THE UNIVERSITY OF MANITOBA

SEARCH FOR ABNORMALLY SYNTHESIZED PROTEINS IN NERVOUS TISSUES OF HAMSTERS AND MICE WITH GENETICALLY DETERMINED MUSCULAR DYSTROPHY

by

Marilyn D. Hayward

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MARILYN DOROTHY HAYWARD

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

For my friend and husband Rolly

with love-

"...let truth be free to make her sallies upon Thee, and Me, which way it pleases God. For who knows how, better than he that taught us first to plough, to guide our mind and pens for his design? And He makes base things usher in Divine...."

-John Bunyan, Pilgrims Progress

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ABSTRACT

Muscular dystrophy is a group of genetically determined diseases characterized by muscular weakness and wasting. As in other inborn errors of metabolism the genetic defect(s) in the muscular dystrophies appear to be expressed in many tissues besides muscle and heart. Assuming that the genetic defect is expressed in(an) altered protein(s) a systematic search was performed for abnormally synthesized proteins in nervous tissues of two animal models of muscular dystrophy, the genetically determined muscular dystrophies in hamster, strain BIO 14.6 and mice, strain 129Rej/dydy.

To investigate the metabolism of the two different animals(diseased and control) simultaneously and to ensure exact reproducibility between the fractionation of diseased and control animal tissues a dual labelling technique was employed. Radioisotopes of leucine, L-(U^{14}C)leucine and L-(4,5^{3}H)leucine, were injected intraperitoneally into age-matched pairs of dystrophic and normal animals respectively. After a set period of incubation with the radioisotope the animals were killed and tissues were excised, combined into one preparation for homogenization and subcellularly fractionated. The dual labelling technique used in this way allows the comparison of the in vivo incorporation of ^{3}H and ^{14}C-leucine into various protein fractions in the normal and dystrophic animals. Proteins were separated by three analytical methods: SDS gel electrophoresis, disc gel electrophoresis and isoelectricfocusing.

A study of brain subcellular fractions of Syrian hamsters(1-6-days-old) by SDS gel electrophoresis and isoelectricfocusing showed no detectable difference between normal and dystrophic hamsters. An apparent abnormality of the particulate fractions was found to be an artifact of the leucine labels and could be removed by acetone extraction.

The dual labelling technique was used to investigate known neural abnormalities of the murine dystrophy. Preliminary work with the brain soluble and particulate fractions(mitochondrial) indicated no detectable abnormality was present in the dystrophic mice(4-12-weeks-old). Extirpated spinal cord homogenates of these mice showed protein abnormalities.

Dystrophic mice which were 29-days-old showed a decreased label incorporation into protein fractions in a region of an approximate molecular weight of 23,000. This may have been a true abnormality ultimately caused by the dystrophic gene. Lubrol solubilized homogenates of the spinal cord fractionated on isoelectricfocusing gels indicated two abnormalities in dystrophic mice of both 47 and 66 days of age. SDS gels of unlabelled spinal cord, spinal roots and sciatic nerve gave reproducible differences between 129Rej/dydy and 129Rej/+? mice; the dystrophic mice showing a decreased quantity of several protein components of high and low molecular weights. Using the 4.5% polyacrylamide gel with SDS but a Tris-EDTA buffer system spinal cord homogenates showed several components altered in concentration in the dystrophic mice compared to their normal littermates.

The pattern of leucine incorporation into various protein fractions of brain from both dystrophic mice and hamsters showed a remarkable identity between normal and dystrophic animals. This can be taken as conclusive evidence that there are no gross defects in any of the more abundant proteins of the brain.

Several abnormalities were detected in the spinal roots and sciatic nerve of dystrophic mice. However, the incorporation of labelled leucine into these fractions was too low to resolve the abnormalities reliably. Future studies, possibly on newborn animals and employing two-dimensional electrophoretic techniques may be required to clarify these findings.

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LIST of ABBREVIATIONS

ACh.....acetylcholine
AChE.....acetylcholinesterase
CAT.....choline acetyltransferase
d.....daltons
DMD.....Duchenne muscular dystrophy
DNA.....deoxyribonucleic acid
EDB.....extensor digitorum brevis muscle
EDTA.....ethylene diamine tetraacetic acid
EGTA.....ethylene glycol-bis(-aminoethyl ether)N,N tetraacetic acid
EDL.....extensor digitorum longus muscle
G-6-PDH.....glucose-6-phosphate dehydrogenase
IEF gel.....isoelectricfocusing gel
HbS.....sickle cell hemoglobin
Leu .....leucine
LDH .....lactate dehydrogenase
mepps ..... miniature end plate potentials
MeV ..... million electron volts
MD ..... muscular dystrophy
MICRO ..... microsomal pellet subcellular fraction
MITO ..... mitochondrial pellet subcellular fraction
mm ..... millimetres
M.W.....molecular weight
NADH ..... nicotinamide adenine dinucleotide
N-DNP-L-LEU.dinitrophenyl-L-leucine
NP ..... nuclear pellet
PA ..... polyacrylamide
PAGE ..... polyacrylamide gel electrophoresis
PM ..... picomoles
PMSF ..... phenylmethylsulfonylfluoride
RBC ..... red blood cell
SC ..... Schwann cell(s)
SD ..... standard deviation
SDH ..... succinate dehydrogenase
SDS.....sodium dodecyl sulfate
SOL.....soleus muscle
spec. act...specific activity
SR.....sarcoplasmic reticulum
SUP.....ultrasupernatant fraction, supernatant
T.D. ..... tracking dye
TEMED.....N,N,N',N'-tetramethylethylene diamine
uF .....microfarad
ul .....microlitre

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INTRODUCTION

The muscular dystrophies are a heterogeneous group of inherited diseases in man and animals. These diseases are characterized by striated muscle weakness and necrosis and usually lack any symptoms of nervous system disease (1). The progressive nature of these diseases is shown most dramatically in the human Duchenne muscular dystrophy. The disease heterogeneity is reflected in the different histological, electrophysiological and biochemical features of each type of muscular dystrophy as well as in their mode of inheritance. Any chance that these diseases have the same primary genetic(DNA) abnormality and biochemical expression of that abnormality at the cellular level is unlikely.

For many years the primary expression of this lesion was thought to occur in the muscle itself though the DNA carrying the muscular dystrophy mutation is contained in every cell (2). In recent times, however, the nervous system has been implicated in these diseases (3-16).

Why isn't the DNA abnormality studied directly, whether studying nervous tissue or striated muscles? The DNA lesion is difficult to study directly for two reasons. The first reason is the number and composition of gene loci present in each cell. In man there are between 40,000 and 100,000 structural gene loci (17). Each gene locus contains the nucleotide base sequence which is transcribed into its corresponding RNA and subsequently translated; resulting in a protein molecule(18). For every amino acid in the protein there is a corresponding triplet of nucleotide bases at the gene locus((19). This would mean that a small protein of 100 amino acids would be coded for by a base sequence of 300 nucleotide bases. The average length of a structural gene locus, however, is between 1000 and 1500 nucleotide base pairs (20). To study the number of bases involved at all the gene loci would be a formidable task.

The second reason there is a difficulty in studying DNA directly is that a point mutation, even a single base nucleotide substitution, can result in a grossly abnormal protein (19). These point mutations may result in changes in the charge(HbS in sickle cell anemia), in the molecular weight(haptoglobin variants), in the stability of a protein (glucose-6-phosphate-dehydrogenase variants), in the rate of synthesis of

of a protein or a complete failure of protein synthesis(thalasssemias) (19). Other mutations like crossing over on homologous chromosomes, or abberrant union after breaks in chromosomes occur, may result in duplication or deletion phenomena which bring about a change in the molecular weight of the protein (19). These mutations may alter the stability of the protein resulting in an altered half-life of the protein.

These various mutations, expressed in the amino acid sequence of a protein, may result in an abnormality which can be detected on the basis of altered molecular weight or altered charge of the protein. Differing rates of synthesis or degradation may also be detected in an analytical system which detects proteins quantitatively; such as polyacrylamide gel electrophoresis with sodium dodecyl sulphate(SDS-PAGE) or polyacrylamide gel electrofocusing(IEF gel).

The dual isotopic labelling technique is useful for studying genetically dystrophic and normal tissues. Dual labelling allows the simultaneous measurement of two different radioactive isotopes in the same sample. The isotopes can be distinguished on the basis of their half-lives or their energy spectrums, and therefore the amount of incorporated label present in a particular tissue or subcellular fraction can be determined. It is assumed that no biological discrimination occurs by the tissues in the utilization of the different isotopes of leucine.

In this study age-matched pairs of normal and dystrophic animals were injected with equimolar concentrations of a radioisotope of leucine (Figure 1). The tissues from the two animals were combined, homogenized and fractionated together therefore no anomalous differences related to differential sample preparation arise. This is an advantage of the dual labelling technique.

Because many thousand proteins exist in a single cell and the functions of only about 1300 are known (21) it may prove impotent to seek out specific cell proteins for analytical study. This would be especially true in genetic diseases where the disease origin is not known. Even when the 'basic defect' or abnormality is thought to be understood, unsuspected discoveries may show our limited knowledge and/or understanding of genetic diseases. In a recent study of cultured Pompe's fibroblasts where the

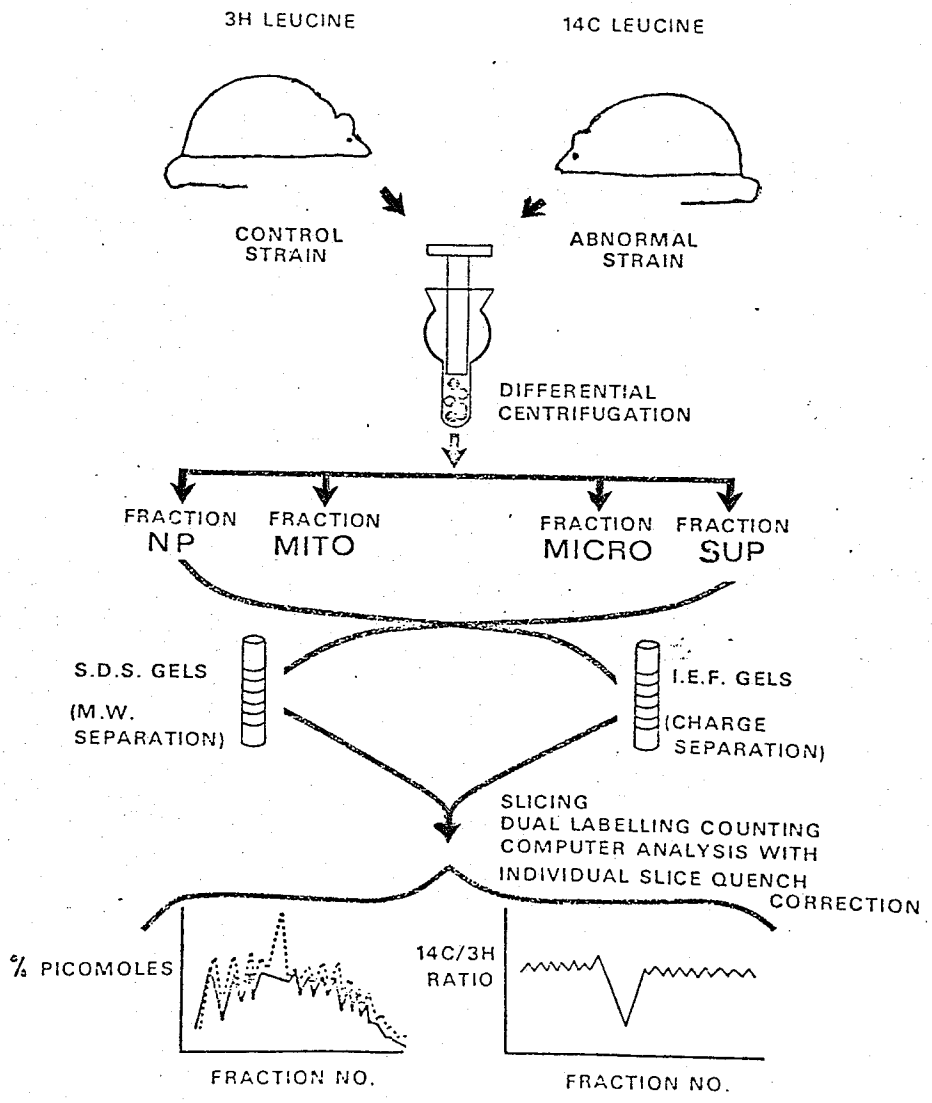


FIGURE 1. Flow scheme of the double isotopic labelling technique.

known basic genetic defect was a deficiency of α-1,4-glucosidase a second abnormality was found (22). This result was obtained using ^{3}H-and ^{14}C-leucine isotopes for labelling the Pompe's and normal fibroblasts in culture. The dual labelling technique has another advantage, then, in that it is unbiased.

This technique will be used to search for abnormal proteins of the nervous system tissues in the dystrophic hamster, strain BIO 14.6, and the murine dystrophy of Bar Harbour 129Rej/dydy mice.

HISTORICAL LITERATURE REVIEW

A. Muscular Dystrophy in Man and Animals

The muscular dystrophies are characterized by striated muscle weakness and necrosis. In the early phases of Duchenne muscular dystrophy (DMD) the patients' muscle appears to increase in mass. This is not due to an increase in muscle fibre material but to a replacement of the necrotic muscle cells by an excessive amount of fat tissue (1). Several histological changes in individual muscle fibre breakdown in DMD have been seen. These have been divided into five basic stages by Cullen and Fulthorpe (23). In Stage 1 the muscle fibres appear normal yet they have an increased volume in the sarcoplasm by 35-80%. A decrease in the mitochondrial volume also occurs. In transverse section the muscle fibres appear rounded rather than polyhedral in shape (23,24). In Stage 2 there is overcontraction of the myofibrils around the Z-line associated with overstretched regions in other parts of the sarcomere. Areas of overstretching are regions where no overlapping of the A and I bands occur. Z-line abnormalities occur and they are no longer at right angles to the longitudinal axis of the muscle fibre (23). Localized contraction continues in Stage 3 with the overstretched areas partly empty of the contractile myofibrils. The 'hyaline degeneration' characteristic of muscle fibres of DMD patients' occurs in Stage 4. As the clumps of myofibrillar elements becomes more condensed retraction clots and retraction caps form. The final stage(Stage 5) occurs with the invasion of the muscle fibre by macrophages. Areas of hypercontraction have also been seen in Duchenne patients which show plasma membrane lesions (25).

Fatty infiltration(lipomatosis) also occurs in the muscle of these patients (26) along with the formation of fibrous tissue (27). An increase in the number of subsarcolemmal nuclei with 'nuclear rowing' is seen in the muscle fibres of patients with muscular dystrophy (24).

Several animals with an inherited muscular dystrophy show similar pathological characteristics to the Duchenne muscular dystrophy and have been used as models in the study of this disease. The hamster strain BIO 14.6 has a form of muscular dystrophy which involves the heart and skeletal muscle(28). The skeletal muscle shows the nuclear rowing and central localization of the nuclei, myolysis and macrophage infiltration and some fat accumulation in the muscle mass as is found in DMD (28). The mouse(murine)dystrophy, strain Bar Harbour 129Rej/dydy, also has these histological characteristics as well as nervous system abnormalities (14, 29,30,31).

These animal forms of dystrophy are inherited in an autosomal recessive pattern in contrast to that of the Duchenne muscular dystrophy inherited in an X-linked recessive pattern (1,32,33). Another animal model for muscular dystrophy, which has some similarities to the Duchenne muscular dystrophy, is that of the domestic chicken. This muscular dystrophy is co-dominantly inherited and affects mainly the fast(glycolytic) muscle fibres of the pectoral muscles of the chicken (34). The pectoralis muscle shows major phagocytic cell infiltration and fat replacement of the muscle mass as well as vacuolization of the muscle fibres (26,35).

Though the animal forms of muscular dystrophy show similarities to DMD it is possible that the primary genetic lesion in these diseases is not the same. The study of muscular dystrophy in these animals may however

lead to an understanding of their particular biochemical abnormalities and thus the differences between dystrophic and normal animal tissue.

B. Evidence for Neurologic Involvement in Muscular Dystrophy

Because of the pathological characteristics of muscle in the muscular dystrophies they have been classified as myopathic diseases. A myopathy is usually defined as a disease with the primary disorder in the muscle (2). There are, however, several characteristics of muscle which are determined by the nerves which innervate them. The velocity of contraction and twitch duration can be altered by changing the nervous supply to the muscle (36). The enzyme and structural protein patterns of muscle can change as a result of a change in nervous stimulation; most specifically those proteins of the sarcoplasmic reticulum (37), myosin light chains in the contractile proteins(38,39) and enzymes involved in energy metabolism of muscle (40,41). The localization of acetylcholinesterase(AChE) activity to the muscle sole plate also does not occur without the nerve synapsing with the muscle fibre (42). The characteristics of muscle in DMD and those in denervated conditions or with altered neuronal influence often ressemble each other in morphology, biochemistry, and electrophysiologically. One might have expected this to lead to the investigation of nervous system involvement in muscular dystrophy and even to the possibility that the dystrophy is neurogenic rather than myogenic in origin. However, it was not until in 1967, when McComas and Mrozek found defective motor innervation of the extensor digitorum brevis (EDB) muscle in the dystrophic mouse, strain 129Rej/dydy, and in 1970 when McComas found a reduction of the number of motor units in the EDB muscle in patients with DMD, that a neurogenic origin for muscular dystrophy

was hypothesized (3,4). The concept behind this hypothesis was that the dystrophic motor neuron supplied insufficient trophic factors to the muscle fibres resulting in their weakening and eventual death (43). These experimental results lead one to ask the question; 'Are there neurotrophic factors responsible for the initiation of, or the development of, muscular dystrophy?' The trophic factor may be a messenger molecule which is transferred at the synaptic cleft, or the result of the impulse mediated(action potential) activity of the muscle (44) or perhaps a factor within the nerve not transferred to the muscle but affecting muscle metabolism (45). Because other tissues (46-61) other than muscle express abnormalities in dystrophic animals a study of the nervous tissues should be emphasized in the investigation of a gene mutation resulting in a general metabolic disease. The following section reviews the evidence for neurologic involvement in muscular dystrophy within ten categories:

1. Muscle transplantation studies
2. Organ culture of muscle
3. Mouse Chimaera studies
4. Altered muscle fibre enzymes
5. Altered lipid composition of muscle membranes
6. Axoplasmic flow abnormalities
7. Histological abnormalities in the nervous system
8. Proteins of muscle under neural control
9. Parabiosis studies in Muscular Dystrophy
10. Electrophysiological studies in Muscular Dystrophy

In spite of many inconsistencies in the assessment of the overall effect that the nervous system plays in muscular dystrophy a further study of the nervous system seems relevant in determining the exact nature of the muscular dystrophies.

1. Muscle transplantation in the animal muscular dystrophies- the effects of normal and dystrophic innervation.

Muscle transplantation experiments with dystrophic and normal hamster muscles did not consistently support neural involvement in muscular dystrophy. It was shown that dystrophic muscle transplants appeared unaltered in a normal healthy host whereas intact normal or dystrophic muscle in a dystrophic host were replaced by connective tissue (62). These results using whole muscle transplants were not corroborated by Neerunjun and Dubowitz using minced muscle transplants (63). The dystrophic hamster also regenerated normal minced muscle. Except for opaque rounded fibres in the dystrophic transplants there was no indication that dystrophic muscle could not regenerate. Since normal innervation would be required for normal regeneration of dystrophic muscle this experiment seems to indicate that there is no abnormality in the nervous system of the dystrophic hamster. The difference between these findings(62,63) may arise from the whole and minced(traumatized) state of the muscle being transplanted. Trauma itself may elicit regenerative behaviour by the muscle.(64).

Experiments using mice muscle transplants have also been carried out. Salafsky, using mouse strain 129Rej, showed that in normal hosts, normal and dystrophic muscle regenerated with normal characteristics (6). The transplants into dystrophic hosts did not regenerate whether the donor tissue was normal or dystrophic. 'Environmental effects', such as motoneuron abnormalities, were suggested as being responsible for the muscle's dystrophic condition.

Similar results were obtained in tissue culture (7). No regeneration or growth of the muscle fibres occurred in culture with the dystrophic spinal cord. Normal regeneration occurred with both dystrophic and normal muscle being innervated by normal spinal cord.

Experiments which strongly support neural involvement and even a neurogenic origin for muscular dystrophy are the transplantation studies on mice by Hironaka and Miyata (8). It was found that in the normal host the twitch contractions of both dystrophic and normal muscle were equal to the fast twitch of unoperated normal muscle. In the dys-

trophic host the twitch contractions of normal and dystrophic muscle were equal to the twitch contractions of unoperated dystrophic muscle which were slower than in the normal mouse. Measurements of the maximum isometric twitch and tetanic tensions for this cross transplantation study also implicate the nervous system in muscular dystrophy. A normal host with a dystrophic muscle transplant showed an increase in weight comparable to that of normal muscle in a normal host; in the dystrophic host both muscle types eventually deteriorated.

2. Organ culture of muscle "under the influence" of dystrophic spinal cord motorneurons.

Paul and Powell in organ culture studies with embryonic spinal cords and adult gastrocnemius muscle of mice, strain 129Rej, found that normal muscle regenerated normally in the presence of either spinal cord type (64). Dystrophic muscle, although it showed regeneration potential, was not cured in the presence of normal spinal cord. The characteristics of dystrophic muscles' were lack of cross-striations, a lack of spontaneous contractions with the muscle producing myoblastic outgrowths which fused and aborted. Perhaps a dystrophic neurotrophic factor was absent in the embryonic spinal cord(14-days-old). Dystrophic muscle pathology has been seen in mice embryos of 19 days of age (65). Older spinal cord may be required to demonstrate whether there is neural involvement in muscular dystrophy with these mice. Muscle fibres in culture usually show spontaneous contractions without innervation, yet with innervation these contractions seem to be more coordinated (66). The dystrophic muscle did not express this normal trait. This may be due the fragility of dystrophic muscle or due to a lack of a neurotrophic factor in normal embryonic spinal cord.

In vitro studies with strain C57BL/6J dystrophic mice showed regeneration of both normal and dystrophic muscle in the presence of either embryonic spinal cord; degeneration of the dystrophic muscles took place after 40-60 days in culture (67). The deterioration of

dystrophic muscle in culture suggests there is an abnormality within the muscle not corrected by normal embryonic spinal cord. The tissue culture experiments remove the effects of other than nerve and muscle on muscle regeneration, but the growth conditions may account for the lack of survival of dystrophic muscle.

3. Mouse chimaera studies-the absence of dystrophic muscle histology in the presence of dystrophic myonuclei.

In mice experiments the chimaera is an animal with the genotype of more than one strain of mice. In Peterson's studies the chimaera were formed by the aggregation of two different genotypic mouse embryos (at the 8 cell stage) which, after differentiating into a single blastocyte, were transferred to the uterus of a pseudopregnant female mouse to complete development of the mosaic animal (9). Malic enzymes were used to detect the genotype of the muscle muclei in the anterior tibialis muscles. The presence of a primarily dystrophic genotype in muscle which appeared normal in histology and the presence of a normal genotype in muscle which appeared dystrophic in histology were the experimental findings of Peterson. These studies indicated an extramuscular factor was involved in the dystrophy of mice strain C57BL/6Jdy^{2J}dy^{2J}. Other chimaeras between normal and dystrophic mice (10) have shown no clinical symptoms of dystrophy with myonuclei of primarily dystrophic genotype. That the presence of dystrophic myonuclei does not result in dystrophic muscle pathology indicates an extramyogenic factor responsible for the state of the muscle and perhaps for the origin of muscular dystrophy. This factor may reside in the neurons innervating the muscle (10).

