The Uptake of 3H(G)L Leucine into Single Muscle Fibers in Charcot-Marie-Tooth Disease

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SUMMARY: *In previous studies, the incorporation of 3H(G)L-leucine into muscles of patients with Charcot-Marie-Tooth (CMT) disease was shown to be increased in comparison with that observed in motor neuron disease (MND). To determine the cause of the increased uptake in CMT, studies of single fiber leucine incorporation have been undertaken. The results of this study indicate that the increased incorporation is into those muscle fibers which are undergoing regeneration following reinnervation. These results do not support the thesis that there is an associated myopathic process in CMT.*

RÉSUMÉ: *Lors d'études antérieures nous avons montré que l'incorporation de 3H(G)L-leucine au muscle de patients atteints de Charcot-Marie-Tooth (CMT) dépassait celle de patients avec atteinte des motoneurones antérieurs. Pour déterminer la cause de cette captation augmentée dans le CMT, nous avons étudié l'incorporation de la leucine dans les fibres uniques. Il nous fut ainsi possible de démontrer que cette augmentation de captation se fait principalement dans les fibres musculaires qui sont à régénérer après une ré-innervation. Ces résultats n'appuient donc pas la thèse de l'existence d'un processus myopathique dans le CMT.*

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INTRODUCTION

In a previous publication we reported a significantly increased uptake of 3H(G)L leucine into muscle proteins in Charcot-Marie-Tooth disease (CMT) (Monckton and Marusyk, 1977). This study showed the increased uptake to be into cytoplasmic proteins; the corresponding uptake of leucine in motor neuron disease (MND) was found to be normal. Since denervation is a primary feature in both conditions, it seemed necessary to account for the different uptake patterns. In considering the reasons for this, the following possibilities were reviewed in relation to Charcot-Marie-Tooth disease: 1) that there is a myopathic component to the disease; 2) that abnormal neurotrophism is responsible for a different and increased synthesis for muscle proteins; 3) that the abnormal uptake is due to the effects of reinnervation and subsequent muscle regeneration.

The purpose of this paper is to provide new data on single muscle fiber 3H(G)L leucine uptake, which we feel can provide a solution to the question.

METHODS

Motor point biopsies were obtained from six patients with Charcot-Marie-Tooth disease, hereditary motor and sensory neuropathy (H.M.S.N. type I) and three patients with typical motor neuron disease. Pieces of vastus lateralis muscle were also obtained from 10 patients undergoing surgery for chronic noninfective orthopaedic problems. Fragments (0.5 gm) of the muscle specimens were placed in an isotope mixture consisting of 0.9 ml human serum and 100 μ Ci of 3H(G)L leucine (Amersham-Searle). The muscle was incubated at 37°C for one hour and rinsed in normal saline. Each specimen was then cut into smaller pieces for routine light and electron microscopy, autoradiography, and scintillation counting. These specimens were fixed in 3% glutaraldehyde in phosphate buffer at pH 7.2 for two hours, and post-fixed for one hour in 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol and embedded in epon 812 (Ladd); 0.5% 2,5 diphenyloxazole (P.P.O.) (Amersham-Searle) was added to the propylene oxide/epon mixture of the embedding procedure. Epon blocks were polymerized at 60°C for 48 hours.

Thick (1 μ) cross-sections were cut and mounted on glass slides previously subbed with an aqueous solution of 0.5% gelatin and 0.05% chrome alum. The slides were dried and dipped in Ilford L4 nuclear emulsion and kept in light tight boxes at 4°C. The slides were developed after three days and the sections stained with a 1% solution of phenylenediamine in methanol.

The sections were photographed, individual fibers identified in three serial sections, and the grains counted over each fiber. The areas of each fiber were determined by tracing on squared transparent paper. The grain counts were averaged over three serial sections to ensure that counts represented all parts of the sarcomere. The average counts were then related to the cross sectional area of the fibers in one section. Fiber types were deduced from mitochondrial counts obtained over unit areas of fibers on electron microscopy at 6000 x magnification and examination of sections after staining thick sections with phenylenediamine in methyl alcohol (Korneliussen, 1972).

