

Investigation of the organelle pathology of skeletal muscle in chronic alcoholism

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SUMMARY The muscle abnormalities associated with chronic alcohol consumption were studied by applying histological and biochemical techniques to tissue obtained by percutaneous needle biopsy from the quadriceps muscles of 41 patients. Measurement of the fibre size showed atrophy of both type I ($p < 0.05$) and type II ($p < 0.001$) fibres. The degree of atrophy was more severe for type II fibres (33% reduction in median diameter) than type I (17%). Marker enzyme activities for the principal organelles were assayed. Compared with biopsy specimens from non-alcoholic controls, no differences were found in the activities of lysosomal, mitochondrial, peroxisomal, cytosolic, sarcolemmal, or sarcoplasmic reticulum enzymes, expressed per μg DNA. A reduction in the protein to DNA ratio was evident in severely atrophic biopsies, and this was associated with a significant reduction of myofibrillary Ca^{2+} -ATPase activity. These results suggest a selective loss of type II fibre myofibrillary protein and do not confirm earlier suggestions of specific mitochondrial damage.

Recent years have seen a considerable increase in alcohol consumption in most countries throughout Europe. This has been accompanied by an increased incidence of alcohol-related physical disease,¹ which now accounts for a substantial proportion of medical illness seen in urban hospitals.^{2,3} Work on primates, including man,⁴ has shown that ethanol, even with a nutritionally adequate diet, causes the fatty change characteristically seen in the livers of heavy drinkers. Skeletal muscle damage in alcoholism was first described as long ago as 1822,⁵ but it has only recently become the attention of detailed investigation. Recent reviews have developed the clinicopathological classification and discussed possible mechanisms of damage.⁶⁻⁹ Acute rhabdomyolysis and chronic symmetrical proximal wasting are quite distinct clinical pictures seen in alcoholics, but some of the ultrastructural and biochemical reports have not clearly distinguished between these two syndromes.

Ultrastructural changes in the mitochondria,¹⁰⁻¹⁶ sarcoplasmic reticulum,¹⁶ and sarcolemma¹⁷ have been reported after acute or chronic exposure to ethanol, but these studies were only qualitative and functional abnormalities in these organelles have not been studied systematically. Some reports^{18,19} have suggested that there is reduced activity of suc-

cinatase and malate dehydrogenases and of cytochrome c oxidase in muscle from heavy drinkers, also implicating mitochondrial damage in this disorder.

This study has differentiated the acute and chronic forms of muscle damage with a view to investigating possible differences in organelle pathology. The investigation of tissue organelles by enzyme analysis of milligram quantities of tissue has proved valuable in the understanding of various human diseases.²⁰ It has been used to study human^{21,22} and experimental²³ cardiomyopathies and muscular dystrophy in the mouse.²⁴ The properties of the principal organelles have recently been studied in normal human muscle;²⁵ assay methods and specific assay conditions have been established for the organelle marker enzymes.

This paper describes the application of these techniques to the study of muscle damage associated with alcoholism. To allow correlation between morphological and biochemical data, muscle biopsies have been classified as showing acute damage or chronic fibre atrophy, and the latter has been quantified.

Material and methods

PATIENTS

Needle biopsies were obtained with a modified

Bergström needle from the vastus lateralis portion of the quadriceps muscle by a standard technique.²⁶ Control biopsies were taken from hospital patients by the same technique, as part of an investigation of muscle or systemic symptoms unrelated to alcohol. Full clinical and laboratory investigation subsequently showed these control patients to have no evidence of muscle disease; their biopsies were histologically normal. Muscle biopsies were taken from a consecutive series of patients receiving treatment for physical or psychological problems associated with the consumption of more than 100 g (men) or 80 g (women) of ethanol daily for at least three years. All patients were studied between one and five days after admission. A few patients were excluded from the study because deranged clotting function made biopsy unsafe. All patients were euthyroid by routine laboratory tests. Most had mildly deranged liver function and macrocytosis, but those with biochemical evidence of osteomalacia or seriously disturbed liver function as reflected by a serum albumin concentration below 25 g/l were excluded from analysis. No patient had the clinical picture of pseudo-Cushing's syndrome

