The Incorporation of ^{3}H (G) L-Leucine into Single Muscle Fibers in Duchenne Dystrophy and Charcot-Marie Tooth Disease

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SUMMARY: *The results are reported of single muscle fiber uptake of tritiated leucine in muscle biopsy material from Duchenne dystrophy and Charcot-Marie Tooth disease. The uptake in the two conditions is compared and suggests that the previously reported increase in synthesis of cytoplasmic proteins in muscular dystrophy are probably related to regenerative efforts by the muscle fibers.*

RÉSUMÉ: *Nous rapportons nos résultats de l'étude de l'incorporation de la leucine tritiée dans les fibres musculaires uniques provenant de materiel de biopsie de la Dystrophie de Duchenne et de la maladie de Charcot-Marie-Tooth. La captation est comparée dans les deux entités. Les résultats suggèrent que l'augmentation de synthèse des protéines cytoplasmique préalablement observée dans la dystrophie musculaire est probablement reliée aux effets régénératifs des fibres musculaires.*

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In an earlier study we showed there was an increased uptake of tritiated leucine into proteins in muscle obtained from patients with Charcot-Marie Tooth disease. Analysis of the uptake into single muscle fibers showed the increased incorporation was into smaller fibers only, and these appeared to be undergoing regeneration following reinnervation (Monckton and Marusyk, 1978).

The search for a primary biochemical defect in Duchenne dystrophy has lead to the demonstration of a wide spectrum of abnormalities, all of which appear to be secondary phenomena. Of the areas of biochemical interest studied, few have received more attention that protein synthesis. In the dystrophic mouse there is an increase in uptake of tritiated leucine into cytoplasmic protein, but a decreased uptake into contractile elements in muscle (Coleman and Ashworth, 1959; Kruh et al, 1960; Monckton and Marusyk, 1975; Nihei et al 1971; Simon et al, 1962).

Although there are no studies of protein turnover in Duchenne dystrophy, the pattern of uptake of tritiated leucine in Duchenne muscle is similar to that seen in the dystrophic mouse, and shows a marked increase in incorporation into cytoplasmic proteins and a reduced uptake into structural protein. We have also shown that the localization of this uptake in progressive muscular dystrophy is similar in the sarcomere to that in the normal human and dystrophic mouse muscle (Monckton and Nihei, 1971; Monckton and Marusyk, 1976, 1978). Because all these observations have been carried out largely by specific activity assays and tissue autoradiography, we thought it desirable to determine whether fiber labelling is uniform in Duchenne muscle. We, therefore, decided to examine individual muscle fiber uptake as in Charcot-Marie Tooth disease.

MATERIALS AND METHODS

The muscle biopsies were carried out in six patients with Duchenne dystrophy and six patients with Charcot-Marie Tooth disease. Control muscle was obtained from nine patients, age 13 to 64 years, undergoing surgery for chronic non-infective orthopedic disorders. Fragments of the muscle obtained were placed in an isotope mixture containing 0.9 ml. human serum and 100 microcuries of ^{3}H (G) L-Leucine (Amersham Searle). The muscles were incubated at 37 degrees C. for one hour and then rinsed in normal saline three times. Each specimen was then divided and prepared for routine light microscopy and autoradiography. These specimens were fixed in 3% glutaraldehyde in phosphate buffer at pH 7.2 for two hours, post-fixed in 1% osmium tetroxide in phosphate buffer for one hour, dehydrated in ethanol and embedded in Epon 812 (Ladd); 0.5%, 2,5-diphenyloxazole (PPO, Amersham Searle), was added to the propylene oxide/Epon mixture of the embedding procedure (Rogers, 1969). The Epon blocks were polymerized at 60 degrees C. for 48 hours. Thick (1 u) 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 degrees C. (Fisher et al, 1971). The slides were developed after ten days. The sections were stained

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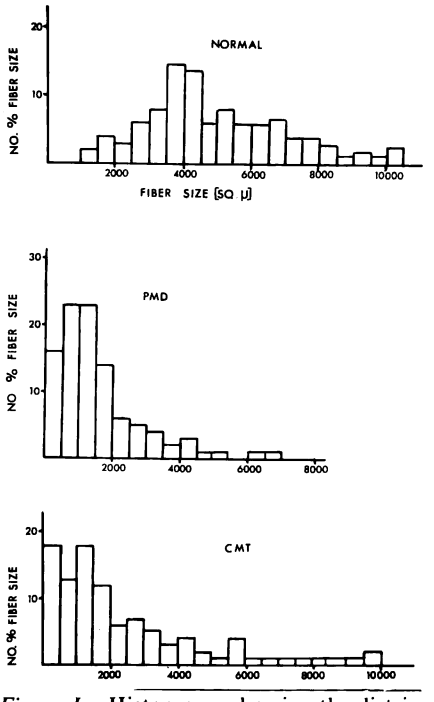