4. Altered enzyme activities in dystrophic muscle fibres.

A characteristic of regenerating and denervated muscle is the spread of acetylcholinesterase activity and acetylcholine(ACh) sensitivity over the entire muscle fibre membrane (42,66). In the dystrophic mouse C57BL/6Jdy^{2J}dy^{2J} both fast and slow muscle fibres were sensitive to ACh outside the motor end plate region; also both muscle fibres were tetrodotoxin resistant(able to generate action potentials in the presence of

an inhibitor of Na^+ permeability)(68). These results were different from the mouse strain 129Rej/dydy which shows no extrajunctional ACh sensitivity or tetrodotoxin resistance (69) indicating that no denervation or regenerative process is occurring.

In the adult dystrophic chicken there is a high extrajunctional AChE activity, the enzyme being similar to the embryonic enzyme which is small in molecular weight and has three isozymes (70). The normal adult chicken shows low activity of the AChE enzyme only at motor end plate regions and the enzyme is a single high molecular weight isozyme. This indicates a neurotrophic or lack of neurotrophic influence on the muscle membrane in muscular dystrophy of the chicken. It has been shown, however, that extrajunctional AChE activity appears only after two weeks of age in the dystrophic chicken(71). The authors feel this AChE data suggests a myogenic rather than a neurogenic origin for the defect in a maturation process of muscle. Several other papers researching the acetylcholinesterase regulation in the dystrophic chicken have indicated that a muscle rather than a neural cause for the abnormality is supported (70-72).

Knowing that the inability to right itself once placed on its back is the most apparent clinical characteristic of dystrophy in the chicken and that this has been known to appear 2-3 weeks after birth(when AChE results were recorded) does not invalidate the idea of a neural root for this abnormal trait. This clinical symptom may be linked with some abnormality at the synapse of the nerve and muscle (73). Albuquerque and Warnick, in fact, suggest a decrease in sodium permeability at the dystrophic presynaptic membrane resulting in altered neurotransmitter release as the cause of this trait in the chicken. The changes in fast twitch muscle fibre electrical properties may then be due to neurotrophic factors deficient in the dystrophic chicken. These 'neurotrophic factors' may result in altered ionic regulation in the muscle membrane. Posterior latissimus dorsi muscles in the dystrophic New Hampshire chicken showed a decreased in fatiguability compared to normal muscle (35). The restoration of the fatigued twitch response by potassium chloride infusion suggested abnormal neuromuscular transmission (35). An alteration in the

type of motor unit(to mostly type C as defined by Edstrom and Kugelberg in 1968) may also be the reason for the fatigued muscle pattern in the dystrophic chicken. The research into these parameters has not been carried out.

The limb bud experiments in the chicken suggest a muscle factor involved in dystrophy, yet no other tissue or humeral factors were tested for. In these experiments, 3 1/2 day old normal and dystrophic transplanted limb buds(in fibre diameter, ACh, LDH, SDH activities and electromyographic patterns) showed that the limb bud rather than the host neural influence was responsible for the characteristics of the muscle (72).

The above findings in the dystrophic chicken seem to strongly support a muscle involvement, even a myogenic origin, of muscular dystrophy. Neural involvement cannot be ruled out as chicken muscular dystrophy responds to treatment with a drug which acts at the nerve muscle synapse (74).

Following specific enzyme markers for oxidative and glycolytic metabolism(succinic dehydrogenase and phosphorylase respectively), it was observed that the fast muscle was more susceptible to muscular dystrophy and showed an inability to mature to its full anaerobic state (75,76). This may be due to a trophic factor(inhibitory or excitatory) involved in maturation which is present(or absent) in the animals with muscular dystrophy. Cosmos and her coworkers suggest however that this is more a property of the muscle fibre membranes themselves than a nervous system disorder (75).

Lactate dehydrogenase(LDH) and glucose-6-phosphate-dehydrogenase (G-6PDH) levels in dystrophic mice were similar to the change in enzyme activity following denervation of normal mice in both gastrocnemius(fast) and soleus(slow) muscles (41). Similar enzyme activities do not result from demyelination. These experimental findings indicate that other nervous system abnormalities are present in murine dystrophy other than a lack of myelin formation. The results are consistent with a neural involvement of muscle fibre characteristics in muscular dystrophy.

5. Altered lipid composition of muscle membranes in dystrophic animals.

Another characteristic of muscle which appears to be partially determined by neural influences is the lipid composition of the muscle fibre membrane. It has been well documented that dystrophic muscle in DMD patients has higher cholesterol and sphingomyelin components and less lecithin and phosphatidyl cholines than normal muscle (77).

The same increase in sphingomyelin components also occurs in mice with dystrophy (78) and the alteration in tissue components was found to be due to the sarcoplasmic reticulum fraction (79).

An altered membrane fluidity(which is affected by cholesterol content) occurs in the dystrophic chicken (47). Sha'afi found an increase in membrane microviscosity which could be determined on the basis of increased cholesterol/phospholipid ratios in the dystrophic chicken muscle membranes. When neural tubes from dystrophic chicken embryos were transplanted into normal hosts, an increase in the cholesterol content of pectoral muscles was found to occur (80). When neural tubes of normal embryos were transplanted into dystrophic embryos a normal cholesterol value was obtained in the pectoral muscles (80). Hence, the conclusion that the high content of cholesterol in pectoral muscle of dystrophic chicken was regulated by the genotype of the neural tube. Here again we find that there is an altered nervous system influence in muscular dystrophy which is acting on the muscle.

6. Axoplasmic flow abnormalities in animals with muscular dystrophy.

Axoplasmic flow abnormalities may be related to the dystrophic condition of muscle by causing an altered amount or type of neurotrophic factor(s) in the nervous tissue innervating muscle. Because of the histological studies indicating peripheral nerve involvement in murine dystrophy axoplasmic flow studies have been undertaken (11,12).

In the murine dystrophy strain 129Rej/dydy a decrease in the amount of slow moving material(1 to 2mm/day) and a marked increase in the amount of fast moving materials(up to 128mm/day) occurred in the sciatic

nerve (11). Choline acetyltransferase was also shown to be transported in lower amounts and at slower velocities in dystrophic mice(129Rej/dydy), and to have increased activity in the sciatic nerve (12). The results in young animals(6 to 7 weeks) indicated that this abnormality is not due to late disease processes in muscular dystrophy. Loss of muscle contact usually shows a decrease in choline acetyltransferase(CAT) activity and results in unaltered axoplasmic flow, so the results here indicate an altered neuronal influence on muscle rather than the reverse (12). It is not known whether this abnormality is independent of the muscular dystrophy in mice (81).

Komiya and Austin showed that leucine metabolites in the dystrophic mice were reduced in amount at high velocitites(200mm/day) and increased in amount at fast flow velocities(500mm/day) (82). Phospholipid and cholesterol flows were also altered in this study. Phospholipid showed an increased quantity moving at slow flow rates(120mm/day), cholesterol showed an increased amount flowing at both slow and high velocities(120 and 1800 mm/day respectively) (13).

The report of reduced axonal transport of dopamine-β-hydroxylase in murine dystrophy indicate that abnormalities lie also in adrenergic nerve cells (83). From colchicine binding studies it was found that the reduced transport of dopamine-β-hydroxylase was not associated with any apparent changes in the tubulin content of the sciatic nerve (83).

A dual labelling study of leucine labelled proteins in mice showed that protein components in the sciatic nerve were increased in amount and had increased flow rates in dystrophic mice compared to their normal littermates(strain 129Rej); lipid components also showed increases in rates of flow in the dystrophic mice (15). Spinal cord proteins of lower molecular weight showed a greater rate of synthesis in dystrophic animals compared to normal (15). These axoplasmic flow studies indicate neural abnormalities in the murine dystrophy of strain 129Rej.

Abnormalities in dystrophic hamster axoplasmic flow were observed by Boegman and Marien (84). They detected a lower rate of accumulation of choline acetyltransferase in dystrophic hamster after sciatic nerve ligation. No inhibitor of CAT was present so it was concluded that either a decrease in the fast axonal transport occurred or a decreased

quantity of CAT was actually being transferred to the synaptic terminal of the nerve.

An altered balance of protein synthesis and transport in the nerve cells may result in changes in muscle metabolism and may be involved in muscular dystrophy.

7. Histological abnormalities in the nervous system of animals with muscular dystrophy.

Histological studies have shown several abnormalities within the nervous tissue of animals with murine dystrophy. Large areas of non-myelinated nerve fibres in both the ventral and dorsal spinal roots and in the proximal portion of the sciatic nerve were found in dystrophic mice, strain 129Rej/dydy, from 3 1/2 weeks to 7 months of age (16). Schwann cell numbers were also reduced. The spinal cords, sciatic nerves, and the dorsal and ventral spinal roots of these dystrophic mice showed a decreased amount of protein some of which may be myelin components (85).

Cranial nerves have also been studied in this mouse strain. Non-myelinated fibres and areas of thin myelination existed in the cranial nerves(III-XII) and also in the lumbar spinal roots (29). Since extra-dural cranial nerves express the same visible abnormalities regarding myelination as the spinal roots and are not in contact with cerebrospinal fluid it is unlikely that the origin of the abnormalities lies in the cerebrospinal fluid (29).

In dystrophic mice, strain C57BL/6J, non-myelinated nerves were also observed within the proximal portion of the sciatic nerve (14). In these mice a lack of Schwann cells(their cytoplasm and basement membranes) was found along with thin myelin sheaths. A lack of myelin sheaths also occurred in all the spinal roots, especially the lumbar and sacral roots in both murine dystrophies (31). Schwann cells, myelin sheaths, and the nodes of Ranvier are abnormal in structure in the dystrophic mice strain C57BL/6Jdy^{2J}dy^{2J} (86). Also the mean number of neurotubules in unmyelinated fibres was less in the dystrophic nerve fibre as compared to the normal nerve fibre. As the neurotubules are thought to be involved in axoplasmic flow (87), their decrease may lead to changes in axoplasmic flow rates and

and thus in altered axonal influence on the muscle. These changes may result in the characteristic fibre necrosis in murine dystrophy. The reduction in the number of Schwann cells(SC) in dystrophic mice, strain C57BL/6Jdy^{2J}dy^{2J},found by Bray and coworkers was shown to be a very early neonatal impairment of SC proliferation (88). Basement membrane abnormalities of the SC also occur in the peripheral nervous system in 1-5 month old dystrophic mice of the same strain (89).

In addition to other components of the neural system anterior horn motoneurons have been studied. However the experimental evidence for or against altered horn cell levels in dystrophic animals has proved inconclusive (90-92).

There is a weight of evidence for neural abnormalities in dystrophic mice but it should be mentioned that none of these nervous system abnormalities are found in the dystrophic hamsters BIO 14.6 or in patients with DMD (16).

8. Proteins of muscle under neural control-in denervated and dystrophic muscle.

Abnormalities in protein synthesis related to denervated muscle might give some indication of where one should look for abnormalities in dystrophic tissue.

Ionasescu and coworkers have shown neurogenic control of in vitro ribosomal protein synthesis in muscle of sprague-Dawley rats (93). A marked decrease in noncollagen protein synthesis occurred on denervation of leg muscles for 3 and 14 days. This is a reflection of both a decreased synthesis and increased proteolysis occurring in the denervated muscle (94). SDS-PAGE showed a decrease in the concentration of the heavy chains of myosin in denervated animals in this study. Light chains of myosin, tropomyosin and actin did not change in concentration. These results suggested that the decrease in noncollagen protein in the denervated animals may be a reflection of the decrease in myosin protein(heavy chains) and that myosin protein synthesis is under neural control. The denervated muscle in motor neuron disease has a marked reduction of label incorporation in myofibrillar proteins but normal incorporation into the soluble protein

fraction (95). Altering the innervation of a fast and slow type muscle fibre has shown the light chains of myosin are altered according to the nerve innervating them (38,40). The lack of neural trophic influences also affects other proteins synthesized by the muscle. An example of this is the acetylcholine receptor which is synthesized by the muscle after denervation (96).

Both mice, C57BL/6Jdy^{2j}dy^{2j}, and chicken with dystrophy show extrajunctional AChE activity (68,70,71). That these characteristics are not seen in the murine dystrophy of strain 129Rej/dydy (69) indicate the heterogeneity of these diseases or the fact they may be very different gene mutations.

The myofibrillar fraction of muscle showed a decreased tritiated leucine incorporation in both Duchenne and Becker(benign) muscular dystrophy patients (952). A decrease in noncollagen protein synthesis was found to occur in muscle cultured from DMD patients (94). while there was a normal amount of heavy chain of myosin synthesized. These latter results, a decrease in noncollagen protein(suspected neural involvement) and a normal myosin synthesis(not expected in cases of neural involvement) do not strongly support a neural influence in the muscular dystrophy. However these abnormalities can be corrected by the addition of a soluble enzyme fraction of muscle(93) and calcium chloride and ionophore (94) the mechanism of which may be under nervous control (973).

In dystrophic hamsters a pH 5 enzymic soluble fraction was shown to be responsible for the decreased polyribosomal in vitro protein synthesis of heart and skeletal muscle polyribosomes (554). This was a general defect present also in the brain, liver, and uterine smooth muscle of the dystrophic hamsters. Myofibrillar proteins of heart and skeletal muscle in both cell-free reconstituted systems and in tissue culture were shown to be decreased in quantity (985). Quantitative decreases in all the myofibrillar components, myosin(heavy and light chains) and actin, were noted. Since a decreased myosin synthesis and a decrease in myofibrillar protein synthesis occurs in denervated rat and motor neuron diseased muscles respectively these results in hamster may mean that there is a neural involvement in this muscular dystrophy. This is the only defect

common to a number of tissues that has been observed in muscular dystrophy.

A soluble enzyme fraction from skeletal muscle of dystrophic mice was also responsible for an inhibition of protein synthesis by normal mouse ribosomes in poly-U directed incorporation of phenylalanyl tRNA labelled with ^{14}C (99). It is not known whether there is a neural induction of this soluble factor's synthesis or activity.

An overall increased rate of amino acid incorporation by a reduced number of large polysomes in dystrophic mouse, strain 129Rej/dydy, was observed by Nihei (100). Along with this a decrease in the amino acid incorporation into myofibrillar proteins occurred. Nihei suggested this was due to a reduced myosin synthesis in the murine dystrophy. Tritiated leucine incorporation into normal and dystrophic mice indicated a decrease in the amount of label incorporated into the myofibrillar fraction (101). An increased amount of label was incorporated into the soluble fraction of the muscle of these same mice. Because of this decrease in noncollagen proteins, especially myofibrillar proteins, one might suspect the involvement of neurotrophic factors in this murine dystrophy.

9. Parabiosis studies in muscular dystrophy-a controversy over a neural involvement.

The parabiosis experiments(two animals joined together surgically) with cross-reinnervation of muscle have given the strongest evidence against the implication of the nervous system in muscular dystrophy. Parabiotic experiments between normal and dystrophic mice, strain 129Rej, showed no alteration in the muscle fibre number or fibre type in the cross-reinnervation of tibialis anterior and extensor digitorum longus muscles after 180 days (102). Cross-reinnervation here is the innervation of the tibialis anterior muscle of the dystrophic mouse by the nerve innervating the extensor digitorum longus muscle of the normal mouse. No evidence for neurotrophic abnormalities occurred in parabiosis experiments with cross-reinnervation in the dystrophic hamster(BIO 14.6) (103).

In younger animals 21-25 days old at the time of parabiosis innervation

and after 28-33 days waiting period for reinnervation to occur, no abnormalities developed in the isometric twitch contractions of the triceps surae or tibialis anticus muscles or in the twitch and tetanic tensions which developed in these muscles (104). Here unoperated normal, normal to normal crosses and dystrophic innervation for normal muscle all appeared normal while unoperated dystrophic, dystrophic to dystrophic crosses and normal innervation for dystrophic muscle all appeared dystrophic in twitch and tetanic contractions. It appears that muscular dystrophy in the mouse is caused by other than neurotrophic factors. However these animals are quite old at the time of parabiosis and may not feel the influence of an embryonic factor or early neonatal factor from the nerves innervating them and which are involved in the dystrophic disease process.

More recent evidence for peripheral motor nerves being normal in murine dystrophy is that of the double cross-reinnervation parabiosis experiments with dystrophic and normal mice (105). The disease process in dystrophic solei muscle is not arrested by normal nerve innervation and the disease does not appear in the normal solei which receives dystrophic innervation (105). In these studies cytochemical, electrophysiological and structural analysis show that donor nerves(whether normal or dystrophic) are able to convert the soleus muscle they innervate into fast-twitch muscle fibres. However they do not affect the normal or dystrophic properties of the muscle.

10. Electrophysiological studies on dystrophic muscle.

The last group of experimental evidence to be reviewed is actually the first which supported a neurogenic theory of muscular dystrophy. These are the electrophysiological muscle experiments in both animal models(murine dystrophies) and human forms of muscular dystrophy.

In 1967, it was found that 27% defective motor innervation of the EDL muscle of the dystrophic mouse, strain 129Rej, occurred (3). An even greater denervation in the tibialis anterior muscle of this dystrophic mouse strain was found by Harris and Wilson (106). They found 70% defective motor innervation of this muscle. The denervation suggested by these authors is a gradual denervation process rather than an abrupt

severing of the nerve(and its trophic influence) from the muscle. A similar decrease in motor innervation occurred in the soleus muscle of dystrophic mice (107).

The amplitudes of miniature end plate potentials(mepps) were found to be lower in dystrophic mice, strain 129Rej, compared to normal mice. It was suggested that a reduction in the amount of acetylcholine released from the presynaptic nerve terminal in murine dystrophy occurred (108). It has been observed that the enzyme choline acetyltransferase(CAT) is transported in reduced amounts and at a reduced velocity in the sciatic nerves of murine dystrophy strain 129Rej (12). This perhaps is an indication that the mepps seen by McComas and Mossawy are in fact due to reduced amounts of ACh being released at the nerve terminal. This is due to either an abnormality in its synthesis or transport along the axon of the nerve(the reduction of flow of CAT may be part of the general phenomenon of decreased amounts of protein being transported along the nerve axon at slow flow rates(13,82)). A decrease in the frequency of release of acetylcholine at the presynaptic terminal has also been found to occur and could be responsible for the decreased mepps (109).

Harris and Montgomery found no evidence of functional denervation in the distal hind limb muscles in strain C57BL/6Jdy^{2J}dy^{2J} (68).

In 1970 it was shown that in DMD patients the extensor digitorum brevis muscle had a greatly decreased number of motor units, normal muscle 199(s.e.$\pm$ 10.2) compared to dystrophic muscle 52(s.e.$\pm$8.7) (4). The amplitude of the evoked muscle potential in the dystrophic condition indicated that the denervation resulted in the complete death of the motor unit rather than being the result of the death of random muscle fibres. This indicated that the problem is neuronal in origin rather than myogenic(4). McComas and coworkers have since found defective motor innervation in the EDB muscle of DMD, myotonic dystrophy (5,110) and in the thenar, hypothenar and soleus muscles of these same muscular dystrophies (111). These muscles unlike the extensor digitorum brevis muscle do not show selective loss of motor units due to trauma (69). The soleus muscle shows the characteristic pseudohypertrophy and shortening contractures in the early and late stages of DMD respectively. While both the soleus and

EDB muscles showed loss of functioning motor units at birth, only in the soleus muscle is there a greater loss in later years (44). The amplitude of the maximum evoked response in the soleus shows a dramatic decline to a third of its initial value(40% of normal), the EDB muscle is reduced in its amplitude only slightly. These results were supportive of a neural involvement in muscular dystrophy.

C. Dual Labelling: a Useful Technique to Investigate Inborn Errors of Metabolism

Dual labelling studies have been used successfully in many forms. The dynamics of human erythrocyte populations have been followed using isotopes of iron, ^{59}Fe(a low erergy radiation emitter) and ^{55}Fe(a low energy X-ray emitter) which can be distinguished on the basis of their half-lives(112). The half life of ^{59}Fe is 47 days while that of ^{55}Fe is five years. These isotopes were measured using a geiger-meuller counter. A selective synthesis of two liver nuclear acidic proteins after glucagon administration in vivo were detected using isotopes of leucine (113). Determination of the radioactivity in these experiments was by scintillation spectrometry after combustion and oxidation of the samples; ^{3}H was collected as water and ^{14}C collected as carbon dioxide. Since tissue solubilizers have been developed for biological samples in polyacrylamide gels the combustion and oxidation techniques can be omitted and ^{3}H and ^{14}C distinguished by using discriminator window settings on a liquid scintillation counter (114).

Other experiments have led to the detection of altered chloroplast membrane polypeptides in mutant(F-54) strains of Chlamydomonas reinhardi (115). A dual labelling experiment in quaking mice showed a decrease in a major glycoprotein moiety of myelin along with a shift in its apparent molecular weight (116). In a dual labelling experiment the rate or extent and the pattern of proteins synthesized can be studied by following the simultaneous incorporation of two different radioactive isotopes into proteins

We have applied the technique of dual labelling to the problem of neural involvement in muscular dystrophy. Age-matched pairs of normal

and dystrophic animals were injected with an isotope of leucine. After a set period of incubation the tissues from the two animal strains are combined and homogenized. Following subcellular fractionation the cell proteins and polypeptides are separated by electrophoresis. By using analytical separation procedures like SDS-PAGE and IEF gels quantitative differences in the proteins being synthesized can be monitored. It was hoped that by using a general protein label like leucine which has a high incorporation into proteins that most of the nervous system proteins would be labelled. The nervous system was selected because of the body of evidence in support of neural involvement in muscular dystrophy, and because it seems to be less severely affected in comparison to the skeletal muscle in this disease. A major advantage is that the in vivo metabolism can be studied rather than in vitro processes. This type of study may allow the detection of an abnormality which actually occurs in the animal and is not due to the fragility of the protein synthetic machinery upon isolation.

MATERIALS AND METHODS

A. Materials

1. Animal strains

Two species of genetically dystrophic animals with normal controls were studied.

a) The Syrian hamster, strain BIO 14.6, with muscular dystrophy and a normal Syrian hamster, strain RB, were used between the ages of 1 and 7 days old. These animals were obtained from the Trenton Experimental Laboratory Animal Company, Bar Harbour, Maine and were bred locally by random mating. Both hamsters breed true, that is, both BIO 14.6 and RB hamsters are homozygous for the dystrophic and wild type alleles respectively. It was hoped that by using young animals fewer secondary effects of the disease process would be present. The expression of the primary biochemical abnormality may then be more visible.

b) The dystrophic mice were those of the more severe murine dystrophy, strain 129Rej/dydy with normal littermate 129Rej/+? as controls. Both strains were received from the Trenton Experimental Laboratory Animal Company, Bar Harbour, Maine at 4 weeks of age. These animals were studied between 4-12 weeks of age. This particular dystrophic strain of mouse cannot normally breed and thus two normal mice carrying the recessive allele for dystrophy must be mated to produce dystrophic progeny. These mice exhibit normal Mendelian inheritance patterns. The dystrophic characteristics both biochemical and clinical do not appear until about 2-3 weeks of age. The shipping time therefore regulated the ages of the animals studied. There are no distinguishing clinical characteristics between the heterozygous and the homozygous normal animals (16).

