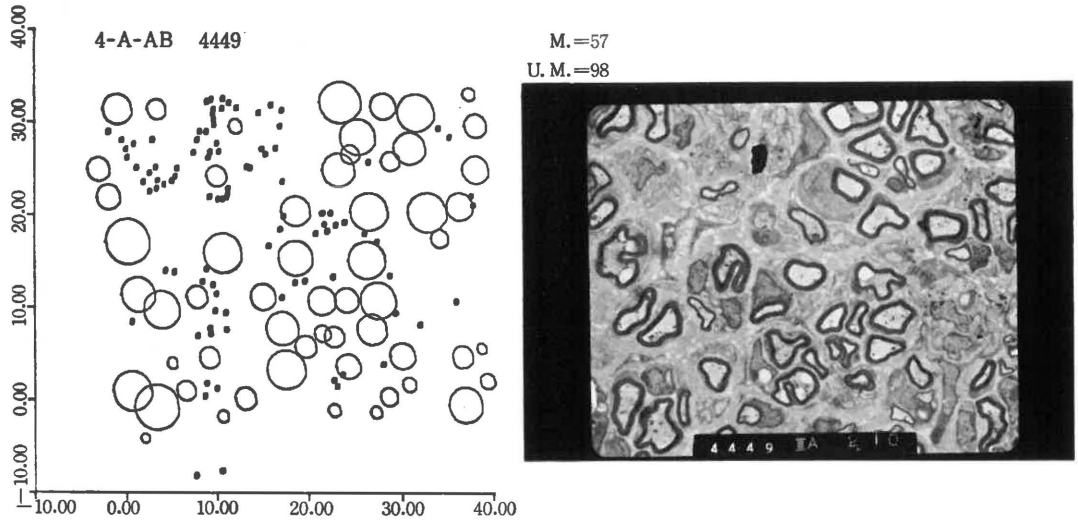

Fig. 4. (4 weeks after graft) Myelinated fiber; $A=0.929$, $X=0.482$, $1/\sqrt{2n+1}=0.099$, random distribution.: Unmyelinated fibers; $A=4.988$, $X=0.833$, $1/\sqrt{2n+1}=0.076$, clustered distribution.

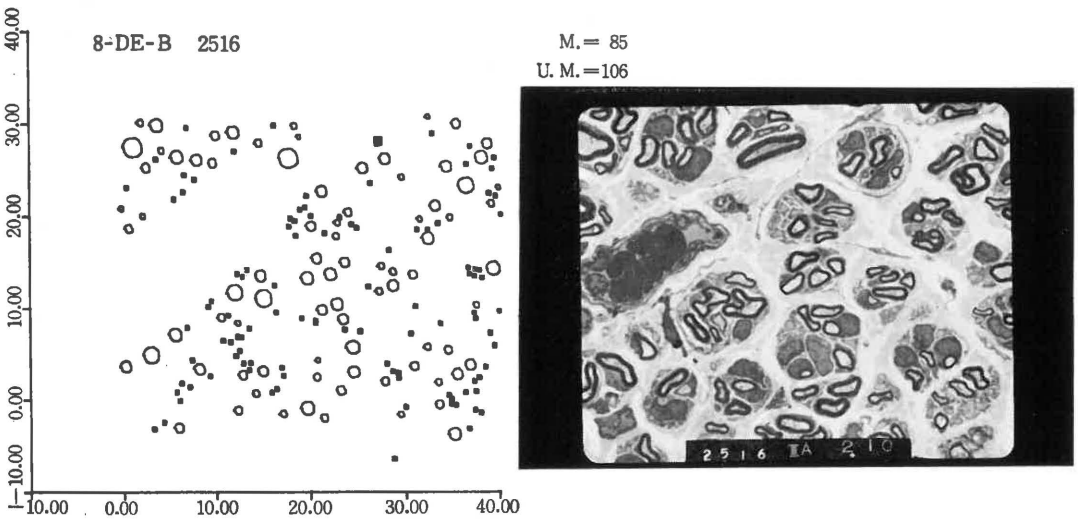

Fig. 5. (8 weeks after graft) Myelinated fiber; $A=1.088$, $X=0.521$, $1/\sqrt{2n+1}=0.079$, random distribution.: Unmyelinated fibers; $A=1.957$, $X=0.662$, $1/\sqrt{2n+1}=0.069$, clustered distribution.