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Table 1

SPECIFIC ACTIVITY

(Counts/min/mg of TCA-Insoluble Tissue)

Normal	C.M.T.	M.N.D.
5,620	20,526	4,014
± 323	± 1,160	± 354

Table 1: The uptake of 3H(G)L leucine into T.C.A. insoluble tissue of normal, CMT, and MND. The figures represent the means ± SEM. A comparison of the means of normal and MND to CMT shows a significant difference $p<0.001$. CMT= Charcot-Marie-Tooth. MND = Motor neuron disease.

Table 2

Leucine Grains/1000 sq. μ

Fiber size (sq. μ)	Normal	CMT	N	P	MND	N	P
2000	20 ± 6	30 ± 11.3	31	<.01	16 ± 3.4	15	<.1
2500	25 ± 8.09	33 ± 11.6	37	<.05	18 ± 3.9	16	<.05
3000	20 ± 6	30 ± 6.5	36	<.001	16 ± 6.2	29	<.1
3500	19 ± 5	28 ± 4.9	51	<.001	14 ± 3.7	43	<.05
4000	20 ± 7.4	28 ± 5.2	51	<.001	15 ± 6.4	38	<.05
4500	23 ± 6	33 ± 7.1	22	<.01	13 ± 2.8	20	<.02
5000	21 ± 8.3	28 ± 8.2	25	<.1	13 ± 2.0	25	<.05
5500	23 ± 7.6	28 ± 6.2	36	<.05	15 ± 2	23	<.02
6000	23 ± 4.6	28 ± 6.6	11	<.05	13 ± 2.6	12	<.02
6500	22 ± 5.3	28 ± 4.7	31	<.05	10.3 ± 4.8	31	<.02
7000	21 ± 4.6	30 ± 4.3	16	<.05	13 ± 2.6	11	<.02
7500	21 ± 4.1	23 ± 2.2	17	>.1	14 ± 0	15	<.02
8000	20 ± 4.0	24 ± 1.5	13	>.1	-	-	-
8500	22 ± 3.4	25 ± 1	6	>.1	14 ± 0	5	<.02
9000	23 ± 4.2	21 ± 4.2	13	>.1	12 ± 3.3	17	<.02

Table 2: The values represent the means ± SD for normal, CMT and MND. The P values of significance were derived by using the Student's T-test. N = number of fibers.

For the specific activity assay, muscle tissue weighing 1 to 3 mg was placed in a vial containing 5% trichloroacetic acid and heated at 70°C for two hours. The samples were removed, air dried on a filter paper for one hour, weighed, and placed in a new vial containing 0.5 ml Protosol (N.E.N.). Each sample was left to digest for several hours at room temperature, after which 10 ml of Econofluor (N.E.N.) was added and the sample counted in a Beckman LS 230 counter.

RESULTS

Table 1 shows the specific activities of muscle from the patients. There is an increase ($p<0.001$) of uptake of tritiated leucine in Charcot-Marie-Tooth disease as compared to the normal and motor neuron disease muscle.

Cross sections of this material were used to determine individual muscle fiber uptake (see Methods). The histograms (Figure 1) show the size distribution of fibers, expressed as percentages of the total number in each of the three groups. In these histograms, the normal material contains no fibers smaller than 2000 μ^2, and the percent distribution of fibers is in accord with that usually seen (Figure 1 top) (Sissons, 1963). In the Charcot-Marie-Tooth disease muscle (Figure 1 middle), there is a marked predominance of smaller fibers, causing a shift to the left in the histogram. The motor neuron disease material shows the same phenomenon (Figure 1 bottom). Figures 2a, b and c show the observed radioactivity by grain counting over each fiber. From these figures, it can be seen that there is a linear relationship of grain count/muscle fiber area. If grain counts are calculated for each fiber by simple proportions to a uniform area of 1000 μ^2, the calculated grain count/unit area (Figures 3a, b, c) shows the comparative level of uptake in the muscle fibers. These histograms show that the normal fibers have a reasonably uniform level of uptake per unit area, irrespective of fiber size. In Charcot-Marie-Tooth disease and motor neuron disease muscle, this is not the case. In both