2. Chemicals

The following chemicals were used in this study. All reagents were reagent grade unless otherwise specified.

a) from Allied Chemical of Chemical Limited(Baker and Adamson quality):
   Glacial acetic acid

b) from Amersham/Searle Corporation:
   L-(4,5^{3}H)leucine (53Ci/mmol)-(57Ci/mmol)

L-($U^{14}C$)leucine (324mCi/mmol),(330mCi/mmol), (47Ci/mmol)
NCS™solubilizer

c) from Analar-British Drug House Chemicals:
Boric acid
Sodium chloride
Sodium hydroxide
Sodium tartrate(CH(OH)-COONa):$2H_2O$
Sucrose

d) from Biorad Laboratories:
Acrylamide(electrophoresis purity reagent)
N,N'-methylenebisacrylamide(BIS)
Coomassie Brilliant Blue R-250

e) from C.I.L. Reagents Limited:
Hydrochloric acid min 37%

f) from Eastman Kodak
Photo-Flo 200 solution
N,N,N,N-tetramethylethylenediamine(TEMED)

g) from Fisher Scientific:
Ammonium persulfate(Certified A.C.S.)
Sodium phosphate dibasic
Sodium phosphate monobasic
Sodium hydroxide
Toluene(Scintanalyzed)
Potassium chloride(Spec. for Calomel cell)

h) from J.T. Baker Company:
Sodium phosphate monobasic

i) from L.K.B. Aminkenu
Ampholines pH 3.5-10.0 dry content 40%

j) from Mallinckrodt Chemical Works:
Sodium phosphate monobasic

k) from Merck Laboratories:
Sucrose(aristar)

l) from New England Nuclear Pilot Chemical Division:
Omnifluor(98%PPO & 2%MSB)

m) from Sigma Chemical Company:
-chymotrypsinogen A(bovine pancreas crystallized salt)free type II

β-galactosidase

β-mercaptoethanol(β-SH)

Cytochrome C(horse heart)

Ethylenediamine tetraacetic acid (EDTA) -free acid

Ethyleneglycol-bis(-aminoethyl ether)N,N'-tetraacetic acid (EGTA)

Phenylmethylsulfonylfluoride(PMSF)

Glycine(crystalline anhydrous)

Lubrol WX

Urea

Tris (hydroxymethyl)aminomethane, Trizma base(TRIS)

Pepsin(hog stomach mucosa)

n) from Schwarz-Mann Research Laboratories:

Albumin

Ovalbumin

N-DNP-L-Leucine(N-dinitrophenyl-L-leucine)

o) from Pharmacia-Uppsala

Sephadex G-25(coarse for gel filtration, dry screen analysis 0.0%,water regain 2.4g H_2O/g dry gel)

p) from Dr. J.H. Wang, Dept. of Biochemistry, University of Manitoba

Phosphorylase A

B. Methods

1. Labelling and Dissection of Tissues:

Age-matched pairs of normal and dystrophic or normal and normal animals were intraperitoneally injected with equimolar amounts of radioisotope , L-(4,5^{3}H)leucine and L-(U^{14}C)leucine respectively, in 100μl 0.9% NaCl. The direction of labelling was reversed(L-(U^{14}C) leucine into normal animals) in some experiments and a control test involving the injection of both labels into a single animal was also carried out. After a set period of incubation(1-3 hours) the animals were killed and the tissues were excised. During the period of incubation RB and BIO 14.6 hamsters(1-6-days-old) were incubated at 32°C. The labelling time for the Bar Harbour 129Rej/dydy and 129Rej/+? mice was three hours at room temperature.

In the hamster dual labelling experiments blood was removed by heart puncture with a heparinized syringe while the animals were anesthetized with ether. The animals were then killed by decapitation. The tissues were removed, rinsed, blotted dry and weighed before homogenization. Ice cold medium A(0.25M sucrose, 10.0mM $MgCl_2$, 80.0mM KCl, and 50.0mM Tris Cl pH 7.8) was the rinsing and homogenizing solution.

Spinal roots and regions both proximal and distal to the sciatic notch of the sciatic nerve were dissected by similar methods to Bradley and Jenkison (16). The spinal cord was removed by dissection after spinal root dissection or by a blow out method of deSousa and Horrocks (117).

2. Homogenization:

Two procedures were carried out in the combining of tissues for homogenization; either equal weights of normal and dystrophic tissues were combined or total tissues were combined . The latter procedure was preferred to eliminate the comparison of dissimilar areas within a single tissue studied.

Homogenization was carried out at 0-4°C by Pierce's modified Potter and Elvehjen method, using a smooth-walled glass tube with a teflon pestle, for the brain(118). The spinal cord, sciatic nerves and spinal roots were homogenized (glass pestle on glass tube) with #22,#21, and #20 Dounce

homogenizers respectively, to get uniform shearing in a small shearing volume. The tissues were combined with 5-10(v/w) of medium A for this step.

3. Differential Centrifugation:

Differential centrifugation was carried out on brain homogenates at 0-4°C in a Sorval centrifuge(RC2B) with a ss34 fixed angle rotor and in a Beckman L3-40 ultracentrifuge with a type 50 fixed angle rotor(Figure 2). With this method the distribution of low and high rate sedimenting particles at the onset of centrifugation may result in contamination of the pellet fractions with particles of lower sedimentation rates simply because they were spatially nearer the bottom of the centrifuge tube(some vesicles with a high sedimentation rate may be left suspended if the centrifugation time is not long enough) (119).

In initial studies and dual labelling experiments the homogenized sample was centrifuged at 59,364 x g x 120 minutes in a Beckman L3-40 ultracentrifuge with a type 40 fixed angle rotor. The supernatants were removed and concentrated by freeze-drying.

4. Removal of Free Leucine

a) G-25 Sephadex chromatography: From the soluble ultrasupernatant fractions the free label was removed by elution on G-25 sephadex columns.

2.5gm of Sephadex G-25 beads were swollen in 70ml of deionized double distilled water(ddw). The solution was stirred and the gel was allowed to settle and the fines were decanted twice in the preparation procedure. Approximately 5ml bed volumes were packed at 0-4°C and equilibrated with 0.01M sodium phosphate buffer pH 7.2. Separation of the protein from free amino acid was achieved using this buffer(Figure 3). Buffers of lower ionic strength, 0.001M sodium phosphate pH 7.2 and distilled water, had free label eluting between two peaks of protein (120).

The protein was monitored by the method of Lowry and coworkers(121).

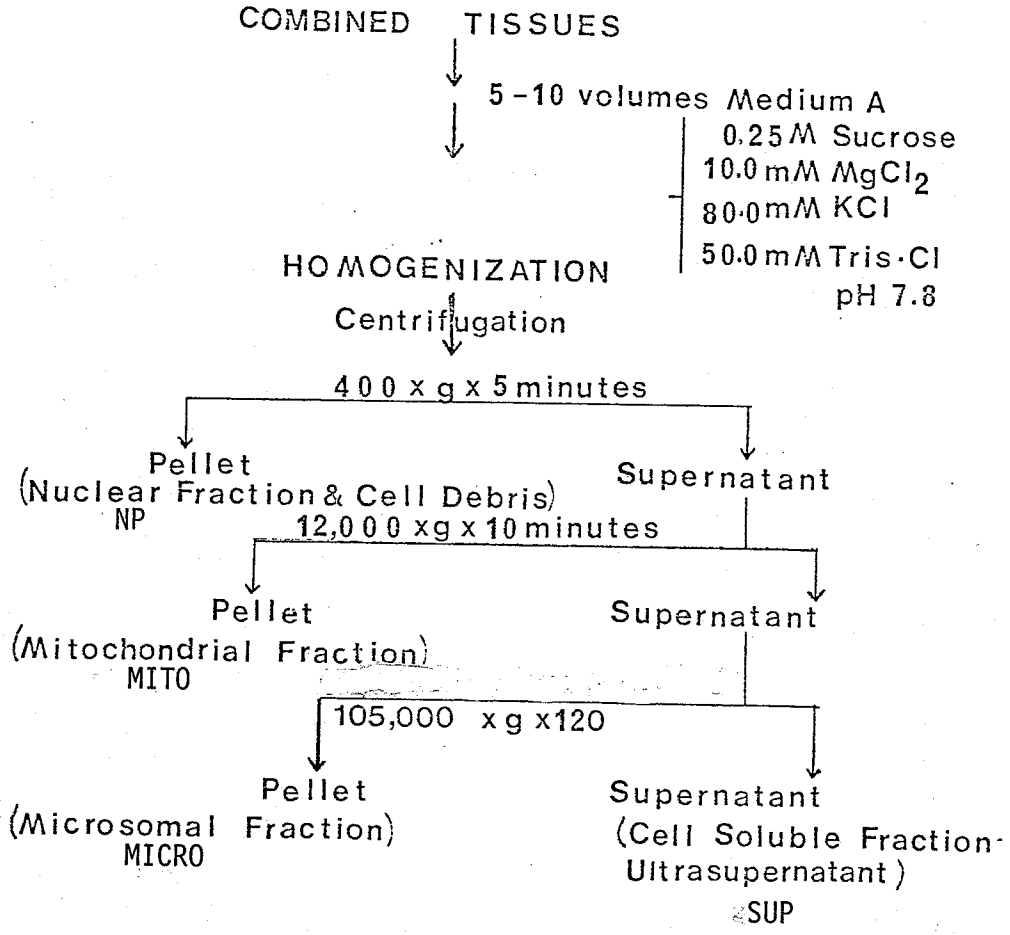


FIGURE 2. Differential centrifugation procedure for subcellular fractionation.

Figure 3. G-25 Sephadex Column Elution Profile

Elution of free radioisotope(L-(4,5^{3}H)leucine) from liver soluble(105,000 x g x 120 minutes) fraction on a G-25 sephadex column at 0-4°C. Protein(●——●) was eluted before the "free" or unincorporated leucine(o---o) with a 0.01M sodium phosphate buffer pH 7.2. Protein measured by method of Lowry in a single beam Photometer Eppendorf 1100. Radioisotope was quantitated by counting on a Beckman Liquid Scintillation Counter LS-350.

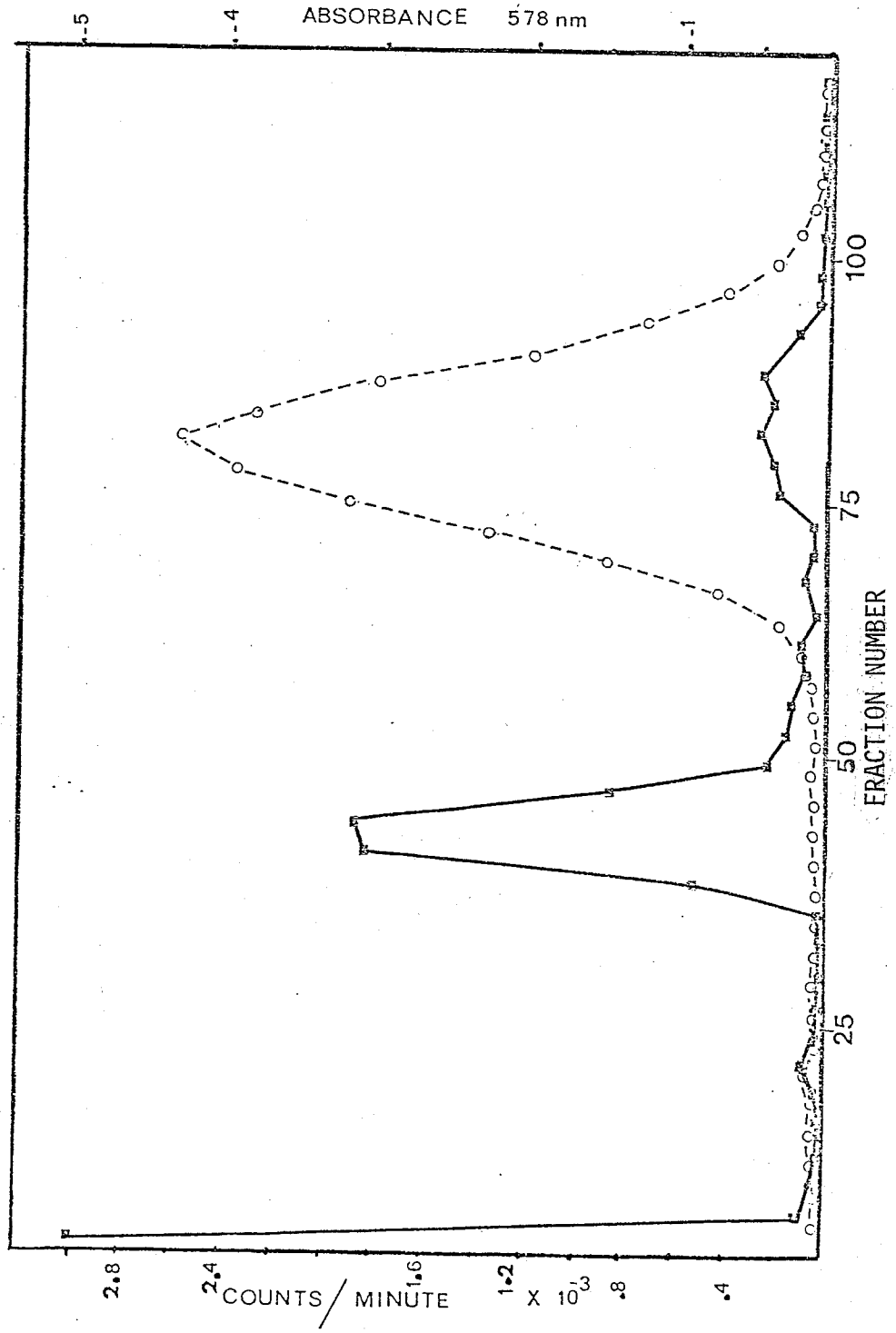

ABSORBANCE 578 nm
.5
.4
.1
100
75
50
25
FRACTION NUMBER
2.8
2.4
1.6
1.2
.8
.4
COUNTS / MINUTE X 10^{-3}

FIGURE 2

Isotopic leucine (L-(4,5^{3}H)leucine) was followed measuring the (radioactive counts-background counts) eluted in each fraction on a Beckman Liquid Scintillation Counter LS350. The columns were initially calibrated with a solution of free labelled ^{3}H-leucine and unlabelled skeletal muscle or liver supernatant protein fractions. After five elutions the columns were checked for close-packing(of the sephadex beads), by the separation of cytochrome-C(red) and L-DNP-leucine(yellow) fractions, before they were further used for labelled protein fractions. This technique proved very useful for the separation of labelled protein components and free label in cell soluble fractions, however, a second method was used to remove free label from particulate fractions.

b) Dialysis with a Spectrapor 3 Membrane: This membrane, with a molecular weight cut off of 3500 daltons, was used in extensive dialysis of SDS and lubrol solubilized cell soluble* and particulate fractions (nuclear-NP, mitochondrial-MITO, and microsomal-MICRO pellets). The dialysate volume was in all cases at least 2 x 10^6 times the volume of the sample with 2 x 2000 ml volume changes(each for 12 hours) of constantly stirred buffer.

The solutions and conditions for dialysis were as follows:

i) for SDS gels: 0.125M Tris Cl pH 6.8 with 0.1% SDS and 0.05% β-mercaptoethanol, dialysis was carried out at room temperature.

ii) for IEF gels with soluble fractions: 0.01M Tris Cl pH 7.4, dialysis carried out at 0-4°C.

iii) for IEF gels with particulate fractions: 0.01M Tris Cl pH 7.4 with 0.1% lubrol-WX, dialysis carried out at 0-4°C.

5. Acetone Extraction Procedure:

The acetone extraction procedure of Ramirez and coworkers was followed (122). Brain nuclear pellet fractions(Figure 2) from a 3-day-old(single RB hamster) and from age-matched 6-day-old(RB and BIO 14.6 hamsters) were disrupted in a 1/1(v/v) 10% SDS solution with 10% Na_2CO_3 at 100°C for 1 minute. The solution was made 20% in β-mercaptoethanol(v/v) and the sample was heated at 100°C for 2 minutes. The sample was ice-cooled after each of the heat treatments. Samples were then placed in

* Cell soluble(SUP) fractions were not lubrol solubilized before isoelectricfocusing.

polyallomer tubes and lyophilized overnight. After this they were resuspended with 0.5ml of water(ddw) in the same tube: 0.225ml of sample was taken for the non-acetone procedure and 0.250ml of sample was kept in the tube for the acetone extraction.

1.8ml of ice-cold acetone was added to the o.250ml sample and mixed thoroughly. The protein precipitate was collected by centrifugation at 12,000 x g x 10 minutes in a RC2B Sorval centrifuge. The precipitate was washed once with 2.0ml of cold acetone and centrifuged again to collect the pellet. The precipitate was finally suspended in 2.0ml of acetone and incubated for 1 hour at 30°C, followed by ice cooling and sedimentation at 12,000 x g x 10 minutes. The pellet was washed once with 2.0ml of cold acetone and a final dry pellet was suspended in 0.5ml 10% (w/v) SDS, 0.05ml 10% (w/v) Na_2CO_3, homogenized, incubated at 45°C x 15 minutes in a tightly closed tube and then heated to 100°C x 3 minutes with 10% (v/v) β-mercaptoethanol. The sample was made to a final volume of 1.0ml with 0.125M Tris Cl pH 6.8 with 0.1% (w/v) SDS in preparation for electrophoresis.

6. Analytical Techniques

The techniques used to resolve polypeptides on the basis of their molecular weight and proteins on the basis of their isoelectric points were the following:

a) Type I-SDS gels
b) Type II-SDS disc gels
c) Type III-SDS,EDTA gels
d) Type IV-IEF gels

a) Type I-SDS gels

The procedure of Weber and Osborn was initially used for SDS gels (123). Separating gels contained 10% (w/v) acrylamide(PA), and 1.08% (w/v) bis-acrylamide(BIS) with 0.1% (w/v) SDS and were polymerized by the addition of 0.05% (v/v) N,N,N'N'-tetramethylethylenediamine(TEMED) and 0.05% (w/v) ammonium persulfate. The gels were cast to a height of 10 cm in 0.6 x 12 cm glass tubes. Sample loads on these gels were usually 100 gm of protein. The electrophoresis buffer was 0.1M sodium phosphate pH 7.2 with 0.1% (w/v) SDS.

Sample preparation after removal of free leucine was as follows: solubilization at 37°C for 2 hours in a 1/1 (v/v) sample to buffer ratio. The buffer contained 0.375M Tris Cl, 0.1% (w/v) SDS and 5% (v/v) β-mercaptoethanol. Samples were overlayed directly on the separating gel and contained 3-5% (v/v) 0.05% (w/v) Bromophenol blue as tracking dye(T.D.) and 10-20% (v/v) 40% (w/v) aristar sucrose.

b) Type II-SDS disc gels

SDS electrophoresis was performed using the discontinuous buffer system of Maizel (124). In the hamster experiments separating gels containing 6M urea, 0.1% (w/v) SDS, 10% (w/v) acrylamide, 0.20% (w/v) BIS were polymerized by the addition of 0.06% (v/v) TEMED and 0.09% (w/v) ammonium persulfate. The stacking gels contained 2.5% (w/v) acrylamide, 0.27% (w/v) BIS and 0.1% (w/v) SDS and were polymerized by the addition of 0.05% (v/v) TEMED and 0.075% (w/v) ammonium persulfate. The electrophoresis buffer contained 50mM Tris Cl pH 8.45, 383mM glycine and 0.1% (w/v) SDS.

In the mouse studies separating gels with 6M urea, 13% or 10% (w/v) acrylamide, and 0.26% or 0.20% (w/v) BIS respectively, were polymerized with 0.06% (v/v) TEMED and 0.09% (w/v) ammonium persulfate. All separating gels contained 0.1% (w/v) SDS. The stacking gels contained 3.0% (w/v) acrylamide, 0.38% (w/v) BIS and 0.1% (w/v) SDS and were polymerized with 0.06% (v/v) TEMED and 0.09% (w/v) ammonium persulfate. The electrophoresis buffer contained 50mM Tris Cl pH 8.3, 383mM glycine and 0.1% (w/v) SDS.

Separating gels were cast to a height of 12 cm or 22 cm in glass tubes, 0.6 x 12 cm or 0.6 x 25.5 cm respectively. All gels were electrophoresed at constant voltage overnight with water cooling. Voltages for electrophoresis were as follows:

10-12 cm gels	20 volts	stacking gel
	35 volts	separating gel
20-22 cm gels	50 volts	stacking gel
	100 volts	separating gel.

Samples were solubilized in 1/1(v/v) solution of 10% (w/v) SDS made 10% (v/v) with 10% (w/v) Na_2CO_3 at 100°C for 1 minute. The sample was then made10% (v/v) with β-mercaptoethanol and heated at 100°C for 3 minutes. Ice-cooling of the samples was carried out after the 1 minute heating period before the addition of β-mercaptoethanol.

Samples after disruption and dialysis and concentration when necessary(with an Amicon microultrafiltration system, model 8 MC with a PM 10 membrane) were made 3-5% (v/v) Bromophenol blue and 10-20% (v/v) of 40% (w/v) sucrose. and were overlayed on the stacking gel surface. Gel buffer was gently overlayed on top of the sample before the electrophoresis chamber was filled.

c) Type III-SDS, EDTA gels

This gel system was a modified buffer system of Fairbanks (125). The separating gel was 4.5% (w/v) acrylamide, 0.168% (w/v) BIS with 1.0% (w/v) SDS and 0.02M EDTA. The gels were polymerized by the addition of 0.025% (v/v) TEMED and 0.15% (w/v) ammonium persulfate.

Samples were prepared as in disc gel system and made 3-5% (v/v) with Pyronin Y and 10-20% (w/v) with sucrose.

d) Type IV-IEF gels

Gel isoelectricfocusing was carried out according to the method of Wrigley (126) using LKB ampholytes pH 3.5-10.0 in polyacrylamide gels which were 5.1% (w/v) in acrylamide and 0.17% (w/v) in BIS-acrylamide. The gels also contained 5% (w/v) sucrose and 1% (v/v) ampholytes. Polymerization of the gel was catalyzed by the addition of 0.0465% (w/v) ammonium persulfate in the gel mixture. The protein sample was polymerized within the gel. Ethanolamine(0.5%) and phosphoric acid(0.5%) were used as the cathodic and anodic solutions respectively. The electrofocusing was carried out in 0.6 x 16 cm glass tubes in a Chrismac Electrophoresis apparatus with water cooling at 4°C, directly about the gel tubes, overnight. The Lauda Thermostat k_x/R#2143 apparatus was used for water cooling. A modified procedure of Klose was followed for power regulation using a pulsed constant power regimen(127):sed:

50 volts-- for 1/2 hour, 50 pulse per second, capacitance 0.5 µf

100 volts-- for 1 hour, 50 pulse per second, capacitance 0.5uf
150 volts-- overnight, 50 pulse per second, capacitance 0.5 uf.

The electrofocusing was monitored by using an aliquot of RBC(red blood cell) hemolysate, containing hemoglobin, in the gel sample. This allowed for visual judgement of the band formation within the gel during the experiment.

pH gradients were monitored by slicing the gel into 2-mm minced fractions and measuring the pH at 0-4°C in 0.5ml(ddw) extracts with a microprobe combination electrode. Gel was sliced and minced by the Gilson Aliquogel Fractionator.

Sample is solubilized in a 1/1 (v/v) of 10% (w/v) lubrol-WX(with 5mM EDTA, 10mM borate, 1mM phenylmethylsulfonylfluoride(PMSF)) and diluted with an equal volume of stock medium(0.01M Tris Cl pH 7.4 with 0.1%(w/v) lubrol-WX). The samples with ice cooling were then sonicated on a Bisonik IV Bronwill sonicator until the solution was clear (for spinal cord homogenate preparation 30 seconds x 30 cycles/second). Samples were then spun at 30,99 x g x 60 minutes on a RC2B Sorval centrifuge(spinal cord samples were spun at 10,000 x g x 13 minutes on an Eppendorf centrifuge). The soluble fraction was then used for isoelectricfocusing. Brain supernatant fractions were used directly after dialysis(and when necessary after concentration of the sample).