PROCESSING OF BIOPSIES

About 30–60 mg of tissue was obtained and divided immediately. One portion was taken for histological study, which included standard histological stains¹⁷ to identify inflammation and acute myopathy and a stain for myosin ATPase at pH 9.4 to distinguish fibre types. The size of fibre types I and II was estimated using a "Magiscan" image analysis system.²⁷ Separate microscope fields were studied to allow measurement of 150 fibres of each type per specimen (Fig. 1). A median diameter and an atrophy factor²⁸ were calculated for each fibre type. The atrophy factor provides a numerical assessment of the size of the tail of small fibres in a histogram constructed of individual fibre diameter measure-

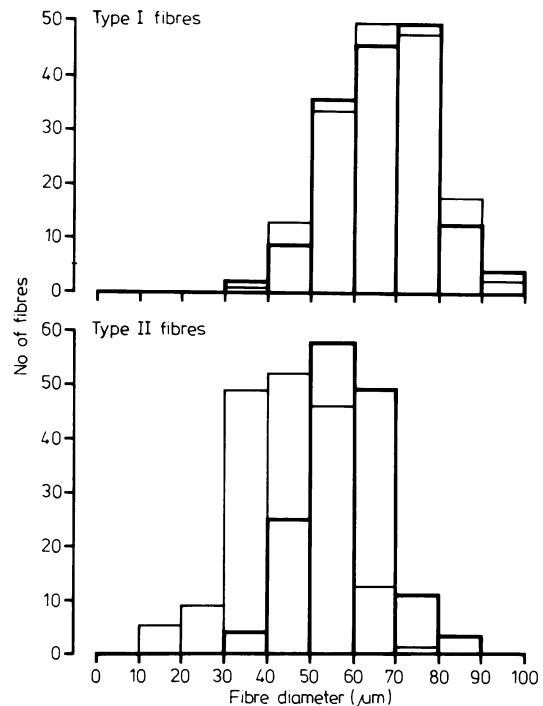


Fig. 2 Histograms constructed from individual fibre diameter measurements of 150 fibres. Normal male control (—), male alcoholic (---).

ments (Fig. 2). The criteria for determining the presence of acute myopathy were characteristic features of an acute rhabdomyolysis.²⁹ The biopsies were then subdivided into five groups: normal patient controls; alcoholic "normal,"—that is, with normal histological appearances and morphometry; alcoholic with acute myopathy; alcoholic with mild type II fibre atrophy; alcoholic with severe type II

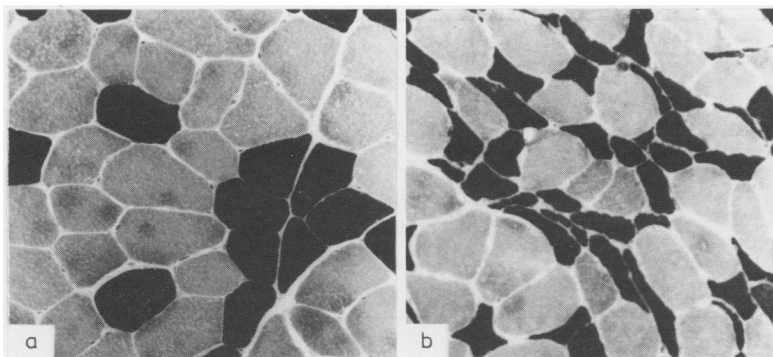


Fig. 1 Photomicrograph of myosin ATPase (pH 9.4) stained section of muscle biopsy taken from (a) a normal male control and (b) a male alcoholic showing atrophy of type II (dark staining) fibres.

atrophy. The group of biopsies showing acute myopathy was excluded from the statistical analysis because of the small number and is discussed separately.