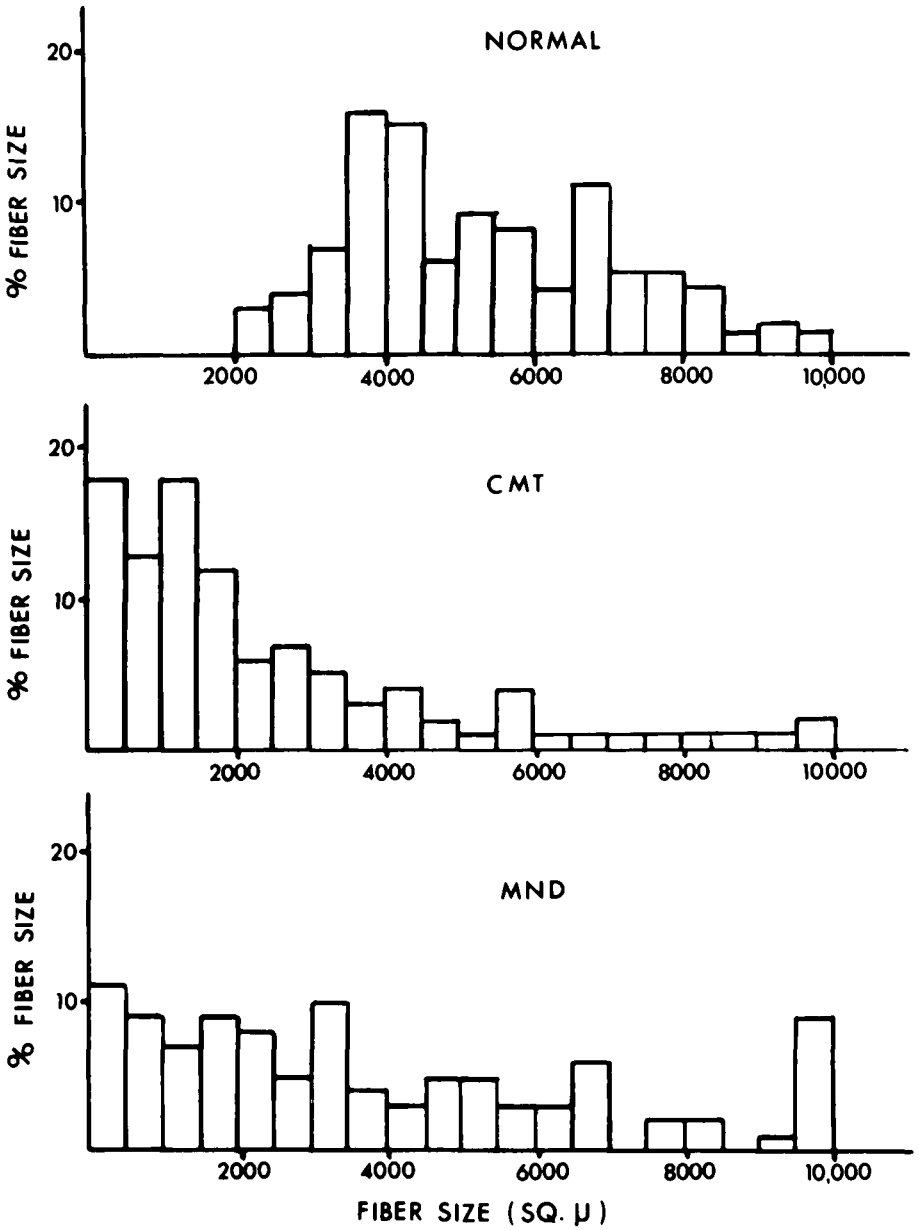

Figure 1—Histograms showing the distribution as a percentage of the total number of fibers in each represented size range. The total number of normal fibers is 237; of CMT, 413; and MND, 115. CMT = Charcot-Marie-Tooth. MND = Motor Neuron Disease.

disorders the smaller fibers are heterogenous as to level of uptake. Fibers have levels of activity from very low to very high. If the means of the levels of fiber activity are calculated for each 500 μ^2 increase in area, the resulting graph, Figure 4, is obtained. In this the means are significantly different from normal for Charcot-Marie-Tooth disease and motor neuron disease. (For levels of significance see Table 2). In Charcot-Marie-Tooth disease, there is an increased level of activity for all fiber size ranges up to 7500 μ^2, after which the activity levels are similar to normal. In motor neuron disease, there is a significant increase in activity of the smaller fibers up to 2000 μ^2, after which the activity falls to normal or subnormal levels. Correlation of the fiber counts expressed as grains/unit area with fiber type shows in the Charcot-Marie-Tooth patients that small fibers with the high activity are usually in association with larger fibers of the same fiber type and tend to group together (Figure 5).