7. Gel Staining and Destaining Procedures

SDS polyacrylamide gels were stained with 0.25%(w/v) Coomassie Brilliant Blue R-250 stain(by Biorad) in 100 ml(91 ml of 50%(v/v) methanol and 9 ml glacial acetic acid) for two hours. Gels were destained in diffusion destainers with a solution of 7.5% glacial acetic acid and 5% methanol in water(ddw) for a period of 1-14 days(due to the intensity of staining the latter was the more usual time for destaining). Activated charcoal was used in the diffusion destainer to remove free stain from the destaining solution.

Type III-SDS,EDTA gels were stained using the method of Fairbanks(125). Gels were stained overnight in a solution of 0.05%(w/v) coomassie blue with 25%(v/v)Isopropyl alcohol, 10%(v/v) glacial acetic acid and water. Gels were destained in a diffusion destainer with a solution of 10%(v/v)

isopropyl alcohol and 10%(v/v) glacial acetic acid.

8. Assay of Radioactivity

a) Gels

Gels were transversely sliced and minced, in 1 or 2 mm slices and extruded into scintillation vials by a Gilson Aliquogel Fractionator. Two methods were utilized for the solubilization of the biological samples within the polyacrylamide gels by the alkaline reagent NCS^{TM}.

i) The samples were dried overnight at 50°C after which 100ul of water(ddw)was added to the sample to give the optimum ratio of solubilizer-water for low quenching and high efficiency counting. NCS^{TM} solubilizer(0.6 ml) was added to the mixture and the vials were capped and heated to 50°C for another 2 hour period. Samples were allowed to cool and glacial acetic acid(20ul) was added to neutralize the basic solubilizer (0.034ml/mlNCS) which often induces chemiluminescence in the liquid scintillation process. 12 ml of scintillation cocktail containing 0.4% Omnifluor in toluene was then added to the sample. This scintillation cocktail contains both primary and secondary scintillator molecules, PPO(2,5-diphenyloxasole) and Bis-MSB(p-bis(O-methylstyryl)benzene) respectively. A secondary scintillator is often necessary to have a photon of a specific series of wavelengths emitted in order to be picked up by the photocathode of the Liquid Scintillation Counter(128,129).

Since this procedure was long and involved just for the solubilization of the samples the effect on counting efficiency and quenching of the following parameters were tested:

-the removal of the 2 hour incubation of the sample with NCS at 50°C

-no addition of the glacial acetic acid to neutralize chemiluminescence of NCS^{TM} in the liquid scintillation process

-a decrease in the volume of omnifluor scintillation cocktail from 12 to 10 ml.

Removal of the 2 hour incubation period with NCS, the glacial acetic acid addition,and the reduction in the volume of omnifluor cocktail gave similar counting efficiencies, as determined by running quench curve standards, as the complete procedure. The following procedure was then used for solubilization of the dual labelled protein samples within

polyacrylamide gels:

ii) Samples of 1-2 mm slices of PA gel were dried overnight at 50°C(to remove water). This was followed by the sequential addition of 80ul water(ddw), 500 ul NCS^{TM} and 10 ml of 0.4% omnifluor in toluene. Samples were allowed to stand in the dark for 12 hours before counting in the Liquid Scintillation Counters LS-150, LS-250, and LS-350 by Beckman. Samples were counted to 5% accuracy in scintillation counters with AQC(automatic quench compensation). Counting efficiencies with narrow ^{3}H and ^{14}C discriminator windows were approximately 38% and 71% respectively.

b) Input Samples

A small volume(ul) of the sample to be layered onto the gel before the addition of bromophenol blue and sucrose was placed in the scintillation vial. To this vial, water(ddw) to a volume of 100ul volume(80ul) was added followed by 600ul NCS^{TM}(500ul), 12 ml omnifluor cocktail(10 ml). Values in parentheses were for the second(ii) solubilization procedure. Samples were counted on Beckman Liquid Scintillation counters to 1% accuracy.

9.Analysis of Data in Dual Labelling

The radioactivity associated with each transverse slice of polyacrylamide gel is expressed as ^{3}H and ^{14}C counts per minute(cpm) within predetermined window settings; quenching and spillover were corrected using the method of Hendler which specifically assays for two β-emitting isotopes (114). The external standard channels ratio was used to monitor quenching in the samples (130). A computer program for the analysis of the data was modified from Boeckx and coworkers (131), published as Scint II program (132). The following data is calculated for each vial after the subtraction of background counts(10-20cpm) in both the ^{3}H and ^{14}C channels, and the application of individual quench correction and spillover:

a) the amount of radioactivity of ^{3}H and ^{14}C in nanocuries per vial

b) the picomoles of each isotope ^{3}H and ^{14}C per vial

c) the ratio $^{14}C/^{3}H$ each expressed in picomoles

d) the normalized ratio(RN) where the individual ratio in each gel

fraction is divided by the average ratio of the total gel.

e) the logarithm of the normalized ratio(log RN)

If the number of counts in a vial(either isotope) falls below a set limit value(two times background counts per minute) the ratio was not computed and the vial was not used in the total picomole values for the gel.

Five computer printed plots can be obtained with the data sheet as well as certain statistical factors (138,22). Basically three of these plots are used for analysis:

a) Plot 1: the picomoles of each label present in each gel fraction (or vial).

b) Plot 3: the 'percentage picomole' plot where the picomoles of each label present in each gel fraction are expressed as a percentage of the total picomoles of that label present on the gel.

c) Plot 4: the normalized ratio plot obtained by dividing each of the individual gel fraction ratios($^{14}C/^{3}H$) by the average ratio of all the gel fractions present.

The criteria for an abnormality in this system will be the following:

1. an abnormal ratio will exceed the mean value by at least two standard deviations
2. the region in which this deviation occurs should correspond to a protein staining peak on a sample gel
3. the deviation from unity of the normalized ratio should be reproducible in different animal pairs
4. the deviant ratio should reverse direction on the reversal of the labelling direction.

Though there are advantages to the dual labelling technique there are also two main difficulties or errors which can occur with this technique. one error(Type I) is that you will see an apparent abnormality when in fact there is no real abnormality present. This could be due to biological individuality between any two animals. A method of checking for this type of error would be to compare as many animal pairs of normal and dystrophic genotypes together as possible. If the abnormality is not reproducible then it is not an abnormality, between normal and dystrophic animals, due to muscular dystrophy. The second error(Type II) with this technique is that

of covering up an abnormality due to a large noise to signal ratio. For instance if a particular protein incorporates little of the radioactive label and migrates with the same relative mobility as one or more components incorporating a greater amount of radioactive label the signal from the smaller component may not be distinguishable from the others. The only way of resolving this type of error is to progressively reduce the components of the system being investigated or by increasing the resolving power of the analytical technique. The latter may mean changing from single-dimensional separation of proteins to a two-dimensional system of separation.

RESULTS AND DISCUSSION

The dual isotope labelling technique was used to search for proteins synthesized in abnormal quantities in the nervous system of hamsters and mice with genetically inherited muscular dystrophies. The results are presented here with some discussion of their meaning in two sections: the first deals with the Syrian hamster with muscular dystrophy, strain BIO 14.6, the second with murine dystrophy in strain Bar Harbour 129Rej/dydy.

A) Nervous System Protein Synthesis in BIO 14.6 Hamsters

1. Time Study for Optimum Incorporation of Labelled Protein Precursor

A time study looking for the optimum period of labelling for high incorporation of the isotope, into brain supernatant proteins, was carried out. Five day old hamsters (strain RB) were intraperitoneally injected with 200uCi L-(4,5^{3}H)leucine and sacrificed after a set time interval. The highest incorporation of the tritiated leucine into this fraction occurred at four hours (Figure 4a). In order to achieve the maximum incorporation of leucine into the soluble proteins of many tissues a labelling time of three hours was selected for the dual labelling experiments (Figure 4a & 4b). Only the supernatant fractions (105,000 x g x 120 minutes) were tested for incorporation of the protein precursor because of the reported abnormalities in this fraction (55,56,101).

2. Application of the Dual Labelling Technique to Proteins of the Nervous System

a) Brain subcellular fractions

i) Brain supernatant fractions

Using radioisotopes of leucine, L-(4,5^{3}H)leucine and L-(U^{14}C)leucine, the pattern of label incorporation into polypeptides was followed with polyacrylamide gel electrophoresis with SDS. In order to detect the minimal variation to be expected from a study of normal and dystrophic

Figure 4. Time Study for Optimum Incorporation of Labelled Leucine

200 Ci of L-(4,5^{3}H)leucine in 100ul of 0.9% NaCl was intraperitoneally injected into 5-day-old RB Syrian hamsters. The animals were then incubated at 32°C(15-180 minutes) or marked and placed in with mother(180-1440 minutes) for a set time period. The picomoles of tritiated leucine incorporated per milligram protein versus time of incubation were then plotted for the soluble fractions of the brain, skeletal muscle(a) and kidney, heart, and liver(b).

Samples had been freeze-dried and resuspended in buffer (o.01M sodium phosphate pH 7.2) and eluted from G-25 sephadex columns with the same buffer. The incubation period for 720 minutes(12 hours) indicated a faulty injection of the radioisotope.

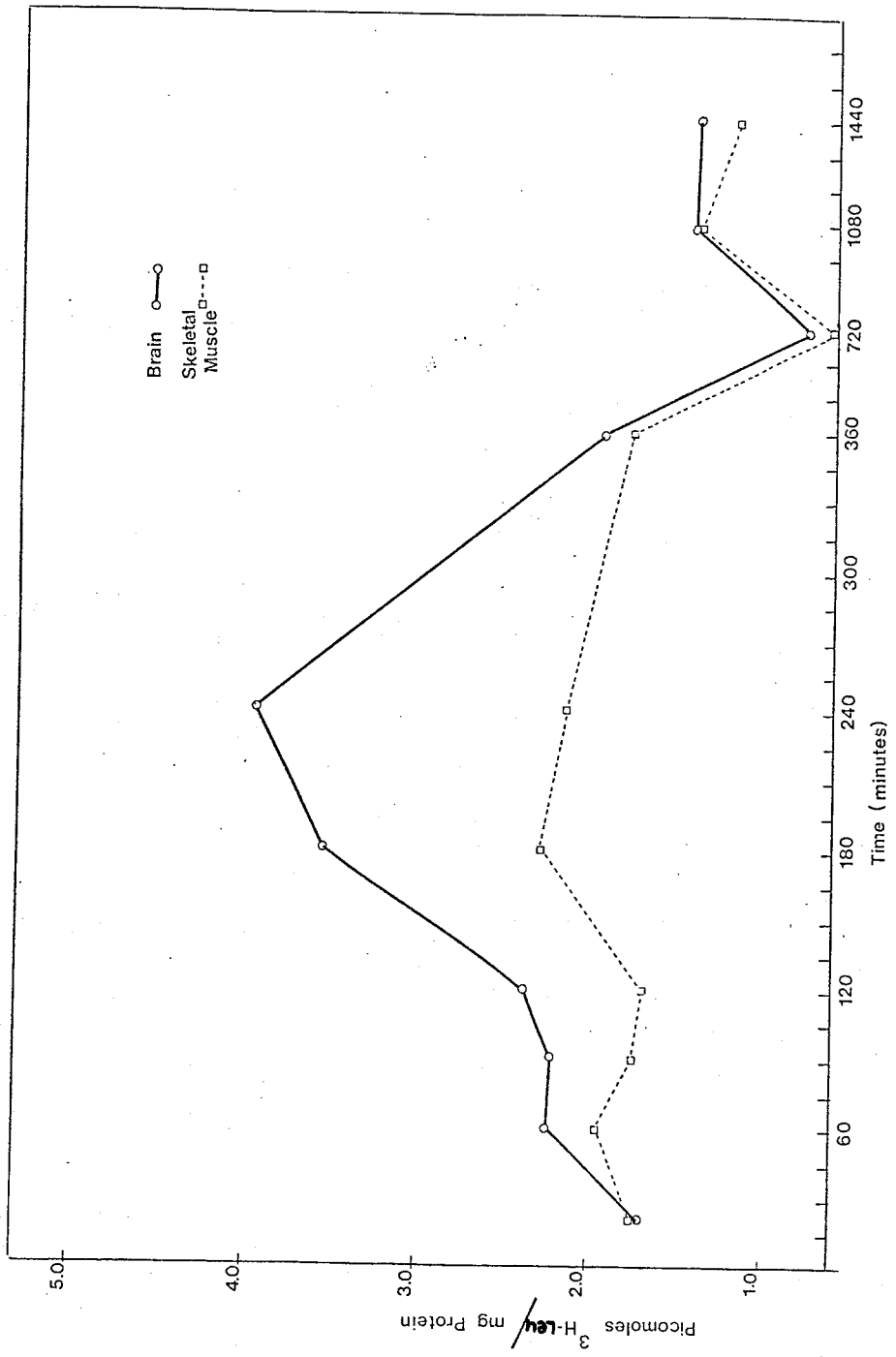

Brain
Skeletal Muscle
Picomoles ^{3}H-Leu / mg Protein
5.0
4.0
3.0
2.0
1.0
60
120
180
240
300
360
720
1080
1440
Time (minutes)

FIGURE 4a

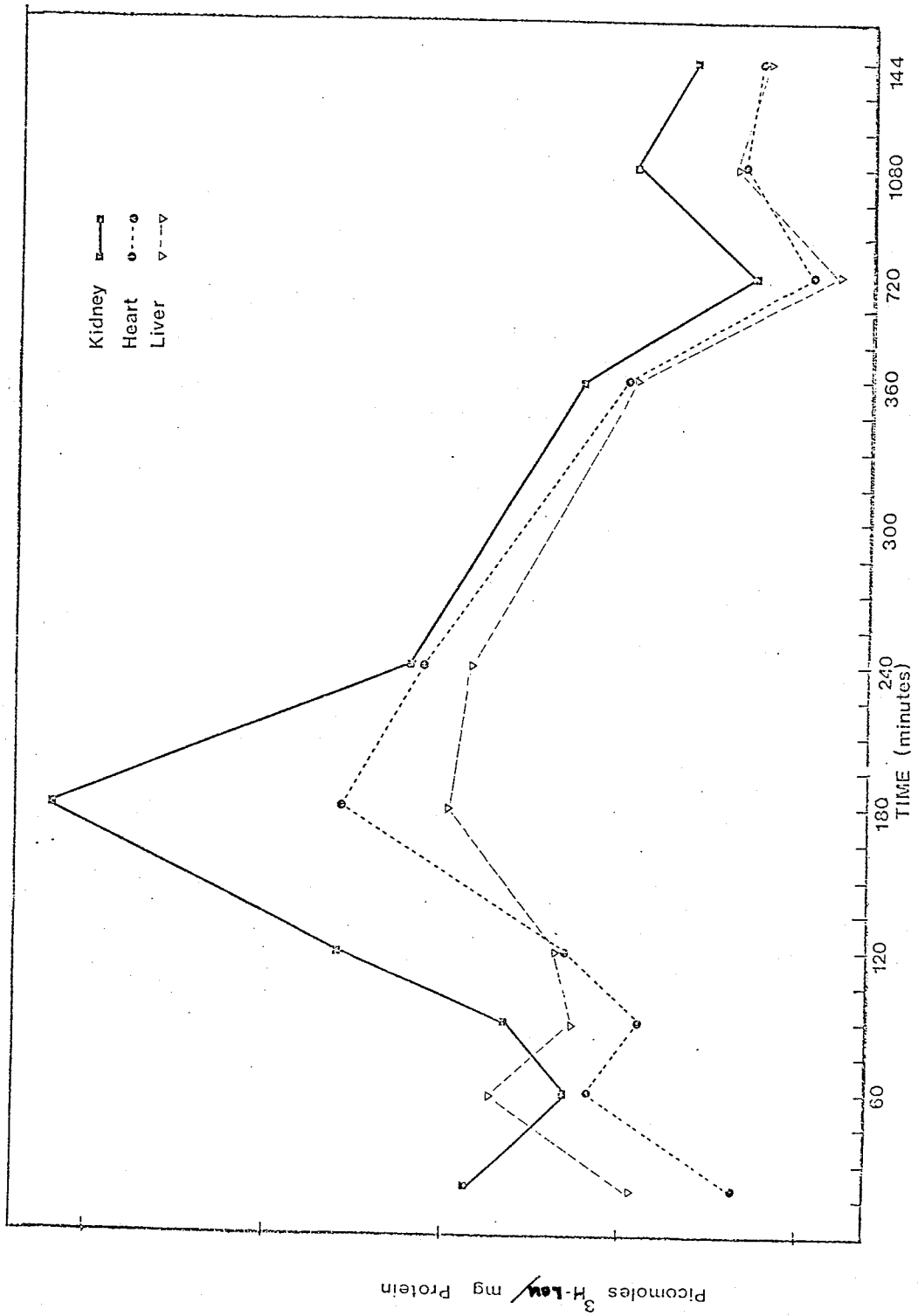

Kidney
Heart
Liver
60
120
180
240
300
360
720
1080
144
TIME (minutes)
Picomoles ³H-Leu/ mg Protein

FIGURE 4b

animals, a dual labelling study of two normal(RB) hamsters was carried out. This should show us any heterogeneity in the quantity of individual proteins synthesized due to biochemical individuality and in mechanical variations in the technique, like incomplete injection of the label and different amounts of tissue combined for homogenization.

The first dual labelling results, then, were of the hamster brain supernatant, where L-(4,5^{3}H)leucine and L-(U^{14}C)leucine, were injected into two different normal Syrian hamsters. After separation of the polypeptide components on 10% SDS gels(Type I) and analysis the following results were obtained. The picomoles of label in each gel fraction(picomole plot) showed similar patterns of incorporation in both normal hamsters but quite a difference in the extent of leucine incorporation into the polypeptide chains(Figure 5). This difference could be due to a true difference in the amounts of protein synthesized by the two animals or more likely to imperfect injections of the label or a difference in the amount of tissue combined for homogenization. If the 'percentage picomole' plot, where the picomoles of label present in each gel fraction are expressed as a percentage of the total picomoles of that label present on the gel, the series of peaks of label incorporation overlap (Figure 6). This indicates that no abnormality was detectable in the cell supernatant. The percentage picomole plot compensates for differences in label injection and unequal amounts of tissues combined for homogenization. The normalized ratio is shown here in Figure 6. Significant deviation ($\pm$ 2 standard deviations) of the normalized ratio from 'one' was used to assess the presence of abnormalities. The normalized ratio was amazingly flat considering the results which might be expected between two different animals.

If extremely low numbers of counts occur within a fraction large variations in the ratio of the isotopes present may occur. In order to avoid this problem an arbitrary number of counts in a vial was chosen below which the ratio was not calculated. Examples of fractions like this can be seen between fractions #64-#72 and #80-#108. Two times the number of background counts(in either ^{14}C or ^{3}H channels) was chosen as the limiting number of counts.

Figure 5. Incorporation of leucine into brain soluble fractions of two age-matched(3-day-old) normal Syrian hamsters(RB).

The hamsters were injected intraperitoneally with equimolar amounts of leucine isotope; one with 1mCi L-(4,5^{3}H)leucine (o); the other with 250uCi L-(U^{14}C)leucine (x); made to equimolar concentrations with L-leucine and made to volume(115ul) with 0.9% NaCl. The specific activity of the tritiated leucine was 1.39Ci/mmol while that of ^{14}C-leucine was 348mCi/mmol.

Separation was on 10% SDS-gels(Type I), migration from left to right. The gel was fractionated into 1-mm slices.

Shown here are the number of picomoles of leucine(both ^{14}C and ^{3}H) incorporated by the brain soluble proteins versus the gel fraction(vial number) (Plot 1) and the ratio of ^{14}C/^{3}H label incorporation in each fraction (Plot 2). The ratio is shown with the experimental mean(x) and the 1SD interval about that mean.

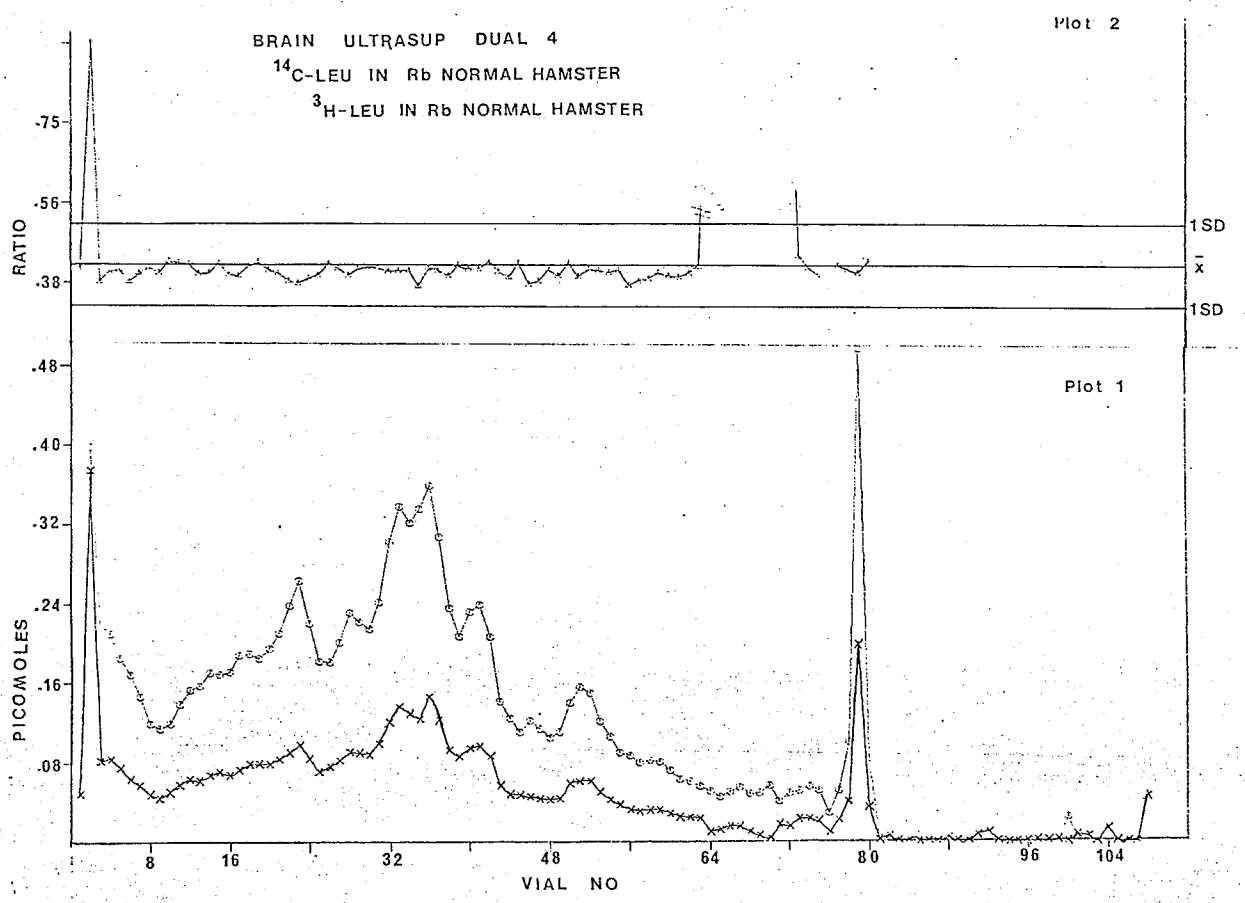

BRAIN ULTRASUP DUAL 4
^{14}C-LEU IN Rb NORMAL HAMSTER
^{3}H-LEU IN Rb NORMAL HAMSTER
Plot 2
Plot 1
RATIO
.75
.56
.38
1SD
$\bar{x}$
1SD
.48
.40
.32
.24
.16
.08
PICOMOLES
8
16
32
48
64
80
96
104
VIAL NO

FIGURE 5

Figure 6. Normalized picomole and normalized ratio plots of leucine incorporation into brain supernatant fractions of age-matched(3-day-old) RB hamsters.