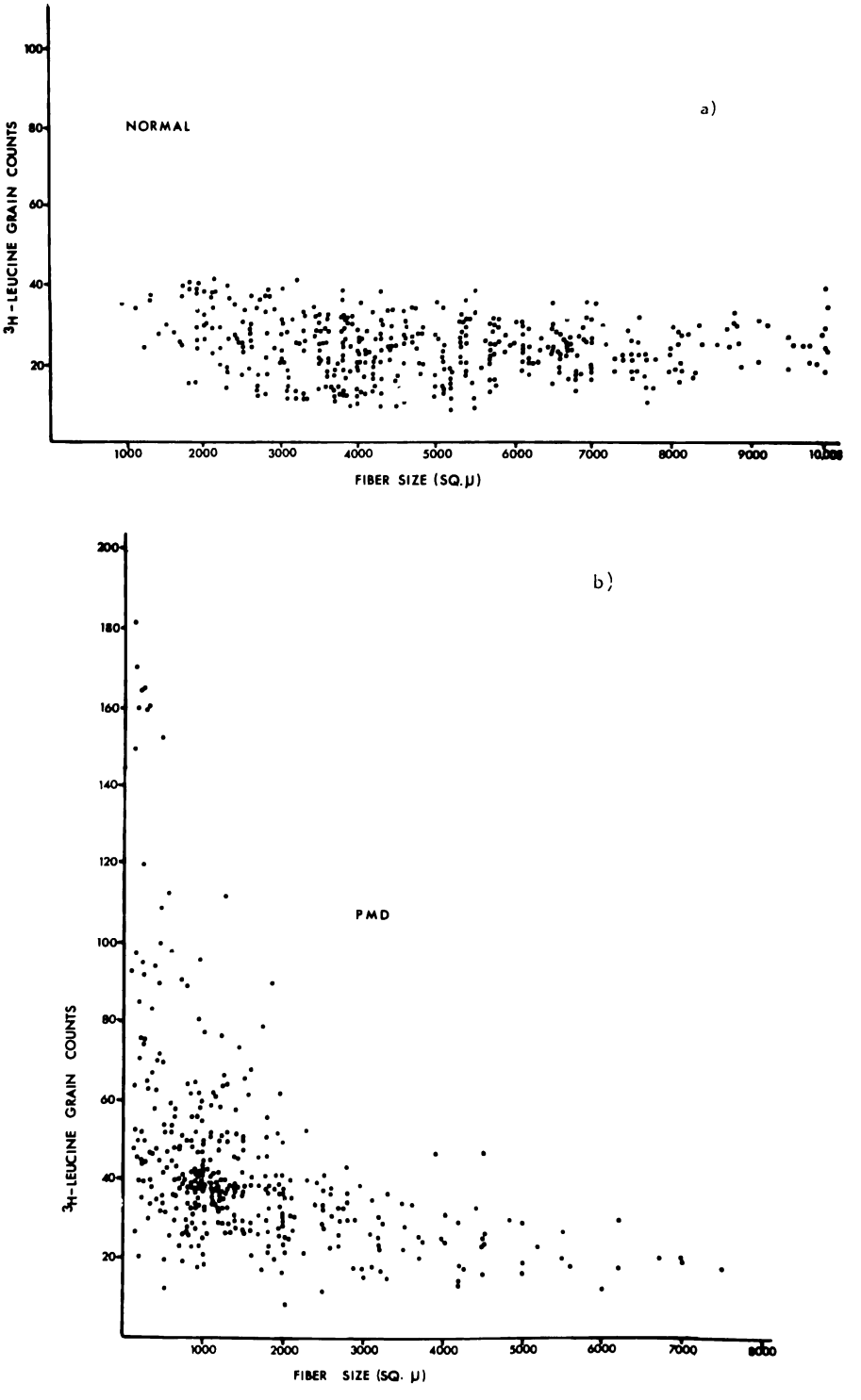
Figure 1—Histograms showing the distribution as a percentage of the total number of fibers in each represented size range. Total number of fibers: Top—normal 394; Middle—Duchenne dystrophy = 341; Lower—Charcot-Marie Tooth disease = 413.

with a saturated aqueous solution of phenylenediamine and examined with the light microscope. Each cross section was photographed and 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 typing was carried out using the method of Korneliussen (1972) in which sections were stained with phenylenediamine dissolved in methyl alcohol and examined by light microscope. Thin sections were also examined by E.M. to confirm fiber types in the sections examined above.