DISCUSSION

The three possible explanations for the increased uptake of tritiated leucine, as outlined in the Introduction and demonstrated in Table 1, can now be considered, in the light of the results reported above.

In our previous paper, we were able to show that the increased uptake of Charcot-Marie-Tooth disease was into cytoplasmic elements alone (Monckton and Marusyk, 1977). The dot histograms of actual grain counts do display linearity, but there is a wide scatter, particularly amongst the smaller fibers. Nevertheless, regression lines fitted to these points exhibit good correlation coefficients. The similar slopes of the Charcot-Marie-Tooth and normal patients in these histograms might tempt one to conclude that the results tend to support a diffuse affection of all muscle fibers in Charcot-Marie-Tooth disease, which would argue for an intrinsic muscle protein synthesis derangement, presumably genetic in nature. In a second series of histograms, obtained by determining the level of uptake/unit area, the true fiber uptake becomes apparent. In the normal fiber histogram, the fibers take up tritiated leucine to similar concentrations irrespective of size in a narrow range. The motor neuron disease fibers follow a similar pattern, except for the smaller fibers where there seems to be a wider scatter between increased and decreased uptake. This scatter might depend on whether the fibers are denervated or reinnervated. This appears to be supported by the observations of Wohlfart (1975) who demonstrated, by histological methods, moderate amounts of axonal branching in motor neuron disease as evidence of attempting reinnervation. Denervated fibers undergoing atrophy will presumably take up less leucine (Goldberg, 1969) than those that are reinnervated and redeveloping. This conclusion is amplified by reference to the graph (Figure 4), which shows the means of grains/unit area in fibers in 500 μ^2 size ranges. Here it can be

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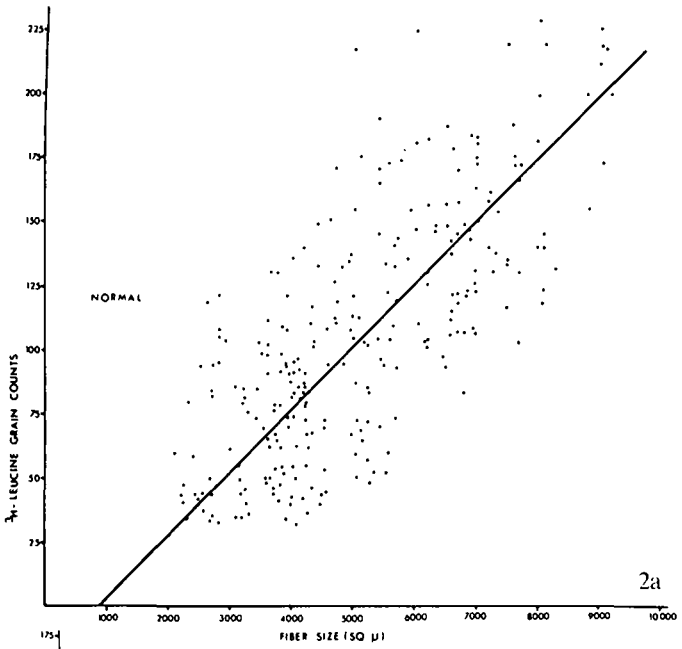
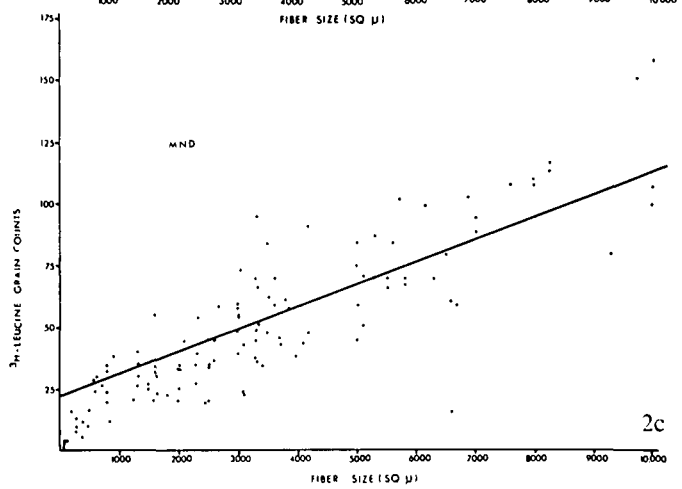