BIOCHEMICAL ANALYSIS

The portion of the biopsy for tissue biochemical analysis was collected into 3 ml ice cold 0.25 mol/l sucrose solution containing 20 mmol/l ethanol and 1 mmol/l disodium EDTA, pH 7.4 (sucrose medium). The sample was then stored at -20°C for up to eight weeks before homogenisation. After it had thawed, the sample was minced with fine scissors and then further disrupted with a small Polyttron homogeniser (Kinematica GmbH, Lucerne, Switzerland) at setting no 2 for 15 s. Homogenisation was then performed with 10 strokes of a loose-fitting (type A) glass pestle in a small Dounce homogeniser (Kontes Glass Co, Vineland, New Jersey, USA) followed by eight strokes of a tight-fitting (type B) pestle. These procedures were performed in ice. The homogenate was then filtered through a $50\ \mu\text{m}$ nylon mesh which removes fibrous debris.³⁰ Enzyme assays were performed within two weeks of homogenisation and the homogenate was stored at -20°C between analyses; samples were not refrozen and thawed more than twice.

ENZYMIC ANALYSES

These were based on fluorimetric or spectrophotometric methods previously adapted for human skeletal muscle biopsies and for which the optimum assay conditions have been determined and enzyme localisations established after subcellular fractionation by sucrose density gradient centrifugation.²⁵ Protein was measured by a modification³¹ of the method of Lowry,³² with bovine serum albumin as standard. DNA was measured fluorimetrically³³ with calf thymus (Sigma type I) as standard.

ANALYSIS OF RESULTS

Means and standard deviations were calculated for each of the groups for each enzyme activity in terms of milliunits/mg protein and milliunits/ μg DNA, where 1 milliunit is the amount of enzyme activity producing one nmol of reaction product at 37°C , with the exception of catalase, which was assayed at 25°C . The distribution of catalase activity showed a positive skew within each group and logarithmic transformation of the results was required to enable comparison of groups by a parametric method.³⁴ For each enzyme the spread within each group was then compared by Bartlett's test of homogeneity of variance.³⁴ If the groups were similar in their degree of spread ($p > 0.05$) a standard one way analysis of variance test was used to compare the groups. If differences were found with these preliminary methods then the group contrast was calculated with group means in order to establish the presence of a linear trend with the degree of atrophy or a difference between the pooled alcohol groups and the control group.³⁵ If the groups showed significantly non-homogeneous variance, as in the case of lactate dehydrogenase and Ca^{2+} -ATPase, then groups were compared using the non-parametric Kruskal-Wallis one way analysis of variance test.³⁶ If this analysis showed significant variance ($p < 0.05$) the groups were compared using a Mann-Whitney test.

Results

Clinical and morphometric details are summarised in Table 1.

MORPHOMETRY

Atrophy of type II fibres was found in 33 of 41 patients studied (Fig. 2). Differences between median fibre diameters were evident with a decreasing diameter from the normal alcoholic to the severe atrophy group. This was true for both fibre types,

Table 1 Clinical details and histomorphometric results from muscle biopsy samples

| Patient groups | Type II atrophy factor | No | Sex | | Age (yr) (Mean) | Median fibre diameter (Mean \pm SEM) | |
|--------------------------------------|------------------------------|----|-----|---|-----------------|--|-----------------|
| | | | M | F | | Type I | Type II |
| Patient controls | <150 Men <200 Women | 13 | 8 | 5 | 21-66 (47) | 60.4 \pm 2.2 | 57.0 \pm 2.2 |
| Alcoholic patients— Normal muscle | <150 Men <200 Women | 8 | 6 | 2 | 37-55 (44) | *63.9 \pm 2.9 | †55.6 \pm 2.0 |
| Mild atrophy | 150-600 Men 200-600 Women | 22 | 13 | 8 | 30-68 (48) | *56.7 \pm 1.9 | †43.4 \pm 1.3 |
| Severe atrophy | >600 Both sexes | 11 | 8 | 3 | 36-66 (52) | *52.5 \pm 1.6 | †36.1 \pm 1.8 |

*Decreasing trend with atrophy ($p < 0.05$).