RESULTS:

Figure I shows the distribution of the muscle fibers by cross sectional area in normal, dystrophic, and Charcot-Marie Tooth patient samples. These histograms show that the majority of fibers in progressive muscular dystrophy and Charcot-Marie Tooth disease are in the smaller size range. The distribution of fibers in the normal group are consistent with that normally seen (Sissons, 1963).

The grain counts over the cross sectional area of each fiber were then calculated out to provide a grain count per 1000 square microns, so that the



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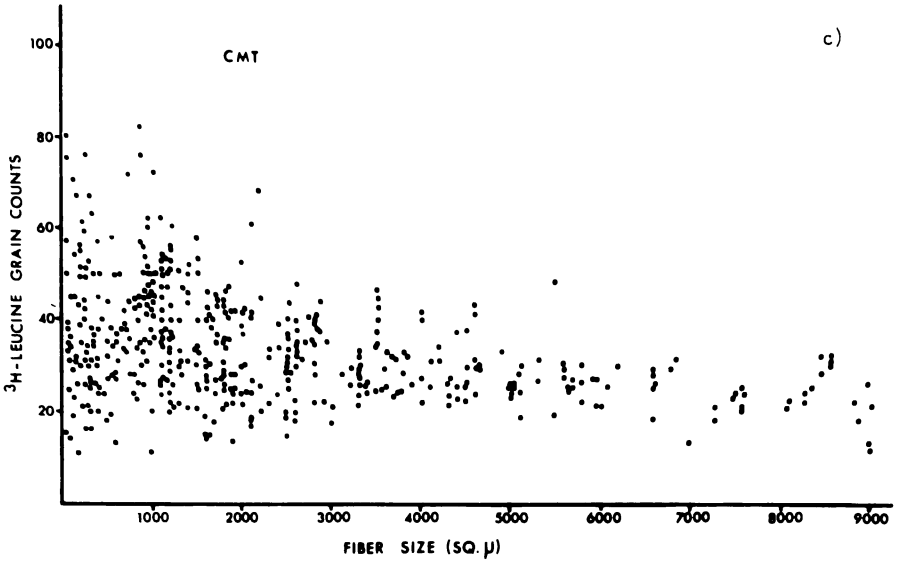


Figure II a, b, c — Histograms showing relationship of grain count/unit area 1000 μ^2 and fiber size. Each dot represents a single fiber. II a — normal. II b — Duchenne Dystrophy II c — Charcot-Marie Tooth Disease.

comparative levels of uptake in the fibers could be obtained. These data were assimilated into dot histograms as seen in Figure II a, b and c. Figure II a shows that in the normal material the uptake/1000 square microns is in a comparatively narrow range for all fiber sizes tested. The histograms from the Duchenne dystrophic and Charcot-Marie Tooth patients show a significant increase in uptake in the smaller fibers, and this abnormally high uptake drops down to the normal range in the larger fibers. In both Charcot-Marie Tooth disease and Duchenne dystrophy it can be seen that the smaller fibers are heterogeneous as to the levels of uptake. Graphs shown in Figure III a, b and c show the means of the levels of fiber uptake for each 500 square microns increase in area. In Duchenne dystrophy this shows the marked increase in incorporation into the smaller fibers, up to 2000 square microns, and a lesser increase in activity up to 4000 square microns. Charcot-Marie Tooth disease patients muscle fibers show a persisting slight, but significant, increase in uptake through to 7500 square microns.