Shown here are the picomoles of each isotope incorporated in each gel fraction expressed as a percentage of the total number of picomoles of that isotope in the gel versus vial number (Plot 3). This is called the normalized or percentage(%) picomole plot. Incorporation of L-(U^{14}C)leucine (x); incorporation of L-(4,5^{3}H)-leucine (o).

The normalized ratio (Plot 4) is the ratio of $^{14}C/^{3}H$ leucine incorporation in each gel fraction divided by the average of all the ratios of isotopic incorporation ($^{14}C/^{3}H \div$ av $^{14}C/^{3}H$). It was arbitrarily chosen that a deviation from the expected normalized ratio of 'unity' is considered abnormal if it exceeds the $\pm$2SD limit. The experimental mean(x) and the 2SD interval about that mean is shown in the figure.

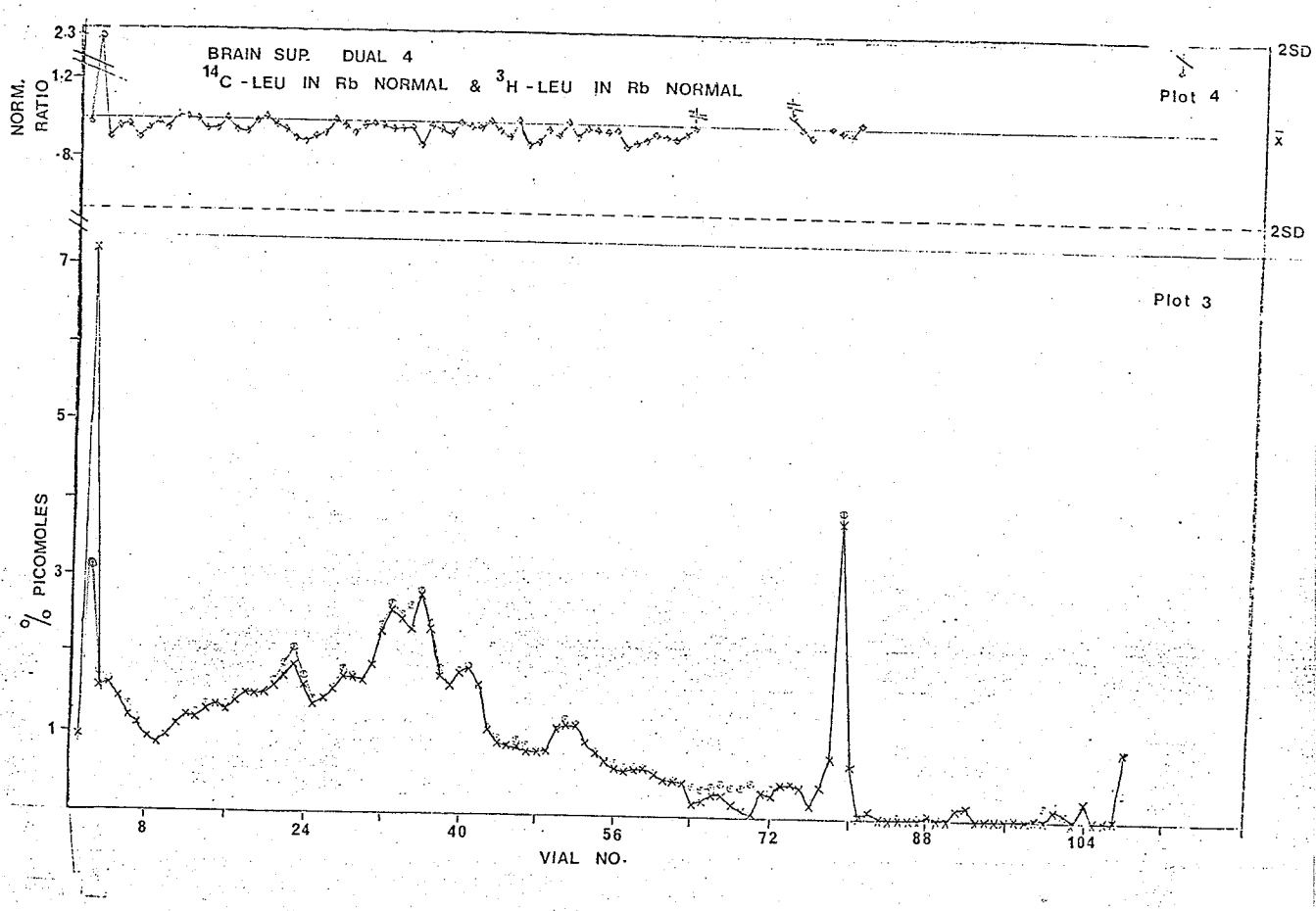

BRAIN SUP. DUAL 4
^{14}C -LEU IN Rb NORMAL & ^{3}H -LEU IN Rb NORMAL
NORM. RATIO
2.3
1.2
.8
2SD
Plot 4
X
2SD
Plot 3
% PICOMOLES
7
5
3
1
8
24
40
56
72
88
104
VIAL NO.

FIGURE 6

At the beginning of the gel(Figure 6) there is an apparent abnormality in fraction # 2. This has been seen by other workers (133) and has been attributed to the presence of a stacking gel, though the actual principles involved are not understood. In this system where no stacking gel has been applied the top portion of the gels in contact with oxygen may not be as firmly polymerized and thus act effectively as a stacking gel (124).

The brain supernatant fractions from two different pairs of normal and dystrophic hamsters were then separated on 10% SDS gels(Type I). Shown here are the experiments with RB(1-day-old) and BIO 14.6(2-day-old) hamsters labelled for 1 hour(Figure 7) and the 1-day-old age-matched RB and BIO 14.6 hamsters labelled for a 2 hour period(Figure 8). * In these brain supernatant fractions there was close superimposition of the pattern of L-(4,5^{3}H)leucine and L-(U^{14}C)leucine incorporation throughout the gels. The normalized ratio seldom differed significantly from 'unity'. There were, however, some areas where the normalized ratio exceeded the $\pm$2SD limits (see vial #51, Figure 7; see vials #86-92, Figure 8). Neither of these results were considered to be significant.

Vial #51 was not taken as a significant abnormality due to the dystrophic condition because the bottom fraction of the gel(anodic) was sliced first by the gel fractionator and some radioactive contamination from a gel previously sliced may have occurred. In fact, the tracking dye bromophenol blue(a very small and highly negative species) had not even reached this end of the gel before fractionation into gel slices.

It is unlikely that contamination from the gel slicer occurred causing the abnormality in fractions #86-92. However the second criteria for distinguishing an abnormality is that the deviation from unity of the normalized ratio should be present in a number of animal pairs. Since the abnormality in this brain supernatant fraction was not reproduced in another pair of animals the same age(Figure 7) the deviation could possibly have been due to biochemical individuality between two animals. It is also possible that these points may be considered in the 5% of the

*Early dual labelling studies were not labelled at the optimum 3 hour period for leucine incorporation.

Figure 7. Normalized picomole and normalized ratio plots of leucine incorporation into brain supernatant fractions of a normal(1-day-old)hamster and a dystrophic(2-day-old) hamster.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine(spec. act. 1.2OCi/mmol) (o); the dystrophic with 200uCi L-(U^{14}C)leucine(spec. act. 240mCi/mmol) (x). The incubation period was 1 hour.

Separation on 10% SDS gels(Type I), migration from left to right, was carried out. The gel was fractionated in 2-mm slices.

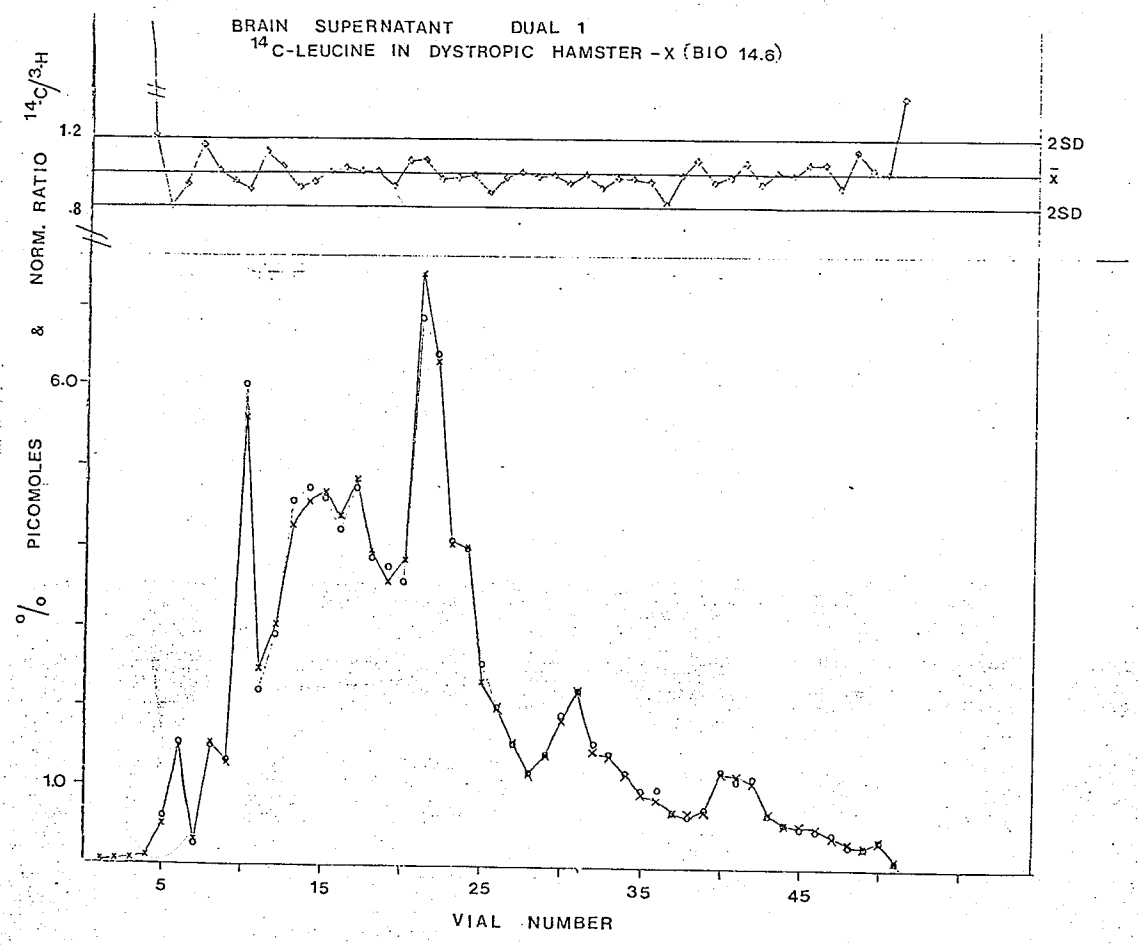

BRAIN SUPERNATANT DUAL 1
^{14}C-LEUCINE IN DYSTROPIC HAMSTER -X (BIO 14.6)
$^{14}C/^{3}H$ NORM. RATIO
1.2
.8
2SD
$\bar{x}$
2SD
% PICOMOLES
6.0
1.0
5
15
25
35
45
VIAL NUMBER

FIGURE 7

Figure 8. Incorporation of leucine into brain supernatant proteins of age-matched(1-day-old) RB and BIO 14.6 hamsters.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine(spec. act. 1.22Ci/mmol) (o); the dystrophic with 200uCi L-(U^{14}C)leucine(spec. act. 240mCi/mmol) (x). The incubation period was 2 hours.

Separation on 10% SDS gels(Type I), migration from left to right, was carried out. The gel was fractionated in 1-mm slices. The % picomole plot(normalized plot) and the normalized ratio plot are shown here.

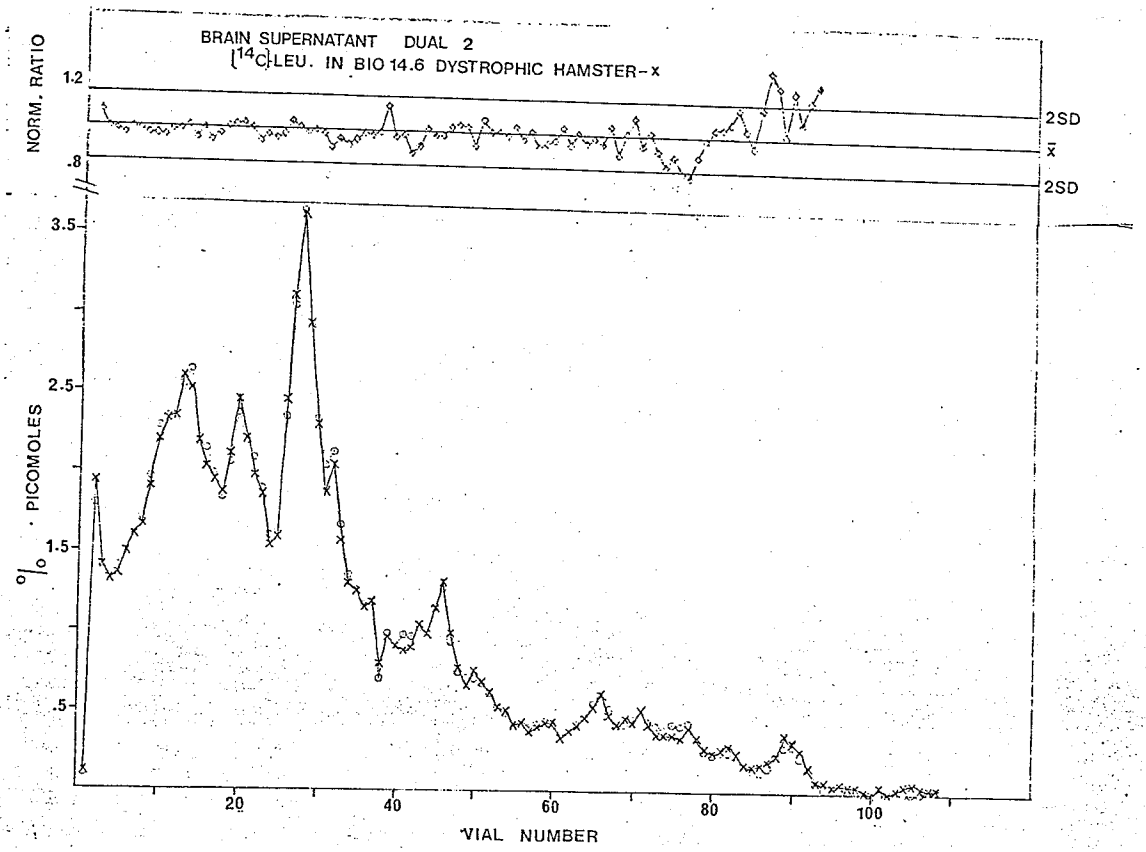

BRAIN SUPERNATANT DUAL 2
[14C]-LEU. IN BIO 14.6 DYSTROPHIC HAMSTER-x
NORM. RATIO
1.2
.8
2SD
x̄
2SD
3.5
2.5
1.5
.5
% · PICOMOLES
20
40
60
80
100
VIAL NUMBER

FIGURE 8

experimental points which lie outside the 2SD interval about the experimental mean.

In order to attain better resolution of the polypeptides a modified disc gel system (124)was used. This method creates sharp starting zones from which the polypeptides can separate (134). Brain supernatant fractions of age-matched(3-day-old and 6-day-old) RB and BIO 14.6 animal pairs were electrophoresed on 10% SDS disc gels(Type II) with a separating gel buffer at pH 8.8 and stacking gel buffer at pH 6.8. The soluble fractions of the brains of dystrophic hamsters showed no abnormalities from normal with regards to the pattern of label incorporation(Figure 9). pair Because no abnormalities were detected, another separation technique, that of isoelectricfocusing, was carried out. In principle this technique can detect single amino acid substitutions in a protein if the result is an altered charge of the protein (19).

Isoelectricfocusing was carried out in polyacrylamide gels using an ampholyte pH gradient from pH3.5-10.0 (Type IV gel). The brain supernatant from age-matched(6-day-old) normal and dystrophic hamsters is shown in Figure 10. No abnormal patterns of leucine isotope incorporation were detected. In fact the normalized ratio showed a very small 2SD interval about the experimental mean.

These results show that there is no detectable difference in the incorporation of leucine by brain soluble fractions in these very young normal and dystrophic hamsters. The proteins were separated on the basis of molecular weight and charge separation, but it is possible that abnormalities could be buried in unresolved portions of the scans.

ii) Brain particulate fractions

Brain mitochondrial and microsomal fractions of RB and BIO 14.6 hamsters(3 and 6-day-old age-matched pairs) were separated on 10% SDS gels(Type I). No abnormality in the pattern of isotopic precursor incorporation was found. Once again poor resolution of the microsomal fractions on this gel system prompted the use of the disc gel system(Type II). The brain particulate fractions(nuclear pellet, mitochondrial and microsomal pellets) of age-matched(6-day-old) normal and dystrophic hamsters were

Figure 9. Incorporation of leucine into brain supernatant proteins of age-matched(6-day-old) RB and BIO 14.6 hamsters.

The normal animal was injected with 2mCi L-(4,5^{3}H)leucine(spec. act. 1.39Ci/mmol) (o); the dystrophic with 500uCi L-(U^{14}C)leucine(spec. act. 347mCi/mmol) (x). The incubation period was 3 hours long.

Separation on 10% SDS disc gel(Type II),with separating gel buffer pH 8.8 and stacking gel buffer pH 6.8,was carried out with migration left to right. The gel was fractionated in 1-mm slices.

The % picomole plot and the normalized ratio are shown.

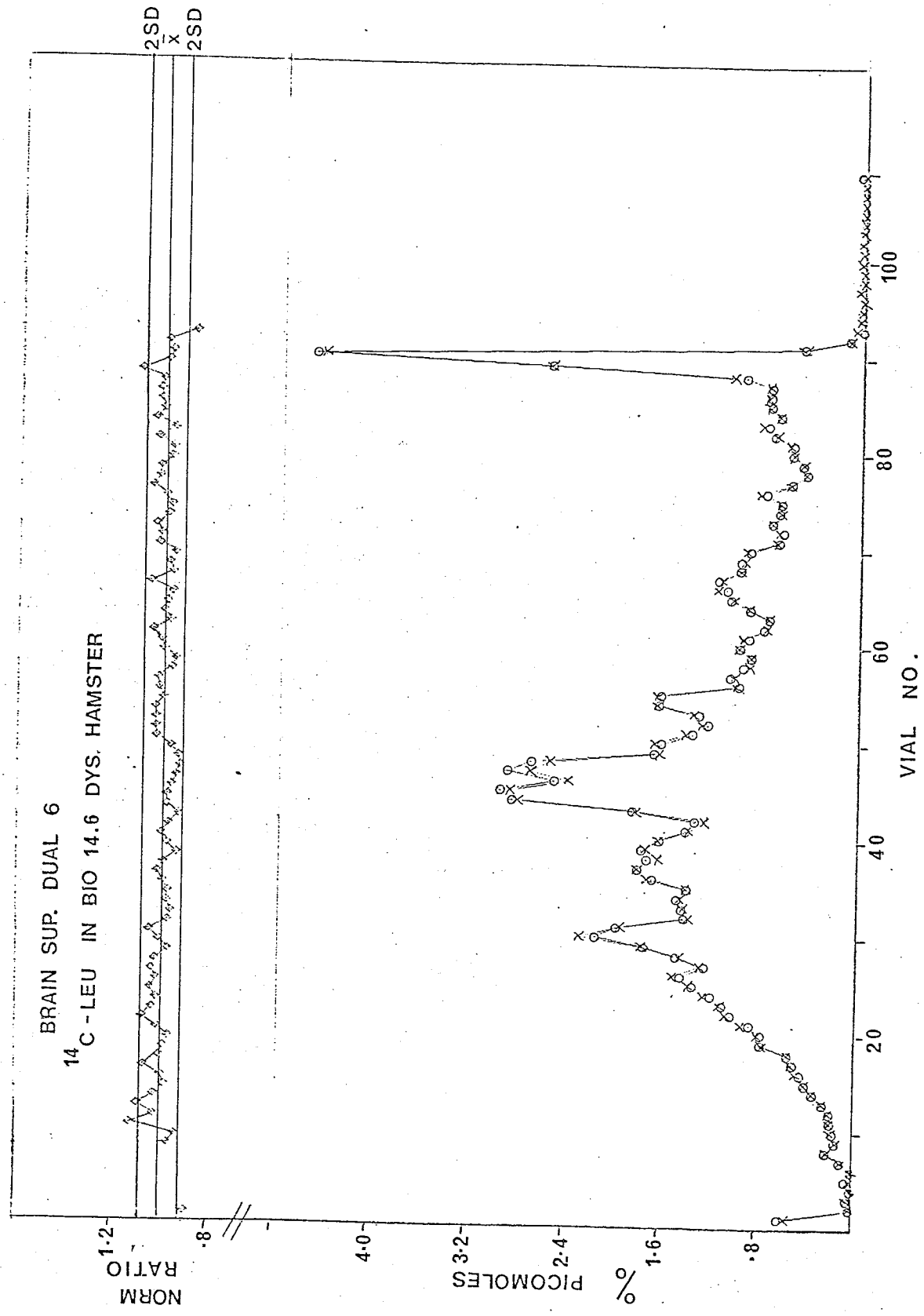

BRAIN SUP. DUAL 6
^{14}C - LEU IN BIO 14.6 DYS. HAMSTER
2SD
$\bar{x}$
2SD
NORM RATIO
1·2
·8
PICOMOLES %
4·0
3·2
2·4
1·6
·8
20
40
60
80
100
VIAL NO.

FIGURE 9

Figure 10. Incorporation of leucine into the brain supernatant fraction of age-matched(6-day-old) RB and BIO 14.6 hamsters.

The normal animal was injected with 2mCi L-(4,5^{3}H)leucine (spec. act. 1.39Ci/mmol) (o); the dystrophic with 500uCi L-(U^{14}C)-leucine(spec. act. 347mCi/mmol) (x).

Separation was carried out on 5.1% polyacrylamide isoelectricfocusing gel(Type IV). pH gradient from pH 3.5-10.0(left to right respectively). The gel was fractionated in 1-mm slices.

The % picomole plot and the normalized ratio plot are shown here.