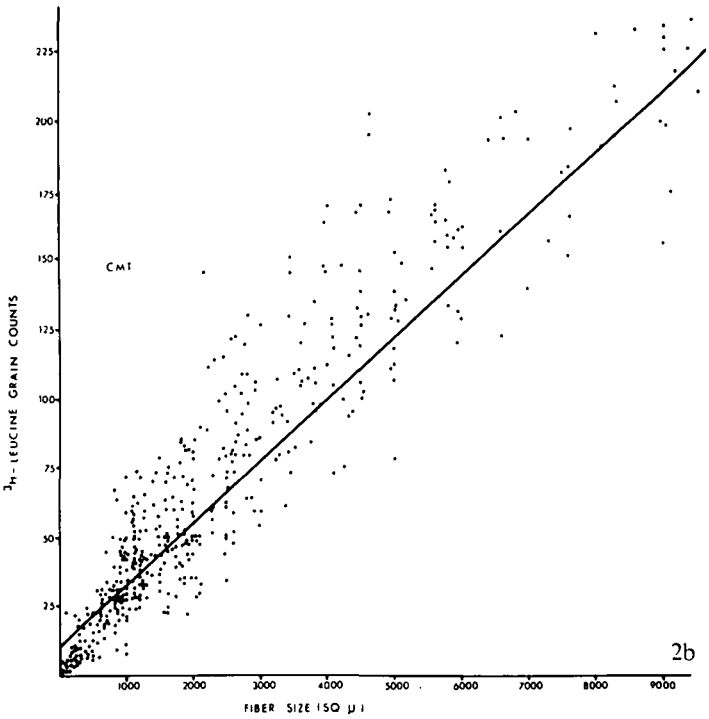
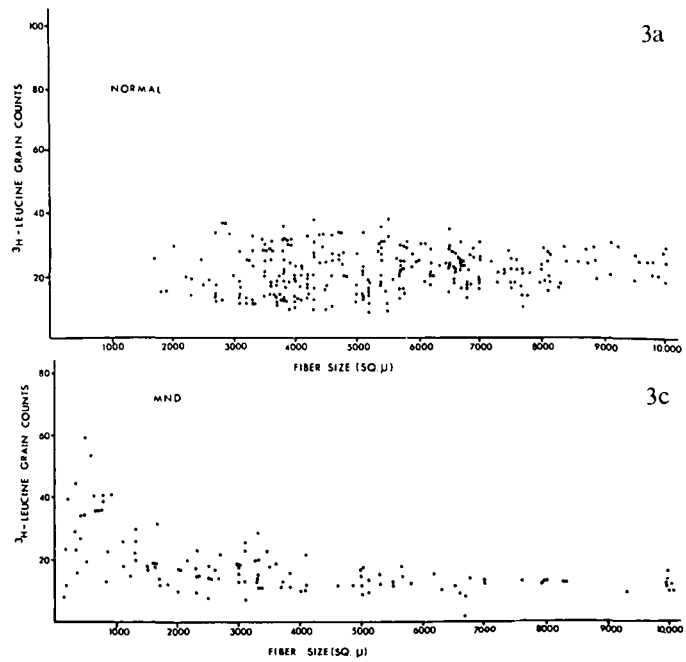


Figure 2a, b, c—Histograms showing the relationship of grains counted over each fiber. The dots represent single fibers. The correlation coefficient of normal is 0.852; of CMT, 0.933; and MND, 0.895. CMT=Charcot-Marie-Tooth. MND=Motor Neuron Disease. 2a — Normal; 2b — CMT; 2c — MND.