†Decreasing trend with atrophy ($p < 0.001$).

though the percentage drop in median diameter from the normal alcoholic group to the severe atrophy group was much larger for type II fibres (33%) than for type I fibres (17%). The individual histograms for each biopsy constructed for each fibre type generally showed a skewed distribution towards the low diameter end. The distribution of fibre diameters was in no case bimodal.

HISTOLOGY

Three patients showed changes of acute myonecrosis. Patient 1, a 41 year old woman, had a type II fibre atrophy factor of 248 and median fibre diameters of 47 (type I) and 35 (type II). Patient 2, a 54 year old woman, had an atrophy factor of 253 and median fibre diameters of 55 and 37, respectively. Patient 3, a 60 year old man, who also appeared severely malnourished, had an atrophy factor of 1600 and fibre diameters of 48 and 31, respectively. Only one patient showed reduction of myophosphorylase stain and this was in relation to a necrotic fibre. Fibrosis or internalisation of nuclei (greater than 2%) was not noted and there was no histological evidence of fibre denervation.²⁹

TISSUE BIOCHEMICAL ANALYSIS (Tables 2, 3, 4)

Table 2 shows the protein and principal organelle marker enzyme activities in the muscle biopsy samples. Neutral α -glucosidase (sarcolemmal reticulum) and 5' nucleotidase (sarcolemma) activities expressed per mg protein showed significant differences between the four groups. On further statistical analysis the mean activities of the patient control and the normal alcohol groups were similar whereas those of the three alcohol groups showed a

significant and increasing trend with atrophy. N-Acetyl- β -glucosaminidase activity per mg protein showed a similar trend, though this was not significant. The pooled results for all alcohol groups, however, showed a significant increase in this enzyme activity compared with patient controls. There were no significant differences in acid phosphatase activities between the various patient groups. None of the above mentioned differences was evident when enzyme activities were expressed per μ g DNA.

Catalase activity was distributed in a positive skew fashion with a few samples in each group having very high activities. This could not be related to the type or severity of myopathy or history of alcohol consumption. It may reflect the degree of red cell contamination of the tissue sample. No significant difference in protein to DNA ratio between the groups was found and, although a decreasing trend with the degree of atrophy was evident, this was not significant.

Table 3 shows that the activities of mitochondrial enzyme markers and lactate dehydrogenase did not differ appreciably between the groups. Assays of ATPase activities are shown in Table 4. Ca^{2+} ATPase activity, both total and azide-resistant, showed a significant reduction per μ g DNA compared with the other patient groups. The azide-sensitive fraction of Ca^{2+} -ATPase did not show significant reductions. Differences were not evident when enzyme activities were expressed per mg protein.

For certain enzymes the mean activity of the group showing acute myopathy was different from the others, but numbers were too small to enable

Table 2 Organelle marker enzyme activities in patient groups.