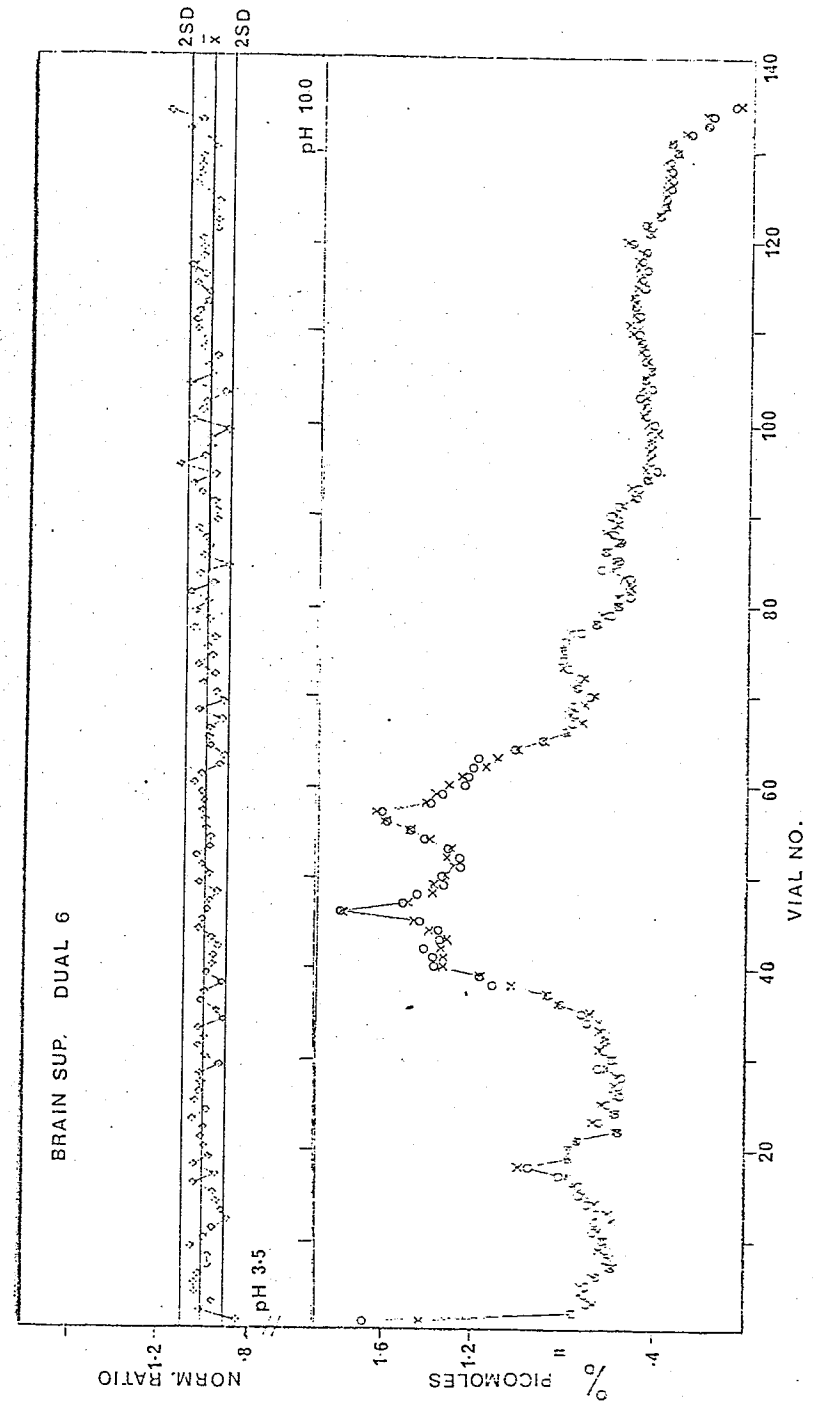

BRAIN SUP. DUAL 6
2SD
x̄
2SD
pH 3·5
pH 10·0
NORM. RATIO
·8
1·2
PICOMOLES
%
·4
1·2
1·6
20
40
60
80
100
120
140
VIAL NO.

FIGURE 10

electrophoresed on 10% SDS disc gels(Type II). An abnormal pattern of incorporation of the two labels was found to occur within the tracking dye region of the gel. The direction of the abnormality(L-($U^{14}C$) leucine having been injected into the dystrophic hamster) indicated an increased synthesis of low molecular weight components by the dystrophic hamster(Figures 11 & 12). This means that the normalized ratio deviates from unity in a positive direction and is outside the 2SD interval so it is considered a significant abnormality. In order to be assessed as a real difference between normal and dystrophic hamsters this apparent abnormality must satisfy two other criteria; reproducibility and reversibility. This difference in incorporation of the labels may also be an experimental artifact(as the abnormality was only seen using the SDS disc gel system with tris,glycine and urea). If the former is true, on reversing the direction of the label(L-($U^{14}C$)leucine being injected into the normal hamster) the abnormality in the normalized ratio should change direction. That is the normalized ratio should deviate from unity in a negative direction. If this defect is reproducible in different pairs of normal and dystrophic animals, it would be considered a true abnormality as well. If the difference is artifactual it is likely that it would not be reproducible in different animal pairs and the abnormality would not reverse direction on reverse labelling. Before testing for reproducibility and reversibility experimental artifacts should be ruled out.

A control test involving the injection of both radioisotopes into the same normal animal(3-day-old) was undertaken to see if there was any discriminatory metabolism of the leucine or the production of artifacts in the preparation procedure of the subcellular fractions. When the nuclear fraction and the mitochondrial fractions were examined by gel electrophoresis, a similar abnormality was found within the tracking dye region of the gel(Figure 13). The direction of the abnormality (increased from unity in the normalized ratio plot) was the same as in the previous experiments(see Figure 11 & 12). This result suggested that the 'defect' was due to the isotopes used in the experiment.

Figure 11. Incorporation of leucine into brain nuclear pellet proteins of age-matched(6-day-old) RB and BIO 14.6 hamsters.

The normal animal was injected with 2mCi L-(4,5^{3}H)leucine (spec. act. 1.39Ci/mmol) (o); the dystrophic with 500uCi L-(U^{14}C) leucine(spec. act. 347mCi/mmol) (x). Incubation period was 3 hours.

Separation was on 10% SDS disc gels(Type II). Gel fractionated in 1-mm slices.

Shown here are the % picomole plot and the normalized ratio. The normalized ratio is shown with the +2SD limit(the -2SD indicated with dashed line). An abnormality in the incorporation of ^{14}C-leucine is seen between fractions #78-#90.

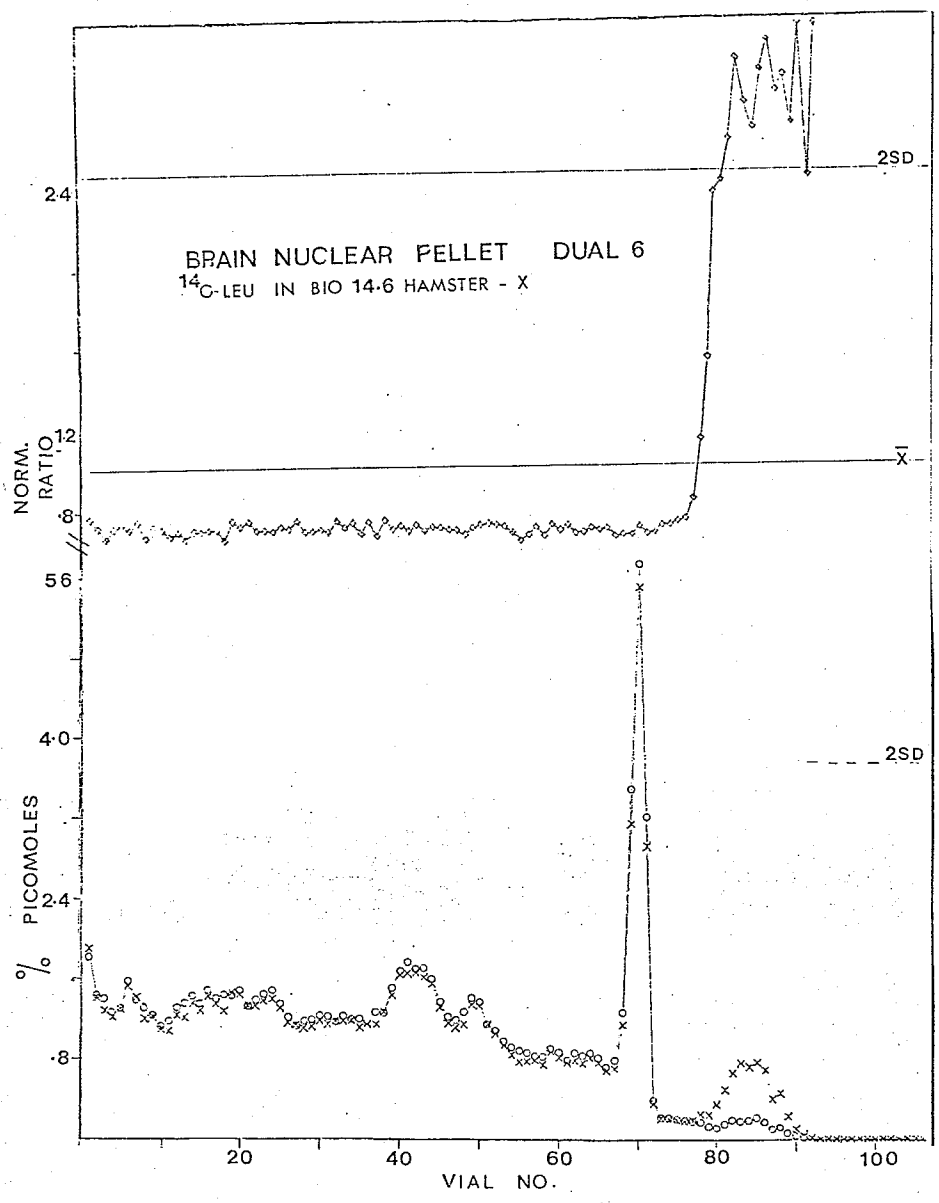

BRAIN NUCLEAR PELLET DUAL 6
^{14}C-LEU IN BIO 14.6 HAMSTER - X
2SD
$\bar{X}$
2SD
NORM. RATIO
PICOMOLES %
2.4
1.2
.8
5.6
4.0
2.4
.8
20
40
60
80
100
VIAL NO.

FIGURE 11

Figure 12. Incorporation of leucine into brain mitochondrial proteins of age-matched(6-day-old) RB and BIO 14.6 hamsters(same pair as Figure 9-11).

Shown here are the % picomole plot and the normalized ratio. L-(4,5^{3}H)leucine was in RB hamster (o); L-(U-^{14}C)leucine in BIO 14.6 hamster (x). The gel was fractionated in 1-mm slices. Separation was on 10% SDS disc gel(Type II).

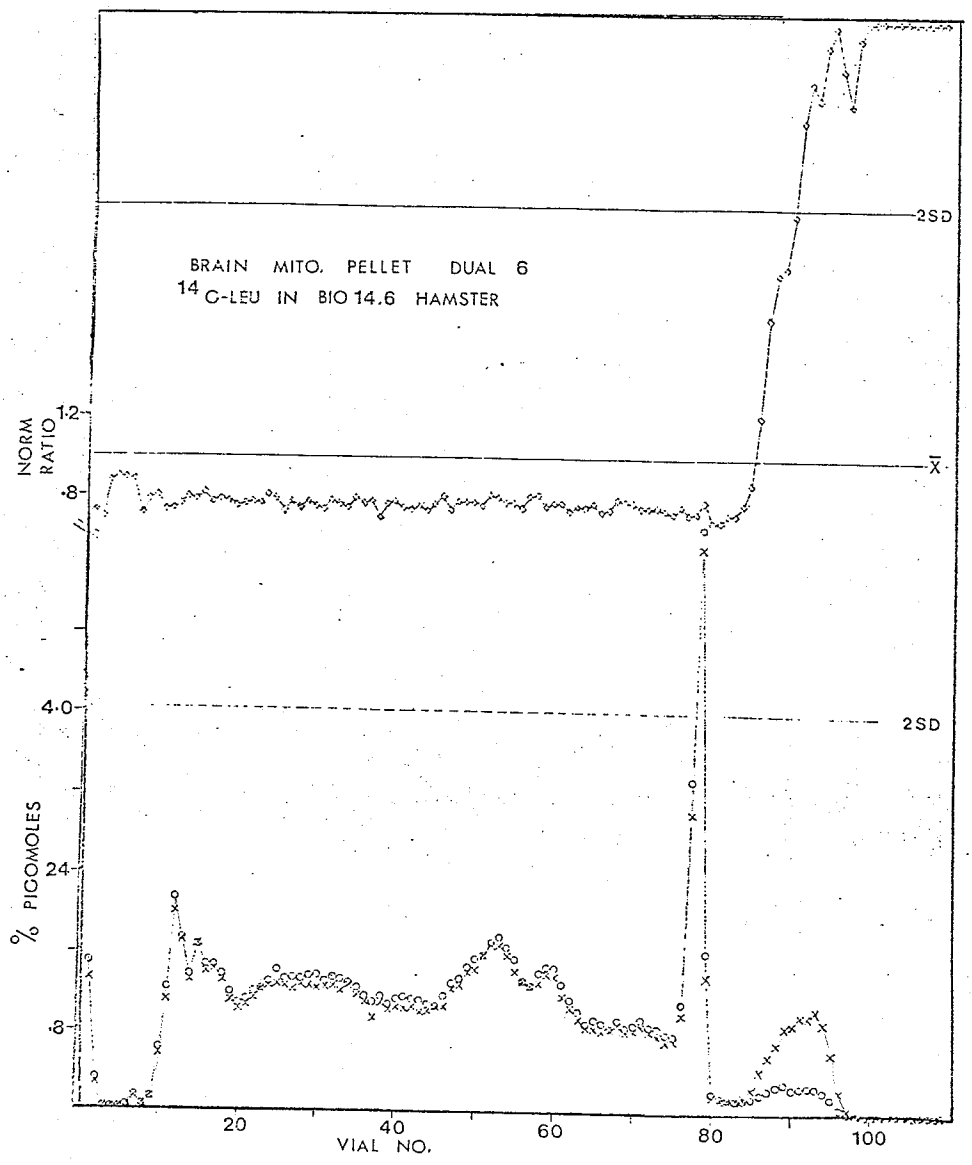

BRAIN MITO. PELLET DUAL 6
14 C-LEU IN BIO 14.6 HAMSTER
2SD
X̄
2SD
NORM RATIO
1.2
.8
4.0
2.4
.8
% PICOMOLES
20
40
60
80
100
VIAL NO.

FIGURE 12

Figure 13. Incorporation of leucine into the brain mitochondrial fraction of a 3-day-old normal hamster.

Both isotopes, 1mCi L-(4,5^{3}H)leucine(spec. act. 1.39Ci/mmol) and 250uCi L-(U^{14}C)leucine(spec. act. 348mCi/mmol), (⊙) and (x) respectively, were intraperitoneally injected into a single hamster. The incubation period was 2 hours.

Separation was on 10% SDS disc gel(Type II), migration from left to right. Shown here are the % picomole plot(normalized plot) and the normalized ratio. A difference in incorporation of ^{14}C and ^{3}H leucines occurred in fractions #42-#45. Gel had been fractionated in 2-mm slices.

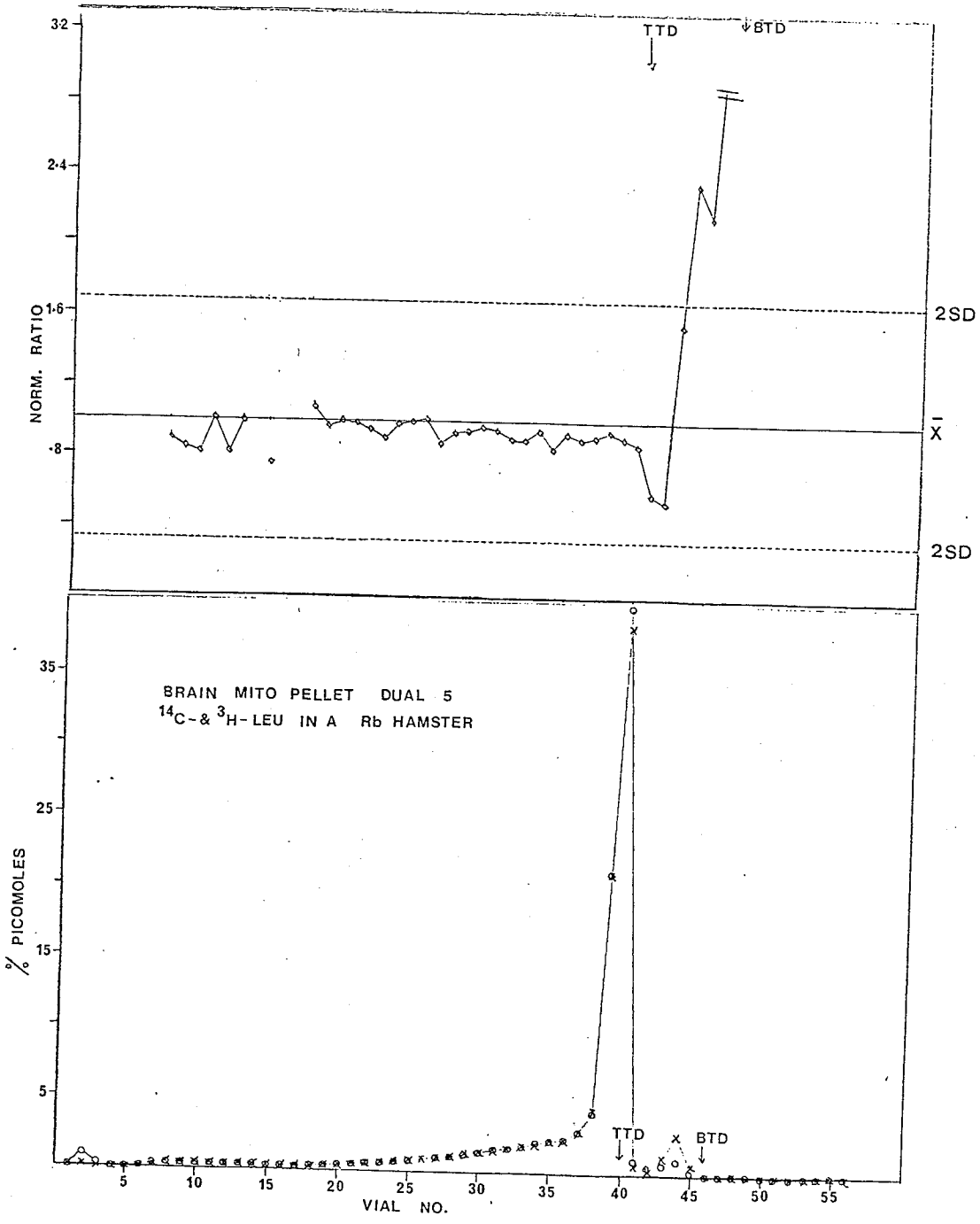

TTD
BTD
3·2
2·4
1·6
·8
NORM. RATIO
2SD
X̄
2SD
BRAIN MITO PELLET DUAL 5
14C-& 3H-LEU IN A Rb HAMSTER
35
25
15
5
% PICOMOLES
TTD
BTD
5
10
15
20
25
30
35
40
45
50
55
VIAL NO.

FIGURE 13

The nature of the radioactive isotope defect could be:
a) due to a contaminant in the leucine label or leucine itself which is not removed by dialysis before the sample is electrophoresed or
b) due to the differential metabolism of the ^{14}C and ^{3}H atoms in the leucine isotopes(L-(4,5^{3}H)leucine and L-(U^{14})leucine).

If the former were true, it would seem that the contaminant 'sticks' to the membrane components of the brain particulate fractions, as the abnormality is not detected in the brain supernatant fractions of either age-matched(3 and 6-day-old) normal and dystrophic hamsters (see Figure 9). The nature of the leucines is different; L-(4,5^{3}H)leucine is specifically labelled at positions #4 and #5 while L-(U^{14}C)leucine is uniformly labelled at carbons #1-6. If the second hypothesis is true it may be that the products of leucine degradation(acetyl CoA and acetoacetate) have differing relative amounts of isotope in them. This is indeed true, as in leucine degradation to acetyl CoA and acetoacetate ^{3}H is lost from both carbon atoms #4 and #5. Thus the $^{14}C/^{3}H$ ratio in the pool of lipid precursors is increased. Lipid moieties in this region of an SDS disc gel have been seen in red blood cell membrane preparations (125) and in brain particulate preparations (122). A proteolipid has also been found to migrate slightly slower than the bromophenol blue T.D. on SDS gel electrophoresis with 8M urea of skeletal and heart muscle of dystrophic animals (135).

iii) Acetone extraction of particulate fractions

In order to verify that this fraction had characteristics of lipid an acetone extraction of the brain nuclear fractions was carried out. Acetone extractions of these fractions from the single normal hamster(3-day-old) and the age-matched normal and dystrophic hamsters(6-day-old) were electrophoresed along with non-extracted samples of the same fraction. It was found that the abnormality was removed in both extractions(Figures 14 & 15). Both ^{14}C(cpm) and ^{3}H(cpm) were decreased by the acetone extraction procedure and thus the % picomole recovery in the tracking dye region were decreased(Table IV). That a decrease occurs in the quantity of both labels recovered indicates that both atoms are incorporated into these compounds but the ^{3}H-atoms are incorporated to a lesser degree. The

Figure 14. Incorporation of leucine in the brain nuclear pellet fraction of a 3-day-old RB hamster.

Both isotopes were injected into the single RB: L-(4,5^{3}H) leucine(spec. act. 1.39Ci/mmol) (o) ; L-(U^{14}C)leucine(spec. act. 348mCi/mmol) (x).

Separation of samples, before and after acetone extraction, was on 10% SDS disc gels(Type II), migration from left to right.

Arrows indicate a region of asymmetric incorporation of ^{14}C- and ^{3}H-leucine which is removed upon acetone extraction of the sample. Shown here are the % picomole plots of each of the fractions.

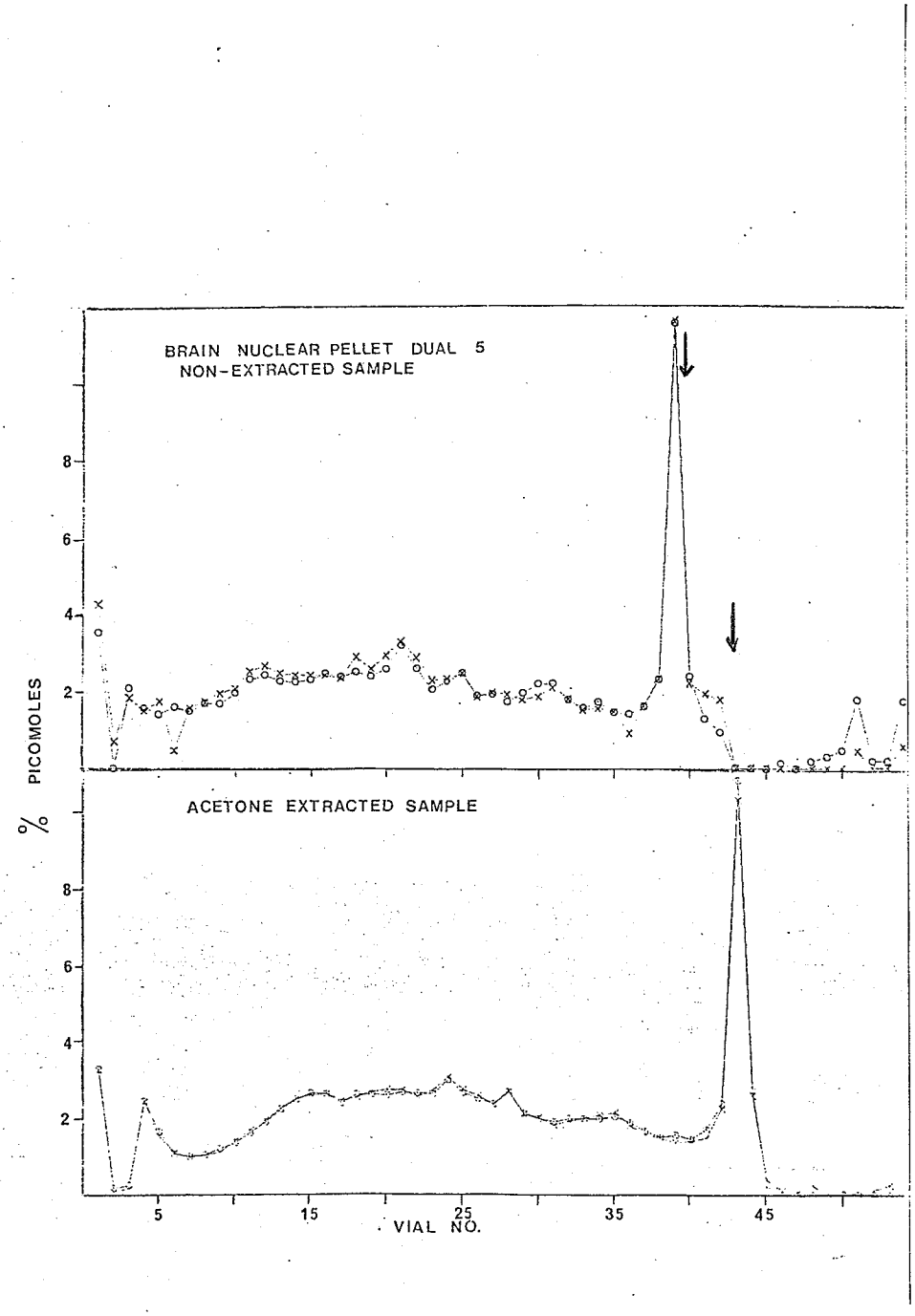

BRAIN NUCLEAR PELLET DUAL 5
NON-EXTRACTED SAMPLE
ACETONE EXTRACTED SAMPLE
% PICOMOLES
2
4
6
8
5
15
25
35
45
VIAL NO.