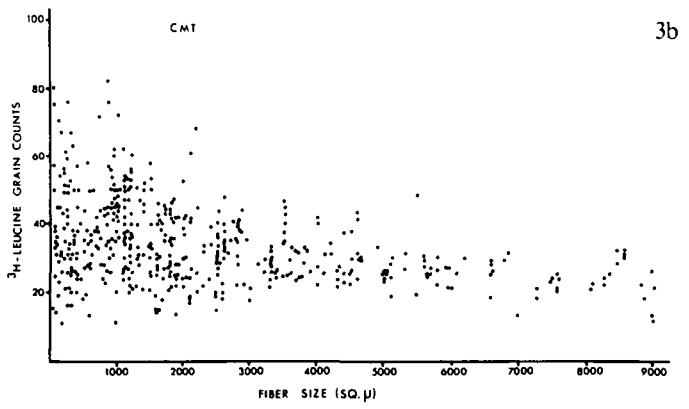


Figure 3a, b, c—Histograms showing the relationship of grain counts/unit area of 1000 μ^2 and fiber size. Each dot represents a single fiber.
CMT=Charcot-Marie-Tooth. MND=Motor Neuron Disease. 3a — Normal; 3b — CMT; 3c — MND.

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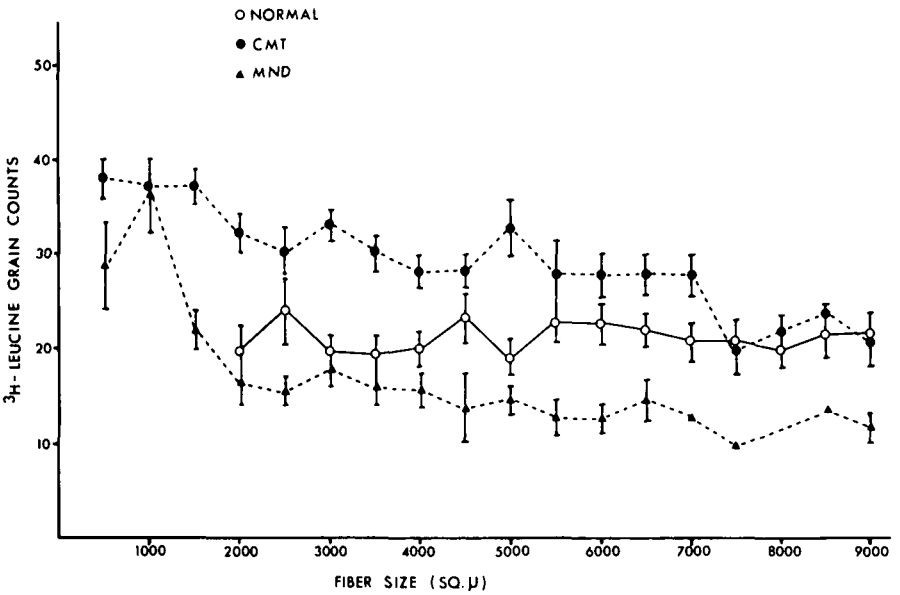

Figure 4—Graph comparing the means ± SEM of fiber activity per unit area and fiber size of normal, CMT and MND. CMT = Charcot-Marie-Tooth, MND = Motor Neuron Disease.

seen that the means are variable, reinforcing the heterogeniety of the fibers in the groups represented in a given range. In Charcot-Marie-Tooth disease the grains/unit area histogram shows a number of small and medium size fibers which have increased uptake, whereas the larger fibers are within the normal range. Reference to the graph in Figure 4 clarifies this observation and shows that the increased activity in these fibers continues through size ranges up to about 7500 μ^2. Beyond that the levels of activity appeared to be normal. If the increased uptake of leucine were an expression of an intrinsic biochemical defect in the muscles of Charcot-Marie-Tooth disease, one might expect this to be manifested in all muscle fibers, irrespective of size. Observation does not show this to be the case, but reveals increased uptake in muscle fibers up to 7500 μ^2 only. Thus, a genetic factor responsible for increased uptake in smaller and medium size fibers would seem to be active only during maturation of the fiber. Possibly the muscle fibers with abnormal uptake were innervated by abnormal nerves, and the changed pattern of tritiated leucine uptake was due to abnormal neurotrophism. It would be equally acceptable to suggest that the abnormality was a normal response of reinnervated muscle undergoing regeneration. If the increased uptake were due to abnormal neurotrophic influences, then they would seem to be effective only during the active rebuilding of muscle. Comparison with the uptake seen in motor neuron disease suggests that reinnervation of muscle fibers is associated with increased uptake of leucine. The difference in the two conditions may perhaps be one of degree rather than nature. The graph (Figure 4) shows that the level of uptake decreases to normal after 7500 μ^2 in Charcot-Marie-Tooth, and this supports the postulate that the synthesis is related to muscle regeneration following reinnervation. In confirmation of this, the trace outlines of muscle fibers with Charcot-Marie-Tooth disease showed that where there were small, highly active muscle fibers these tended to be associated with groups of fibers of the same type, strongly suggesting neuronal branching and subsequent "adop-