| Enzyme (organelle) | | Specific activities (Mean \pm SEM for (n) samples) | | | | |
|--|---|---|--------------------|----------------------|-----------------------|--------------------|
| | | Patient controls* (11) | Alcoholic patients | | | |
| | | | Normal (5) | Mild atrophy (16) | Severe atrophy (7) | Myonecrosis (3) |
| Neutral α -glucosidase ($\times 10^3$) (sarcolemmal reticulum) | a | 40.2 \pm 4.4 | †38.3 \pm 4.7 | †51.7 \pm 3.8 | †57.1 \pm 8.9 | 63.0 \pm 17.0 |
| | b | 18.6 \pm 1.5 | 20.8 \pm 3.5 | 22.3 \pm 1.6 | 19.5 \pm 2.8 | 23.0 \pm 3.2 |
| 5' Nucleotidase (sarcolemma) | a | 0.250 \pm 0.031 | †0.223 \pm 0.038 | †0.406 \pm 0.054 | †0.470 \pm 0.067 | 0.616 \pm 0.061 |
| | b | 0.118 \pm 0.022 | 0.124 \pm 0.027 | 0.186 \pm 0.027 | 0.162 \pm 0.022 | 0.340 (2) |
| N-Acetyl- β -glucosaminidase (lysosome) | a | 0.068 \pm 0.006 | ‡0.076 \pm 0.010 | ‡0.099 \pm 0.009 | ‡0.102 \pm 0.011 | 0.119 \pm 0.046 |
| | b | 0.043 \pm 0.003 | 0.049 \pm 0.005 | 0.055 \pm 0.007 | 0.053 \pm 0.005 | 0.064 \pm 0.003 |
| Acid phosphatase (lysosome) | a | 2.07 \pm 0.23 | 2.02 \pm 0.19 | 2.20 \pm 0.24 | 2.61 \pm 0.37 | 2.57 \pm 0.46 |
| | b | 1.02 \pm 0.12 | 1.02 \pm 0.13 | 1.07 \pm 0.13 | 0.83 \pm 0.19 | 1.19 \pm 0.11 |
| Catalase (log) (peroxisome) | a | 1.12 \pm 0.13 | 0.803 \pm 0.079 | 0.951 \pm 0.094 | 1.28 \pm 0.17 | 0.857 \pm 0.246 |
| | b | 0.715 \pm 0.126 | 0.535 \pm 0.098 | 0.532 \pm 0.081 | 0.731 \pm 0.188 | 0.642 (2) |
| Protein/DNA (mg/ μ g) | | 0.476 \pm 0.039 | 0.530 \pm 0.083 | 0.482 \pm 0.054 | 0.351 \pm 0.049 | 0.500 \pm 0.098 |

*Control specific activities (protein) are taken from Martin *et al.*²⁵

†Increasing trend with atrophy ($p < 0.05$).

‡Combined alcohol groups increased compared with controls ($p < 0.05$).

a = specific activities expressed as mUnits/mg protein.

b = specific activities expressed as mUnits/ μ g DNA.

Table 3 *Organelle marker enzyme activities by patient groups*

| Enzyme (organelle) | Specific activities (Mean \pm SEM for (n) samples) | | | | |
|--|---|------------------------------------|--|--|------------------------------|
| | Patient controls* (11) | Alcoholic patients | | | |
| | | Normal (5) | Mild atrophy (16) | Severe atrophy (7) | Myonecrosis (3) |
| Malate dehydrogenase (cytosol and mitochondria) | a 1160 \pm 104 b 337 \pm 23 | 1110 \pm 200 310 \pm 62 | 1380 \pm 150 342 \pm 41 | 1240 \pm 150 213 \pm 29 | 1280 \pm 71 405 \pm 9 |
| Glutamate dehydrogenase (mitochondria) | a 0.473 \pm 0.148 b 0.427 \pm 0.121 | 0.329 (n = 2) 0.279 (n = 2) | 0.385 \pm 0.070 0.354 \pm 0.097 | 0.376 \pm 0.134 0.291 \pm 0.039 | 0.699 (2) 0.252 (2) |
| Succinate dehydrogenase (mitochondria) | a 9.18 \pm 1.72 b 2.13 \pm 0.49 | 8.10 \pm 0.90 3.02 \pm 0.38 | 6.68 \pm 0.90 1.61 \pm 0.25 | 7.72 \pm 1.74 2.99 \pm 1.63 | 5.73 \pm 1.62 3.06 (2) |
| Lactate dehydrogenase (cytosol) | a 184 \pm 18 b 74.3 \pm 9.6 | 163 \pm 22 87.4 \pm 11.8 | 211 \pm 26 112 \pm 14 | 161 \pm 39 58.0 \pm 3.1 | 232 \pm 59 154 \pm 46 |