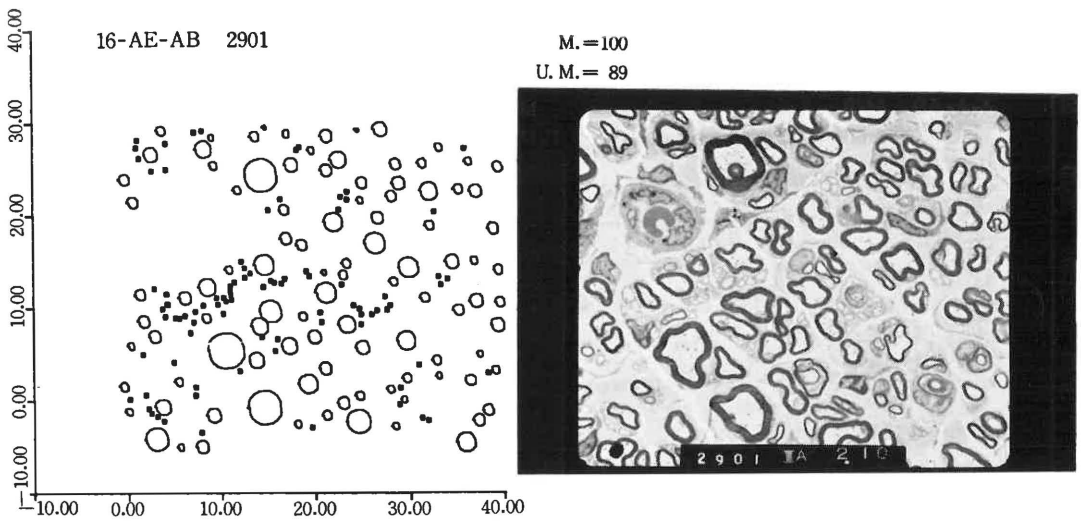

Fig. 6. (16 weeks after graft) Myelinated fiber; $A=0.675$, $X=0.403$, $1/\sqrt{2n+1}=0.071$, regular distribution.: Unmylinated fibers. $A=2.477$, $X=0.712$, $1/\sqrt{2n+1}=0.075$, clustered distribution.

Table 1. Findings of 10 photos sampled from the respective stages of 4, 12, 16 weeks after nerve graft and normal. Test of spatial pattern was made foreach stage.

distribution	classification		
section	regular	random	clustered
4 weeks	0	2	8
12 weeks	2	7	1
16 weeks	5	5	0
normal	8	2	0

duction into the fields of medicine and biology is still quite limited. Although this is the state, it is a fact that computers have become accessible, and we feel that it will not be too long before such methods of analyss as described here will be employed routinely in research. Some of the problems involved in spatial analysis of peripheral nerves will be described.

First, the input data must all be traced with a Graph/Pen, which requires great effort. With the present state of the art in electronics, it is possible to enter the data automatically, but such equipment is expensive and cannot be afforded by the general researcher. In our peripheral nerve graft experiment we have taken about 1,200 electron microscope photos, but more than 1 hour required per photo to imput data for anlysis of the spatial pattern. Therfore, it is practically impossible from the standpoint of expenses and time to input the data contained in all of the photos. We must await the development of improved units to process this vast volume of data at low cost and less labor. Thus, the photo samples introduced in this report are those which were subjectively selected from among our collection and analyzed on the basis of their characteristc images representing the various stages of regeneration.

The second problem is that there is a limit to the size of the data which can be input. This necessitates splitting up the data for analysis. Thus, theoretically in carrying out analysis of the spatial pattern, the degree of error should be small if data of the whols tranverse section of the nerve were collected and analyzed as a whole, or cmprehensively by site such as the central area and periphery. However, one is confronted with a dilemma when performing such analyses because it is necessary to use photograph taken at as low magnification as possible to input a wide area of data as there is a limit to the effective area of the currently available tablet (350×350 mm, Scientific Accesories Corporataion). As the pattern becomes very small, the error will increase, and the coordinates will be rendered inaccurate. Also the unmylinated nerve fibers will become impossible to identify.