FIGURE 14

Figure 15. Incorporation of leucine in the brain nuclear pellet fraction of age-matched(6-day-old) RB (o) and BIO 14.6 (x) hamsters.

The normal animal was injected with 2mCi L-(4,5^{3}H)leucine (spec. act. 1.39Ci/mmol); the dystrophic with 500uCi L-(U^{14}C)leucine(spec. act. 347mCi/mmol). Separation of the brain nuclear pellet proteins before and after acetone extraction was on 10% SDS disc gels (Type II).

Arrows indicate regions of asymmetric isotope incorporation; the abnormality is reduced on acetone extraction of the sample. Shown here are the % picomole plots(normalized ratio plots not shown).

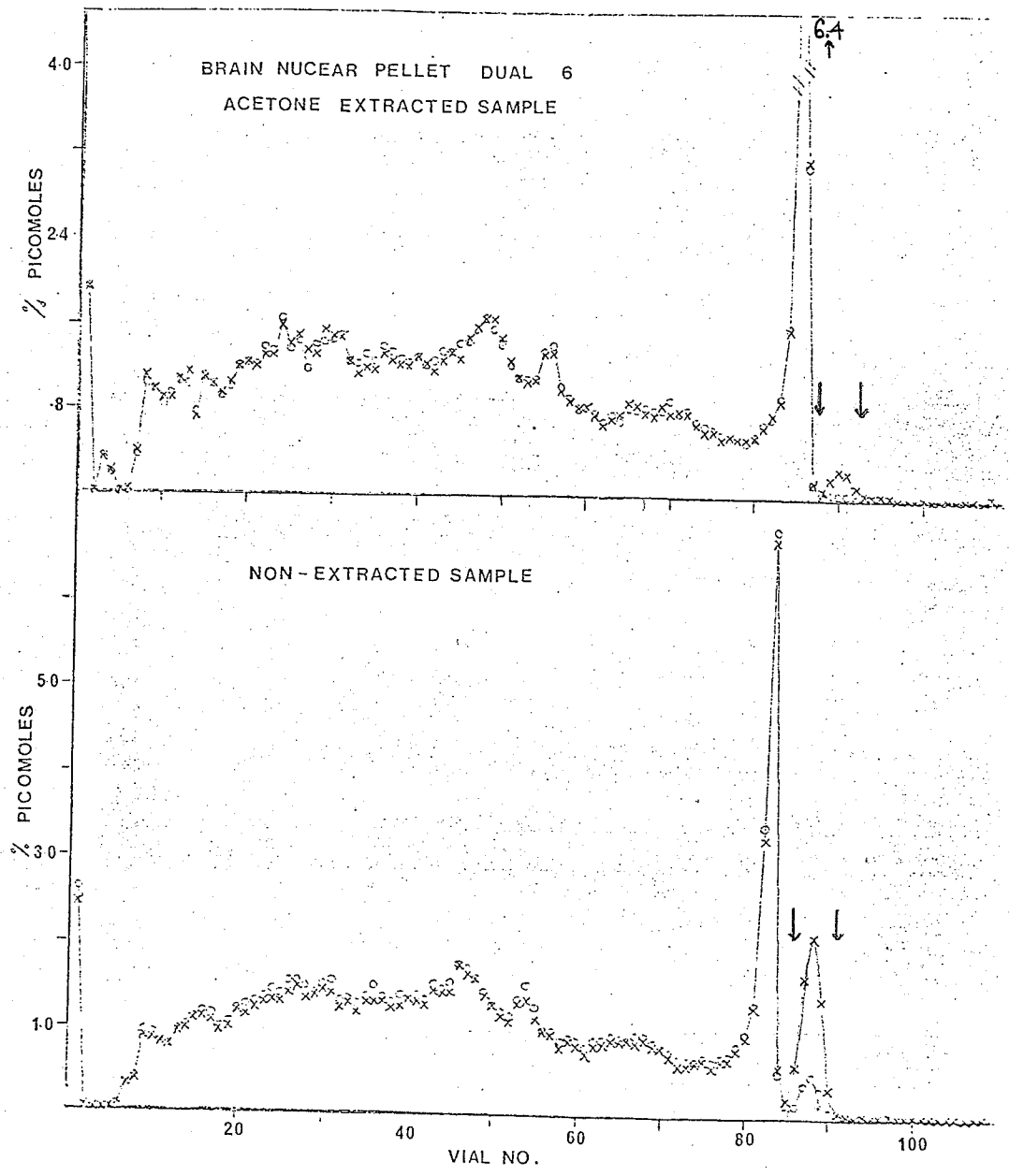

BRAIN NUCEAR PELLET DUAL 6
ACETONE EXTRACTED SAMPLE
6.4
4·0
2·4
·8
% PICOMOLES
NON-EXTRACTED SAMPLE
5·0
3·0
1·0
% PICOMOLES
20
40
60
80
100
VIAL NO.

FIGURE 15

TABLE I

PERCENTAGE RECOVERY of RADIOACTIVE LABEL
IN TRACKING DYE REGION

Age in Days	Hamster Subcellular Fraction	% ^{14}C	% ^{3}H
6	Brain mitochondria	8.72	2.97
	Brain Nuclear pellet	6.23	1.77
3	Brain mitochondria	4.90	2.17
	Brain mitochondria	4.78	1.23
3	Brain Nuclear pellet Acetone Extracted	0.92	0.16
	Brain Nuclear pellet Non-extracted	3.80	2.30
6	Brain Nuclear pellet Acetone Extracted	1.15	0.36
	Brain Nuclear pellet Non-extracted	6.29	1.40

All gels were 10% SDS disc gels(Type II) with 6M urea.

removal of the 'defect' was not complet and could be due to the presence of lipids which are not extracted by acetone.

iv) Brain particulate fractions-Isoelectricfocusing separation

In order to separate membranous proteins from hydrophobic interactions with membrane lipids in isoelectricfocusing a non-ionic detergent must be used. Lubrol solubilized nuclear and mitochondrial fractions of age-matched(6-day-old) RB and BIO 14.6 hamsters were separated by gel isoelectricfocusing(Type IV gel). The incorporation of L-(4,5^{3}H)leucine and L-(U^{14}C)leucine were superimposable in both patterns of separation (Figures 16 & 17). The normalized ratio plots indicated that little deviation from unity occurred in each of these fractions. In fact the variation of the normalized ratio from its experimental mean(x) value is very small as can be seen by the two standard deviation interval about that mean.

b) Spinal Cord proteins in normal and dystrophic hamsters

In the hamster with muscular dystrophy, little evidence has accumulated to suggest any neural involvement in the disease process. Studies on the number of anterior horn cells in the dystrophic hamster have proved inconclusive (90,92) therefore it was decided to study the spinal cord combined with the spinal roots of these animals with the dual labelling technique.

Two pairs of age-matched(30-day-old) hamsters, normal and dystrophic, were injected with isotopic leucines in two dual labelling experiments. In the first L-(U^{14}C)leucine was injected into the dystrophic hamster while in the second L-(U^{14}C)leucine was injected into the normal hamster (Figures 18 & 19). Because these were reverse labelling experiments a decision could be made on whether any observed 'defect' was a true abnormality. After a five day labelling time with the leucine precursors the spinal cord was removed by the method of de Sousa and Horrocks (117). and combined with spinal roots for homogenization. SDS-soluble fractions were electrophoresed on 13% SDS disc gels(Type II). No abnormalities within either of these fractions were detected. However a highly enlarged variance of the normalized ratio occurs because of the large incorporation of label within the tracking dye region of the gel. This region shows the

Figure 16. Incorporation of leucine in the brain nuclear pellet fraction of age-matched(6-day-old) RB (o) and BIO 14.6 (x) hamsters.

Leucine isotopes injected as in Figure 15. The lubrol soluble fraction was separated in 5.1% polyacrylamide isoelectricfocusing gel with a pH gradient from pH 3.5-10.0(left to right respectively). The gel was sliced in 1-mm slices.

BRAIN NUCLEAR PELLET DUAL 6
LUBROL EXTRACTED
NORM. RATIO
1·2
·8
pH 3·5
pH 10 0
2SD
x̄
2SD
% PICOMOLES
1·6
1·2
8
·4
20
40
60
80
100
120
140
VIAL NO.

FIGURE 16.

Figure 17. Incorporation of leucine in the brain mitochondrial fraction of age-matched(6-day-old) RB (o) and BIO 14.6 (x) hamsters.

Leucine isotopes injected as in Figure 15. The lubrol soluble fraction was separated by isoelectricfocusing in a 5.1% polyacrylamide isoelectricfocusing gel with a pH gradient from pH 3.5-10.0(left to right respectively). The gel was fractionated in 1-mm slices.

BRAIN MITO. PELLET DUAL 6
LUBROL EXTRACTED SAMPLE
NOR/A. RATIO
1·2
·8
2SD
x̄
2SD
pH 3·5
(+)
pH 10·0
% PICOMOLES
1·6
1·2
·8
·4
10
30
50
70
90
110
130
150
VIAL NO.

FIGURE 17

Figure 18. Incorporation of leucine in spinal cord homogenate(with spinal roots) of age-matched(30-day-old) normal and dystrophic hamsters.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.32Ci/mmol) (o); the dystrophic with 250uCi L-(U^{14}C)leucine(spec. act. 330mCi/mmol) (x). The labelling period was 5 days.

Separation was on 13% SDS disc gel(Type II), migration left to right. The gel was sliced in 2-mm slices. Only the SDS-soluble fraction of the spinal cord homogenate was electrophoresed on a 20cm gel. The SDS-soluble fraction was obtained by centrifugation at 10,000 x g x 10 minute on an Eppendorf Centrifuge after the sample had been disrupted and dialyzed.

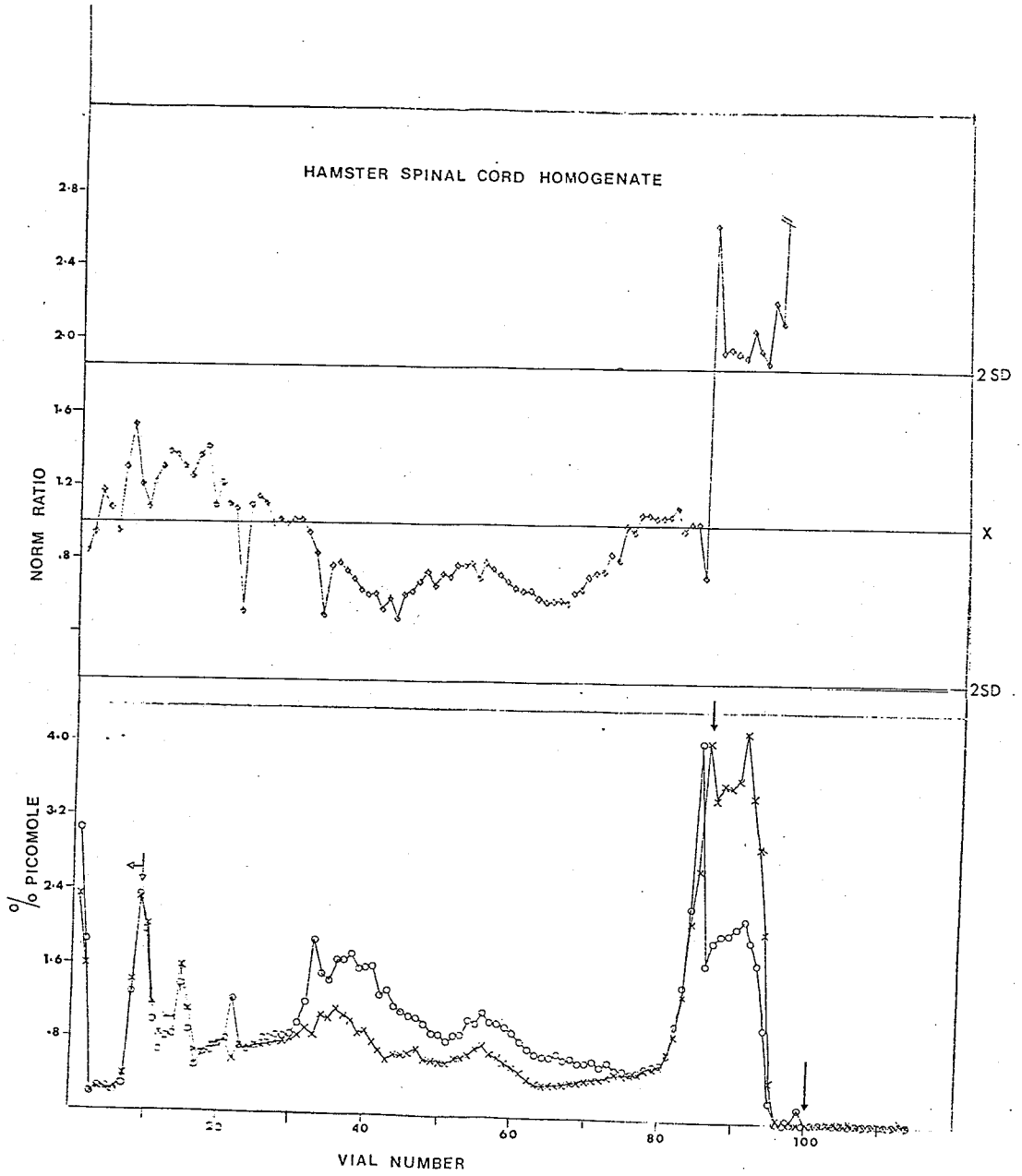

HAMSTER SPINAL CORD HOMOGENATE
NORM RATIO
2·8
2·4
2·0
1·6
1·2
·8
2SD
X
2SD
% PICOMOLE
4·0
3·2
2·4
1·6
·8
20
40
60
80
100
VIAL NUMBER

FIGURE 18

Figure 19. Incorporation of leucine in the spinal cord homogenate(with spinal roots) fraction of age-matched(30-day-old) normal and dystrophic hamsters.

The normal animal was injected with 250uCi L-(U^{14}C)leucine (spec. act. 330mCi/mmol) (x); the dystrophic with 1mCi L-(4,5^{3}H)leucine(spec. act. 1.32Ci/mmol) (o). The labelling period was 5 days.

Separation was on 13% SDS disc gel(Type II), migration from left to right. Gel was sliced in 2-mm slices. Only the SDS-soluble fraction(as in Figure 18) was electrophoresed.

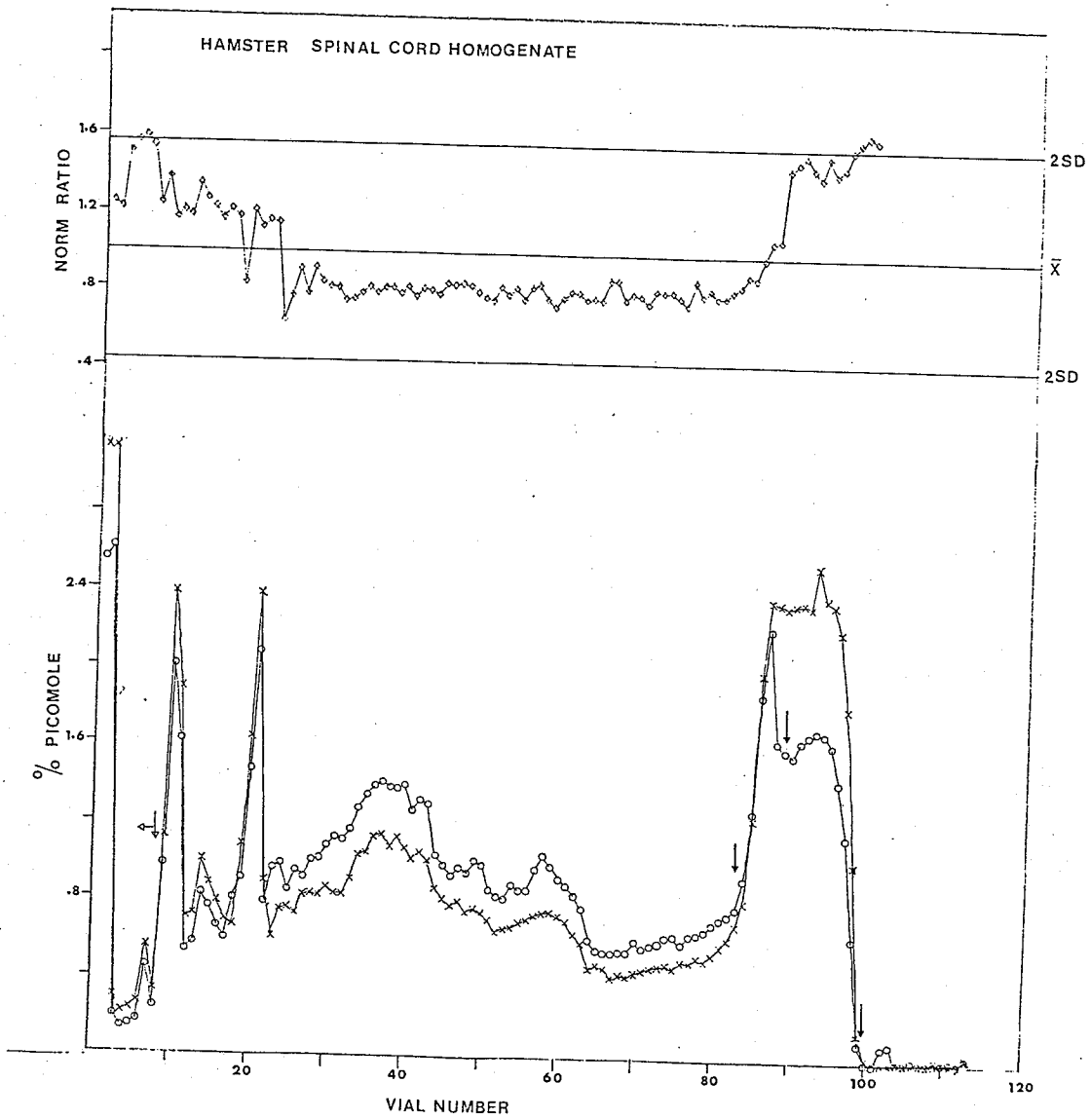

HAMSTER SPINAL CORD HOMOGENATE
NORM RATIO
1.6
1.2
.8
.4
2SD
X̄
2SD
% PICOMOLE
2.4
1.6
.8
20
40
60
80
100
120
VIAL NUMBER

FIGURE 19

'lipid defect' which is an artifact of the type of labels used in the study. By removing the data from this region and reanalyzing the charts a more accurate normalized ratio and 2SD interval about the ratio could be obtained. Though trends in the ratio occurred in the first pair of animals(L-(U^{14}C)leucine into BIO 14.6) showing a decrease in the incorporation of label by the dystrophic hamster the ratio did not deviate from unity be greater than 2 standard deviations. The ratios seen in this pair of animals were not seen in the second pair of animals indicating that these areas are not likely to be 'true abnormalities' due to the dystrophic condition of one of the animals.

B) Nervous System Proteins in Bar Harbour 129Rej/dydy Mice

Since no defects were found in the proteins synthesized by BIO 14.6 hamsters compared to control animals(RB) it was decided to test whether the dual labelling technique would detect abnormalities known to exist in the nervous tissues in murine dystrophy using Bar Harbour 129Rej/dydy mice.

1. The Dual Labelling Technique Applied to the study of Nervous System Proteins in Dystrophic Mice

a) Brain subcellular fractions

i) Brain supernatant fractions

The brain supernatant fractions were examined on SDS disc gels(Type II) in three different dual labelling experiments after injection of leucine isotopes. In the age-matched(29-day-old and 47-day-old) normal and dystrophic mice, the brain supernatants showed no abnormalities in the pattern of precursor incorporation. Though there are gel fractions which indicate slight 'defects' in these gels(see vial #28,97 & 98,Figure 20) they are not considered to be significant. The criterion of reproducibility between animal pairs was not met in these fractions because the 29-day-old mice pair(Figure 20) and 47-day-old mice pair(Figure 21) show different fractions deviating from the normalized ratio by greater than two standard deviations. Therefore, these points are likely due to biochemical individuality or are among the 5% of the experimental points expected to lie

Figure 20. Incorporation of leucine into the brain supernatant fraction of age-matched(47-day-old) normal and dystrophic mice.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (o); the dystrophic with 250uCi L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation was carried out on 10% SDS disc gel(Type II), migration left to right. The gel was fractionated in 1-mm slices.

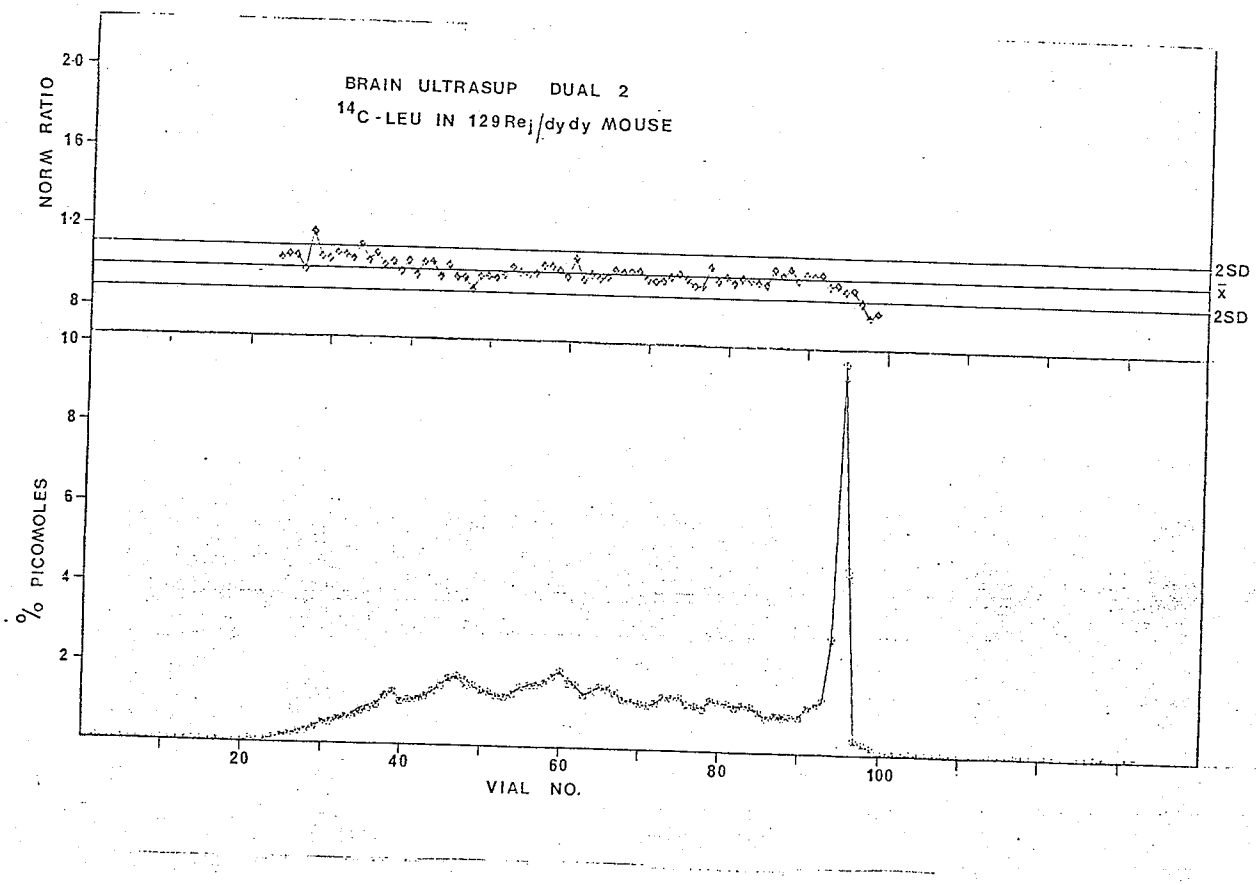

BRAIN ULTRASUP DUAL 2
^{14}C-LEU IN 129Rej/dydy MOUSE
NORM RATIO
2·0
1·6
1·2
8
10
2SD
x̄
2SD
% PICOMOLES
8
6
4
2
20
40
60
80
100
VIAL NO.