*Control specific activities (protein) are taken from Martin *et al.*²⁵

a = specific activities expressed as mUnits/mg protein.

b = specific activities expressed as mUnits/ μ g DNA.

conclusive information to be obtained. There was, however, considerably reduced activity of myofibrillary azide-resistant Mg^{2+} -ATPase, in contrast with the Ca^{2+} -ATPase activities, which were not reduced (Table 4). There was also an increase in 5' nucleotidase activity in tissue from patients with acute myopathy (Table 2).

Discussion

The use of quantitative morphometry has allowed the characterisation of muscle from chronic alcoholics into normal, mild, and severe type II atrophy. The results of this study confirm the finding of an

earlier series¹⁷ that the major histological abnormality of muscle in chronic alcoholics is the presence of a selective atrophy of type II fibres—that is, the fast twitch fibres.

A systematic study of vastus lateralis muscle in normal adult men (aged 22 to 65 years)³⁷ has shown that the proportion and mean diameter of type II fibres decreases with age. Such a change in diameter was not evident in type I fibres. Other workers have also reported type II atrophy in elderly subjects.^{38, 39} The exact mechanism of this change is not known, and it could be related to changing patterns of physical activity, nutritional factors, or endogenous changes of gene expression. The differences in age

Table 4 *Organelle marker enzyme activities by patient group*

| Enzyme (organelle) | Specific activities (Mean \pm SEM for (n) samples) | | | | |
|--|---|-----------------------------------|------------------------------------|------------------------------------|-----------------------------|
| | Patient controls* (8) | Alcoholic patients | | | |
| | | Normal (5) | Mild atrophy (13) | Severe atrophy (6) | Myonecrosis (3) |
| Total Ca^{2+} -ATPase | a 18.1 \pm 2.5 b 4.93 \pm 0.56 | 17.0 \pm 3.7 5.94 \pm 0.84 | 20.3 \pm 4.4 5.32 \pm 0.67 | 13.4 \pm 2.4 †2.68 \pm 0.27 | 19.9 (2) 6.88 \pm 1.10 |
| Azide-resistant Ca^{2+} -ATPase (myofibrillar) | a 16.6 \pm 3.2 b 4.93 \pm 0.51 | 12.9 \pm 1.9 5.20 \pm 0.81 | 13.9 \pm 3.0 4.33 \pm 0.50 | 12.2 (2) †2.99 \pm 0.49 | 17.2 (2) 5.52 \pm 0 |
| Azide-sensitive Ca^{2+} -ATPase (mitochondria) | a 1.1 \pm 0.2 b 0.97 (2) | 3.2 \pm 0.7 ND | 2.86 \pm 0.60 0.93 \pm 0.32 | 2.71 (2) 0.68 (2) | 2.86 (2) 1.52 (2) |
| Total Mg^{2+} -ATPase | a 8.09 \pm 1.4 b 4.77 (2) | 5.80 (2) 5.00 (2) | 10.8 \pm 2.7 (4) ND | 7.45 (1) 4.18 \pm 1.32 | 3.90 (1) ND |
| Azide-resistant Mg^{2+} -ATPase (myofibrillar) | a 7.72 \pm 1.95 | 4.90 (2) | 7.74 \pm 1.31 | 5.95 (1) | 2.00 (1) |
| Na^+ , K^+ -ATPase (sarcolemma) | a 3.92 (2) b 1.83 (2) | 7.05 (2) 3.97 (2) | 4.61 \pm 1.08 1.96 \pm 0.37 | ND ND | ND ND |

*Control specific activities (protein) from Martin *et al.*²⁵

†Significant reduction compared with patient controls and normal alcoholic groups ($p < 0.05$).

a = specific activities expressed/mg protein.

b = specific activities expressed/ μ g DNA.