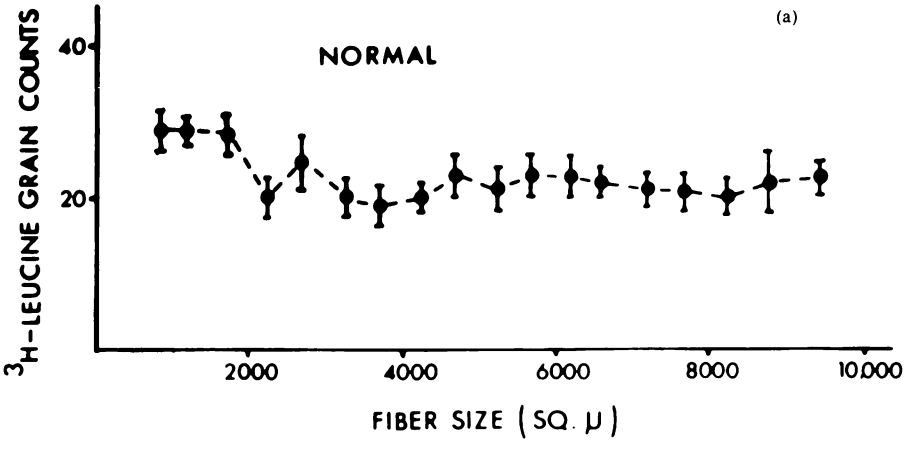
In the Charcot-Marie Tooth patients, as indicated in our previous study (Monckton and Marusyk, 1978), high uptake small muscle fibers are usually seen in association with normal uptake larger fibers of the same type. In the Duchenne dystrophic patients, the fiber type pattern, in general, was normal and there did not seem to be any consistent correlation with fiber type and high grain counts. However, occasional exceptions were seen, as in Figure IV where a very high uptake fiber can be seen in association with other normal range uptake fibers of the same type.

DISCUSSION

At the outset it is recognized that isotope uptake studies in biopsies are subject to a number of intrinsic errors. The only safeguards are the use of standard methods, and in this series considerable care has been taken to ensure the uniform handling of the material and the assay methods.

In discussing the significance of the abnormal uptake into muscle proteins in Charcot-Marie Tooth disease, we considered three possible bases for this: 1) that there might be a defect of muscle protein synthesis affecting the uptake of leucine, 2) that abnormal neurotrophism is responsible for a different and increased uptake, and 3) that the abnormally high uptake is related to reinnervation of muscle fibers. The abnormal uptake is observed to be in the smaller fibers, and these were seen in association with groups of larger fibers with normal activity of the same type, suggesting that the smaller fibers were undergoing regeneration secondary to reinnervation. Of these possibilities we considered the last to be the most reasonable (Monckton and Marusyk, 1978).

In Duchenne dystrophy the present study demonstrates that the bulk of the increased uptake is into small muscle fibers with a small cross sectional area. Interpretation of this requires consideration of some of the factors involved in the uptake of ^{3}H leucine into muscle fibers. The penetration of isotope from the incubation medium in the muscle fibers, in our hands, appears to be uniform and there is no evidence of irregular penetration in terms of superficial fibers and those deep inside the biopsy. The smaller fibers were scattered throughout the biopsy in random fashion and were not confined



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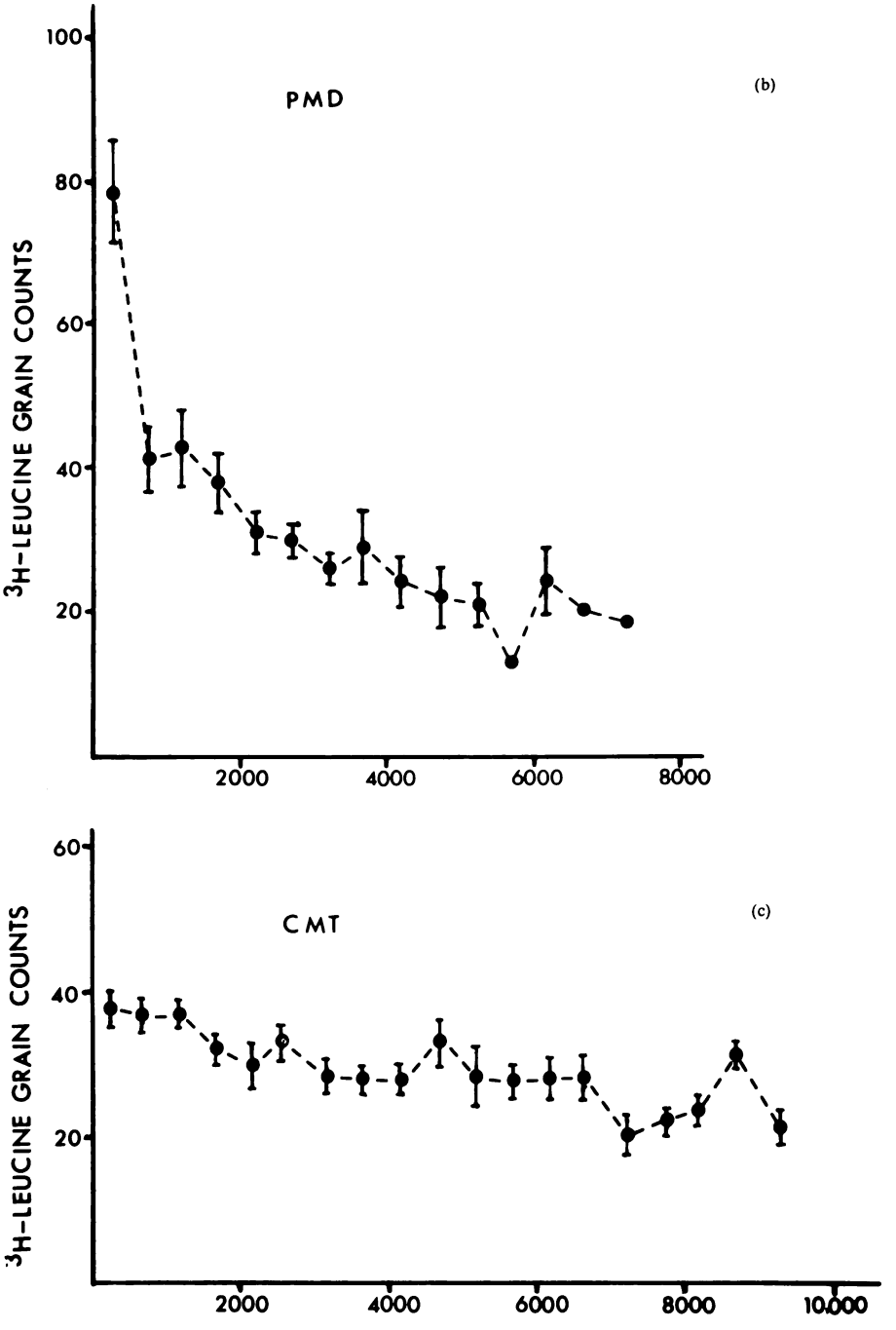