Therefore, there was no alternative for us,

but to select sample areas and analyze them as parts of the whole, after which the data was compiled and an overall evaluation was made. There is the question as to whether this method is appropriate, but as it is impossible to input the data of the whole area with a Graf/Pen, it is not possible to test the findings by the 'distance' method. Therefore, a composite photo of the whole transverse section was broken up into areas of 10×10 μm using graph paper, and the number of axons in each all was counted. Test was made using a separate index, that is, ratio of mean value to variance (V/m), by which it was also determined that when $V/m=1$ it implied random distrebution, >1 meant clustered and <1 signifiied homogenous. In order to bring the level of confidence to 95% or more, it was necessary to have photos of 10 sites for mylinated fibers and 12 for unmyelinated fibers[6]. In veiw of the above degree of precision and funds available, we were obliged to use 10 sampled areas.

As mentioned above, the analysis covered in this report is only a small portion of the approximately 1, 200 phtos taken by the authors. We have found through our study to date that the changes in the spatial pattern during the regeneration process following perphral nerve graft showed the distributaion of myelinated fibers to be in the order of clustered-random-regular, thus approaching the normal pattern. While in the case of the unmylinated fibers, the pattern assumed by most during both the normal and regenerating periods was clustered distribution, and further review by use of the index of Hopkins and Skellam also failed to show clear changes in pattern as in the case of myelinated fibers. The same tendencies are noted within the grafted sections and also at the distal cut ends, thus we are currently unable to demonstrate any difference by site of sample.

Available as analytical methods for spatial patterns in other areas are 'R' of Clark and Evans[1], 'α' of Pielow and Mountford[8], 'V/m ratio' of David and Moore, 'Index of cluster m' of Lloyd[5], 'Indes of despersion I_δ' of Morisita[7] and 'Eberhardt's static A' of Hines and O'hara Hines[3]. We used primarily, the index of Hopkins and Skellam, but our third problem is to ascertain what type of index would give us the most appropriate coefficient to express the regenerative process of the peripheral nerve.

In this study, we analyzed the myelinated and unmyelinated axons separately, but it naturally should be possibale to analyze both combined, that is not making distinction between the two. It has been confirmed that the changes in pattern can be ascetain by Hopkins and Skellam Index. However, it is felt that it would be more meaningful if the data were input separately and the interrelationship between myelinated-unmyelinated axons and blood vessels could be analyzed. That is, determine the degree of unmyelinated axons clustering around myelinated axons and the extent of regaenerated axons clustering ground blood vessels. As there are myelinated and unmyelinated fibers, Schwann cells, blood vessels, collagen fibers, fibroblasts etc. within the nerve bundle, this requires multivariate analysis, but it apprears the analysis cannot be performed with the indices reported to date. This is the fourth problem involved in the analysis of spatial patterns of the peripheral nerves. Thus, it is necessary to develop new indices for multivariate analysis of the spatial pattern.

The diameters and number of axons have been used as coefficients to describe the extents of degeneration or regeneration of peripheral nerves. As we do not yet have a sufficient volume of data, we are unable to make any statements on the relationship between these indices and spatial pattern. This, too, is another problem to be resolved in the future.

We have carefully observed the various panorama-like changes in the histlogical pattern with time during our experiments. Our motive for undertaking this study was to devlop a method which would enable us to express these changes in pattern as coefficients. The apparatus used in this study was a machine on the market made to analyze movie films of cardio-angiographic procedures. This type of equipment is being acquired for general use at a remarkably high pace. We learned that with the development of programs, it would become quite readily possible to carry out such new research as this. In this report, we introduced the fact that spatial pattern analysis can be applied to perperal nerves.

CONCLUSION

Analysis of the spataial pattern of the peripheral nerve was attempted for the first time using the experimental histological specimens of grafted nerve to the common peroneal nerve of rats. The method of analysis was 'distance method of Hopkins and Skellam.

In the normal nerve, the myelinated fibers showed regular distribution while the unmyelinated fibers pressented clustered distribution. It was found that changes in the spatial pattern during the regenerative process was clustered to random to a regular distribution which approximated normal. In the unmyelinated fibers, the pattern throughout the period of regeneration was clustered, and failed to indicate any tendency of pressenting well defined changes as in the case of myelinated fibers.

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