ND = not determined.

distribution of the groups reported here are small and it would seem unlikely that they would introduce significant age related changes.

The size of both fibre types is smaller in women than men.²⁸ In our study the proportion of women in each group was roughly similar and was least in the group with the smallest fibre sizes (Table 1). It seems unlikely therefore that different sex ratios between the groups gave rise to the morphometric differences.

Could physical inactivity explain the findings? Our patients were ambulant and mainly in full time employment. Immobilisation of a limb generally produces preferential type I atrophy,^{40,41} but a mixed fibre atrophy has been reported in association with inactivity.⁴²

Three patients had changes of myonecrosis, identified by standard histological criteria. Although the finding can only be preliminary, there was a considerable decrease in activity of myofibrillary Mg^{2+} -ATPase in the one patient studied. This finding agrees with previous studies¹⁶ of an individual patient and of experimentally induced changes in human volunteers and baboons chronically exposed to ethanol. Those studies showed both reduced Ca^{2+} uptake by sarcoplasmic reticulum in vitro and reduced myosin Mg^{2+} -ATPase activity. Although striking ultrastructural abnormalities of the mitochondria and sarcoplasmic reticulum were noted, fibre size was not assessed.

In a previous study of chronic alcoholics¹⁸ studied within one to two days of an acute debauch, significant reductions in the activities of lactate, malate, and succinate dehydrogenases were reported. Enzyme activities returned to control values within six days. No histological assessment of the biopsies was made, so direct comparison with our patients may not be appropriate. Kiessling *et al*¹⁹ found reduced activities of lactate and triose phosphate dehydrogenases in biopsies from chronic alcoholics. They also reported type II fibre atrophy in the group as a whole and were able to correlate the specific activities of those enzymes with the reduced amount of fast twitch glycolytic, type II fibres. They also showed reduced mitochondrial volume fraction and reduced activity of cytochrome oxidase in these patients, but did not report type I fibre atrophy. We found some atrophy of type I fibres but failed to confirm any reduction in four mitochondrial enzyme activities in the muscle biopsies.

The major enzyme abnormality, particularly when the results were expressed/ μ g DNA, was the increasing reduction in myofibrillary Ca^{2+} -ATPase with increasing atrophy. The activity of this enzyme is higher in type II than type I fibres.⁴³ Thus the reduc-

tion may reflect the selective loss of contractile elements from type II fibres. The other abnormalities found in our studies may also be explained on the basis of a selective atrophy of myofibrillary protein, with relative preservation of other cell protein, and specifically a preservation of the sarcolemma and sarcoplasmic reticulum so that enzyme activities associated with these organelles show an apparent rise when expressed in terms of protein content but not in terms of DNA content. This underlines the importance of using a suitable reference parameter for the expression of enzyme activities. In this type of study, wet and dry weight or tissue protein will all tend to change in the same direction as myofibrillary atrophy and thus possibly obscure significant changes.

The increased activity of one lysosomal enzyme, N-acetyl- β -glucosaminidase, without significant change in the other lysosomal marker enzyme is of doubtful pathological importance. Increased lysosomal enzyme activity has previously been reported in human muscle dystrophy.⁴⁴ Neurogenic atrophy of human muscle is associated with increased numbers of lysosomes⁴⁵ and in experimental animals with an increased activity of acid hydrolases.⁴⁶ The normal catalase value also contrasts with the increased activity of this enzyme recently reported in muscular dystrophy⁴⁷ and in the type II atrophy caused by starvation or denervation of rat skeletal muscle.⁴⁸ The observed atrophy in our patients may therefore have a different pathogenic mechanism from type II atrophy occurring in other conditions.

These findings suggest that gross organelle damage is not involved in the pathogenesis of the muscle damage associated with chronic alcoholism. The selective atrophy of fast twitch fibres is of interest and suggests that further attention should be paid to the physiological role and metabolic properties of these fibres, and the effects of ethanol thereon.

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