Figure III a, b, c — Graphs showing means ± S.E.M. of fiber uptake per unit area and fiber size by 500μ^2 steps in normal, dystrophic and Charcot-Marie Tooth disease.

to superficial areas. It seems that there was no difficulty in penetration of isotope to the core of the portion of biopsy incubated. A further factor of significance is that of the surface to volume ratio in determining uptake of radioisotopes. In smaller fibers there is an increased surface to volume ratio that theoretically increases the area of access of isotopes and would thus facilitate penetration. In our cases it will have been observed that many of the smaller fibers had normal levels of uptake and one must presume that those with increased levels had this for some other reason than surface to volume ratio.

Fiber typing in the Duchenne dystrophic cases failed to show a pattern consistent with reinnervation as we have discussed with increased isotope uptake in Charcot-Marie Tooth disease.

If it is assumed that muscle necrosis in muscular dystrophy occurs as a result of plasmalemmal defect (Bodensteiner and Engel, 1977; Mokri and Engel, 1975), and that repair occurs segmentally, then those segments will be variably less than normal in cross sectional area and will demonstrate increased levels of protein synthesis. We believe that our studies are entirely in support of such an hypothesis and that the increased uptake of tritiated

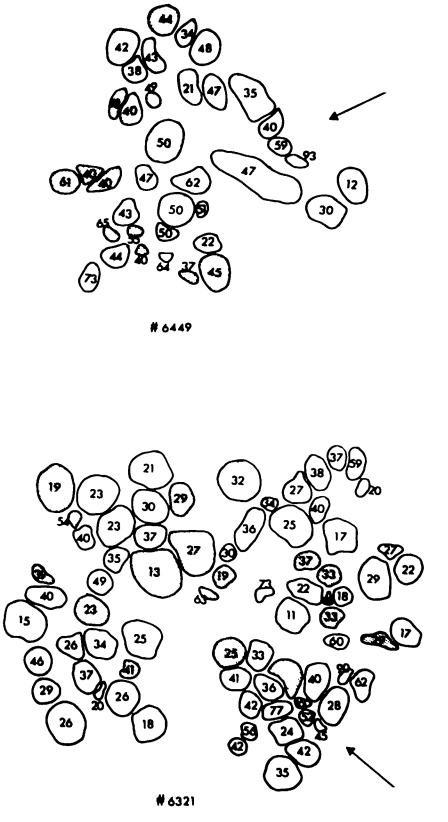

Figure IV — Tracings of two biopsies from patients with Duchenne dystrophy. #6449 shows random fiber type distribution with evidence of reinnervation as judged by a group of Type II fibers, one of which has very high grain counts. Biopsy 6321 shows similar changes — see arrows.

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leucine is in response to the regenerative effort of damaged muscle fibers. Nevertheless, we are unable to say whether this response is in itself normal in degree.

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