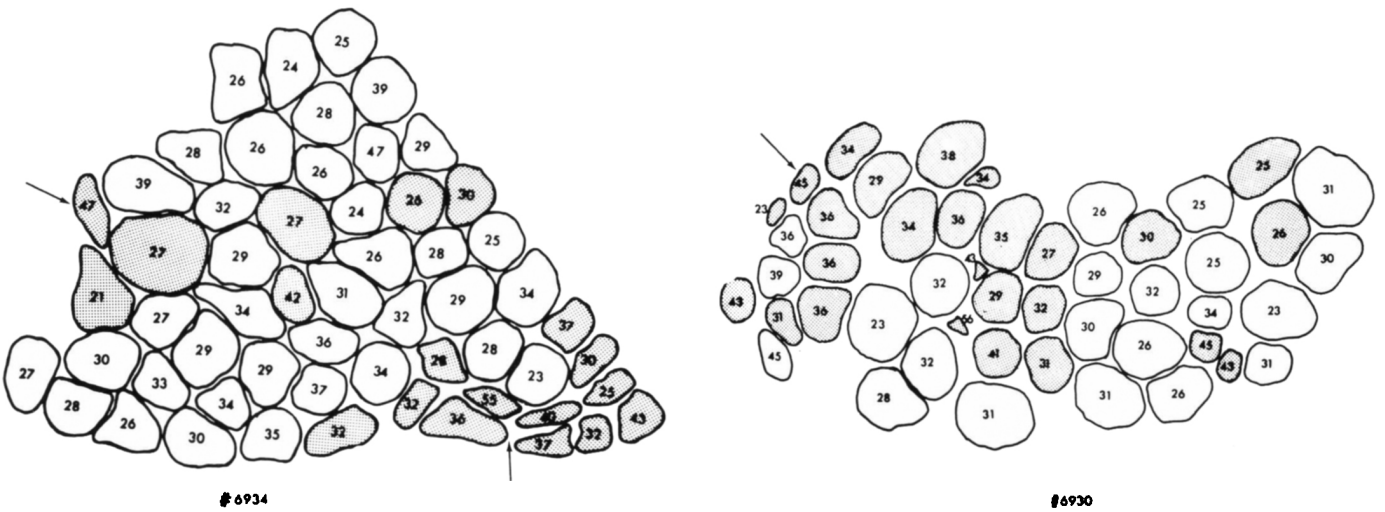

Figure 5a, b—Tracings from two biopsies of CMT muscle fibers stained with p-phenylenediamine showing type I (dark) and type II (white) fibers. Note small fibers with increased activity grouped with low activity large fibers of the same type (arrows). CMT = Charcot-Marie-Tooth, MND = Motor Neuron Disease.

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tion" of the small fibers with regeneration. These findings support the observations of Coers (1976) of an increased terminal innervation ratio in Charcot-Marie-Tooth disease and those of Tomé and Fardeau (1976) of an increased ribosomal activity in the smaller muscle fibers.

In conclusion, the argument as to whether there is a genetic myopathic disorder in association with the neuronal component of Charcot-Marie-Tooth disease as suggested by a number of authors (Haase and Shy, 1960; Engel, 1976b; Mumenthaler, 1970) cannot be substantiated by the method of autoradiography which, on the contrary, firmly suggests an active regenerative effort by the muscle fibers following reinnervation.

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