FIGURE 20

Figure 21. Incorporation of leucine into the brain supernatant fractions of age-matched(66-day-old) normal and dystrophic mice.

The normal animal was injected with 250uCi L-(U^{14}C)leucine (spec. act. 330mCi/mmol) (x); the dystrophic with 1mCi L-(4,5^{3}H)leucine(spec. act. 1.23Ci/mmol) (o).

Separation was carried out on 10% SDS disc gel(Type II), migration from left to right.

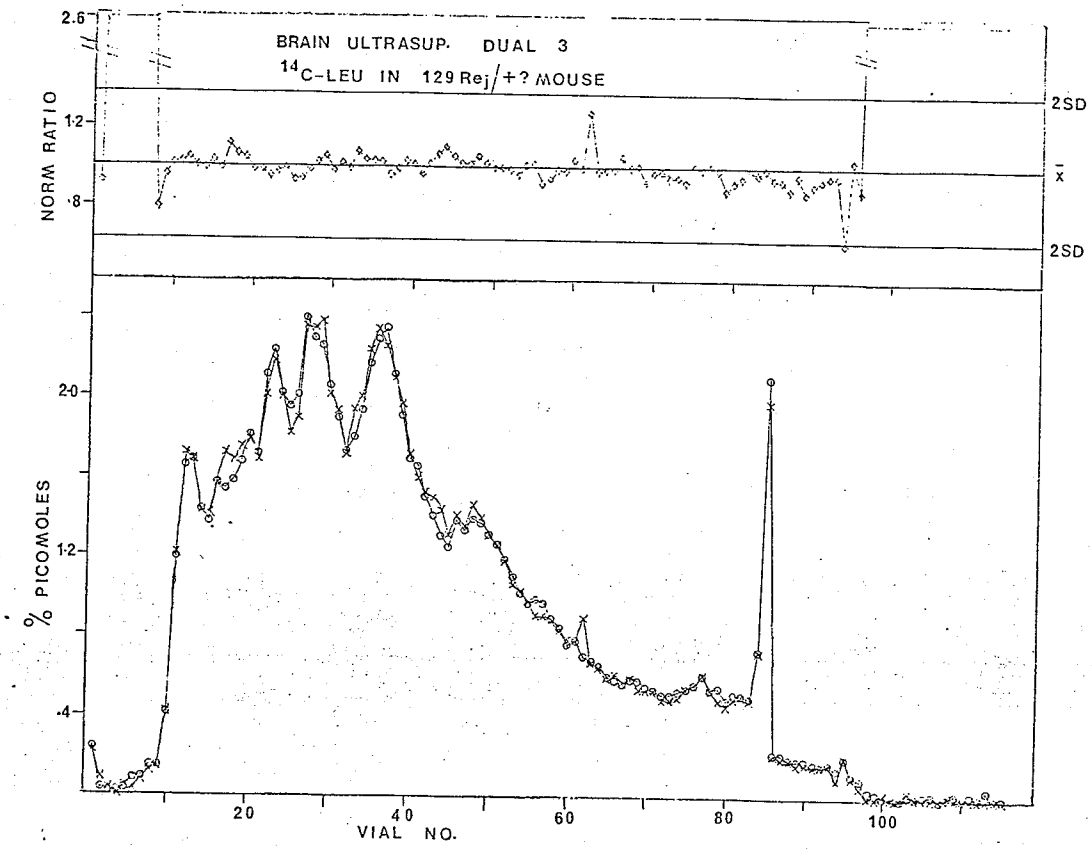

BRAIN ULTRASUP. DUAL 3
^{14}C-LEU IN 129 Rej/+? MOUSE
NORM RATIO
2.6
1·2
·8
2SD
$\bar{x}$
2SD
% PICOMOLES
2·0
1·2
·4
20
40
60
80
100
VIAL NO.

FIGURE 21

outside the two standard deviation interval. A second reason for not considering these points as true differences between normal and dystrophic mice is it was not possible to demonstrate reversibility. The reverse labelling experiment was carried out on age-matched(66-day-old) normal and dystrophic mice. Here the brain supernatant is shown electrophoresed on a 13% SDS disc gel(Figure 21). No abnormalites were detected.

ii) Brain mitochondrial fractions

The brain mitochondrial fractions(12,000 x g x 10 minutes) from the same three dual labelling experiments on age-matched mice pairs, strain 129Rej/dydy and 129Rej/+?, were electrophoresed on SDS disc gels(Type II) (Figures 22,23,&24). The abnormality showing a greater ^{14}C-atom incorporation within the tracking dye region of the gel was the only abnormality visible in this subcellular fraction. The direction of the abnormality was the same whether L-(U^{14}C)leucine was injected into the dystrophic mouse (Figures 22 & 23) or into the normal littermate(Figure 24). This indicated that it was similar to the abnormality found in the hamster particulate fractions explained by the way leucine is incorporated into lipids.

b) Spinal Cord(with spinal roots) proteins

i) Spinal cord polypeptides separated by SDS-PAGE

In the first experiment, on age-matched 29-day-old normal and dystrophic mice, there was a decreased incorporation of ^{14}C-leucine into some of the spinal cord polypeptides of the dystrophic mouse(Figure 25). This region of the gel, containing proteins of an approximate molecular weight of 23,000(Figure 27) showed a decreased normalized ratio but did not exceed the defined limit for an abnormality(2SD). Normally, this small deviation in the ratio would not have been considered abnormal. However since at least 8 fractions had deviant ratios further investigation was warranted. There were large peaks of incorporation of ^{3}H and ^{14}C leucines within and at the top of the tracking dye region. The normalized ratio is distorted because of the 'lipid artifact' where an excess of ^{14}C-atoms appear in lipid moieties compared to ^{3}H-atoms. The calculations were

Figure 22. Incorporation of leucine into the brain mitochondrial fractions of age-matched(29-day-old) normal and dystrophic mice.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (o); the dystrophic with 250uCi L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation on 11.7% SDS disc gel(Type II) was carried out with migration from left to right. Gel was fractionated in 1-mm slices.

Arrows indicate the artifact due to the labelling of lipid moieties which run with the leading ion/trailing ion boundary.

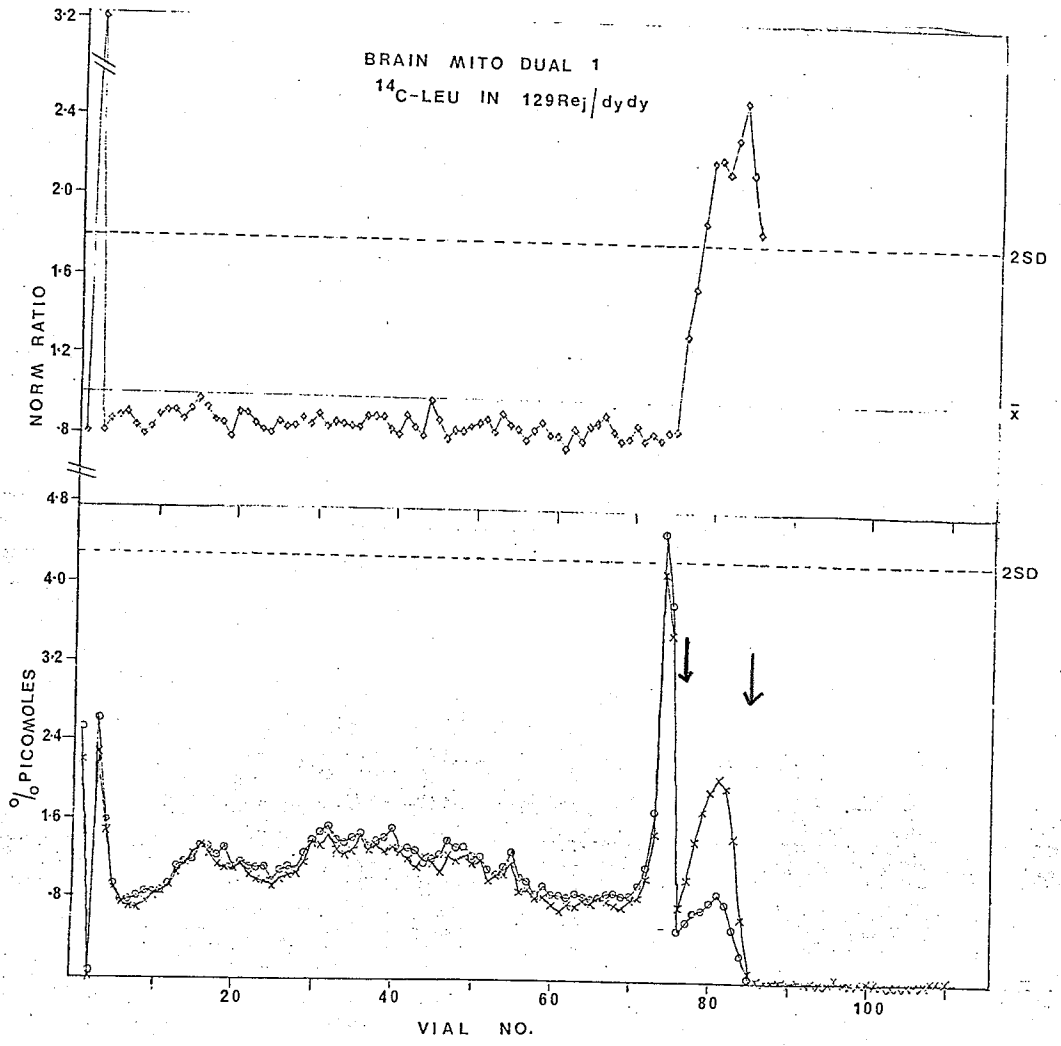

BRAIN MITO DUAL 1
^{14}C-LEU IN 129Rej/dydy
NORM RATIO
3·2
2·4
2·0
1·6
1·2
·8
2SD
x̄
4·8
4·0
3·2
2·4
1·6
·8
2SD
% PICOMOLES
20
40
60
80
100
VIAL NO.

FIGURE 22

Figure 23. Incorporation of leucine into the brain mitochondrial fractions of age-matched(47-day-old) normal and dystrophic mice.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (o); the dystrophic with 250uCi L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation on 10% SDS disc gel(Type II), migration left to right, was carried out. Gel was fractionated in 2-mm slices.

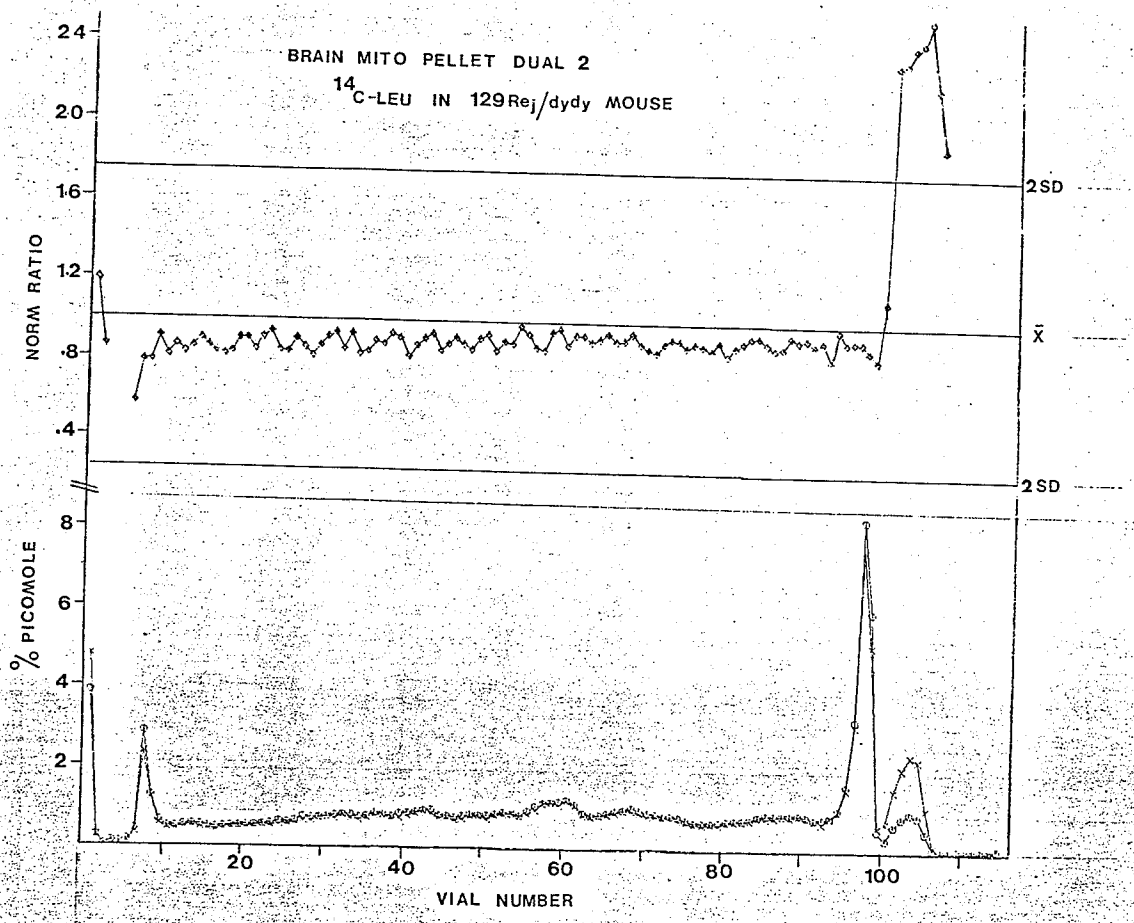

BRAIN MITO PELLET DUAL 2
^{14}C-LEU IN 129Rej/dydy MOUSE
NORM RATIO
2.4
2.0
1.6
1.2
.8
.4
2SD
X̄
2SD
% PICOMOLE
8
6
4
2
20
40
60
80
100
VIAL NUMBER

FIGURE 23

Figure 24. Incorporation of leucine into the brain mitochondrial fractions of age-matched(66-day-old) normal and dystrophic mice.

The normal animal was injected with 250uCi L-(U^{14}C)leucine (spec. act. 330mCi/mmol) (x); the dystrophic with 1mCi L-(4,5^{3}H)leucine(spec. act. 1.23Ci/mmol) (o).

Separation was carried out on 13% SDS disc gel(Type II), migration left to right, and gel was sliced in 2-mm slices.

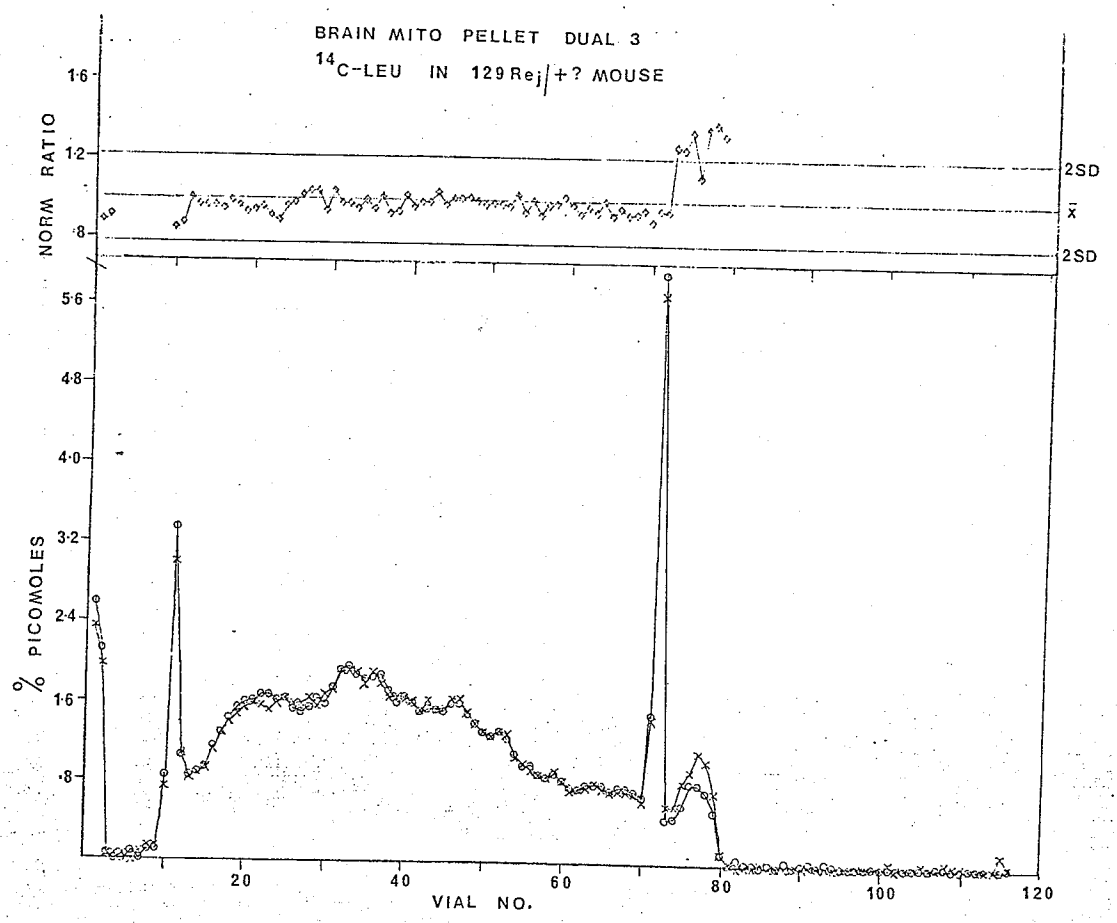

BRAIN MITO PELLET DUAL 3
^{14}C-LEU IN 129Rej/+? MOUSE
NORM RATIO
1·6
1·2
·8
2SD
$\bar{x}$
2SD
5·6
4·8
4·0
3·2
2·4
1·6
·8
% PICOMOLES
20
40
60
80
100
120
VIAL NO.

FIGURE 24

Figure 25. Incorporation of leucine into the spinal cord homogenate fraction (with spinal roots extirpated) of age-matched(29-day-old) normal and dystrophic mice.

The normal animal was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (o); the dystrophic with 250uCi L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation was carried out on a 13% SDS disc gel(Type II), migration from left to right. Gel was sliced in 2-mm slices.

Shown here are the % picomole plot, arrows(↓) indicate region of lower ^{14}C-leucine incorporation by the dystrophic mouse, and the normalized ratio plot with the experimental mean and the 2SD interval about that mean. Tracking dye region marked with open arrows(⇩).

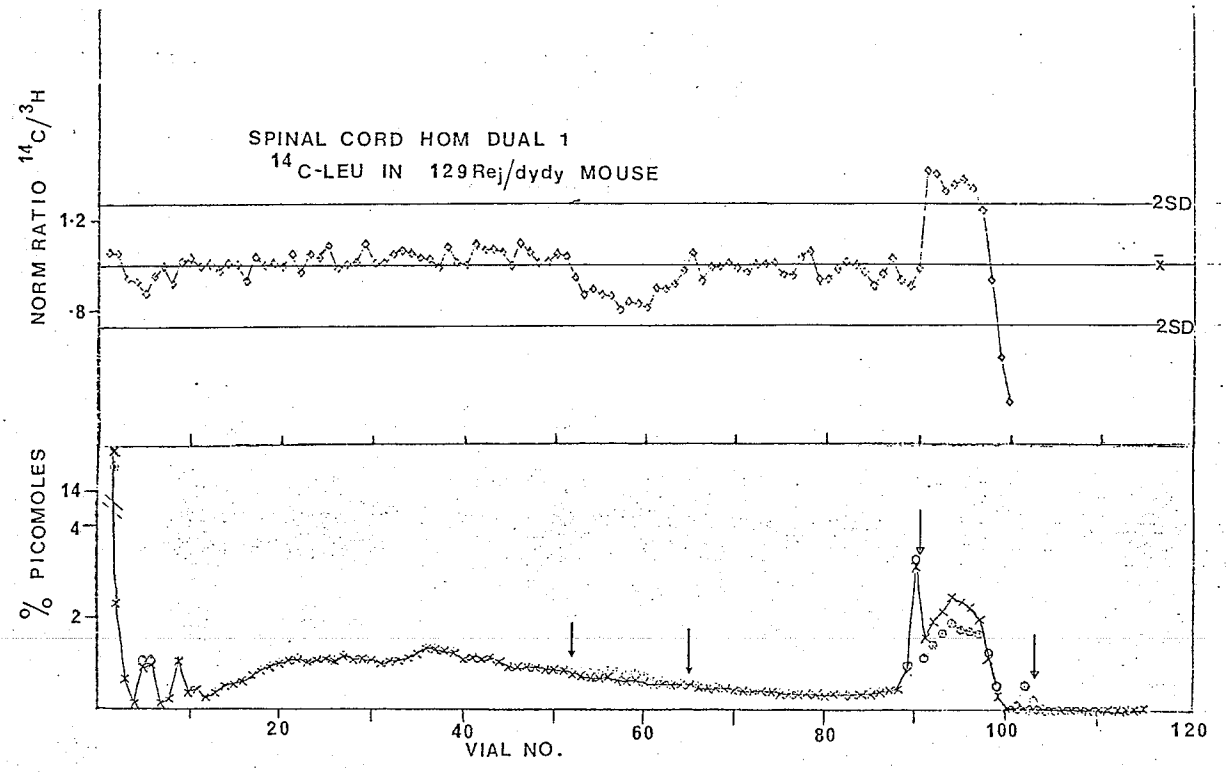

SPINAL CORD HOM DUAL 1
^{14}C-LEU IN 129Rej/dydy MOUSE
NORM RATIO $^{14}C/^{3}H$
1·2
·8
2SD
2SD
% PICOMOLES
14
4
2
20
40
60
80
100
120
VIAL NO.

FIGURE 25

(110)

Figure 26. Incorporation of leucine into the SDS-soluble spinal cord homogenate fraction(spinal roots extirpated) as in Figure 25.

Arrows indicate region expressing an abnormality in the % picomole plot. Data was recalculated for % picomole incorporation minus the stacking gel and tracking dye regions.

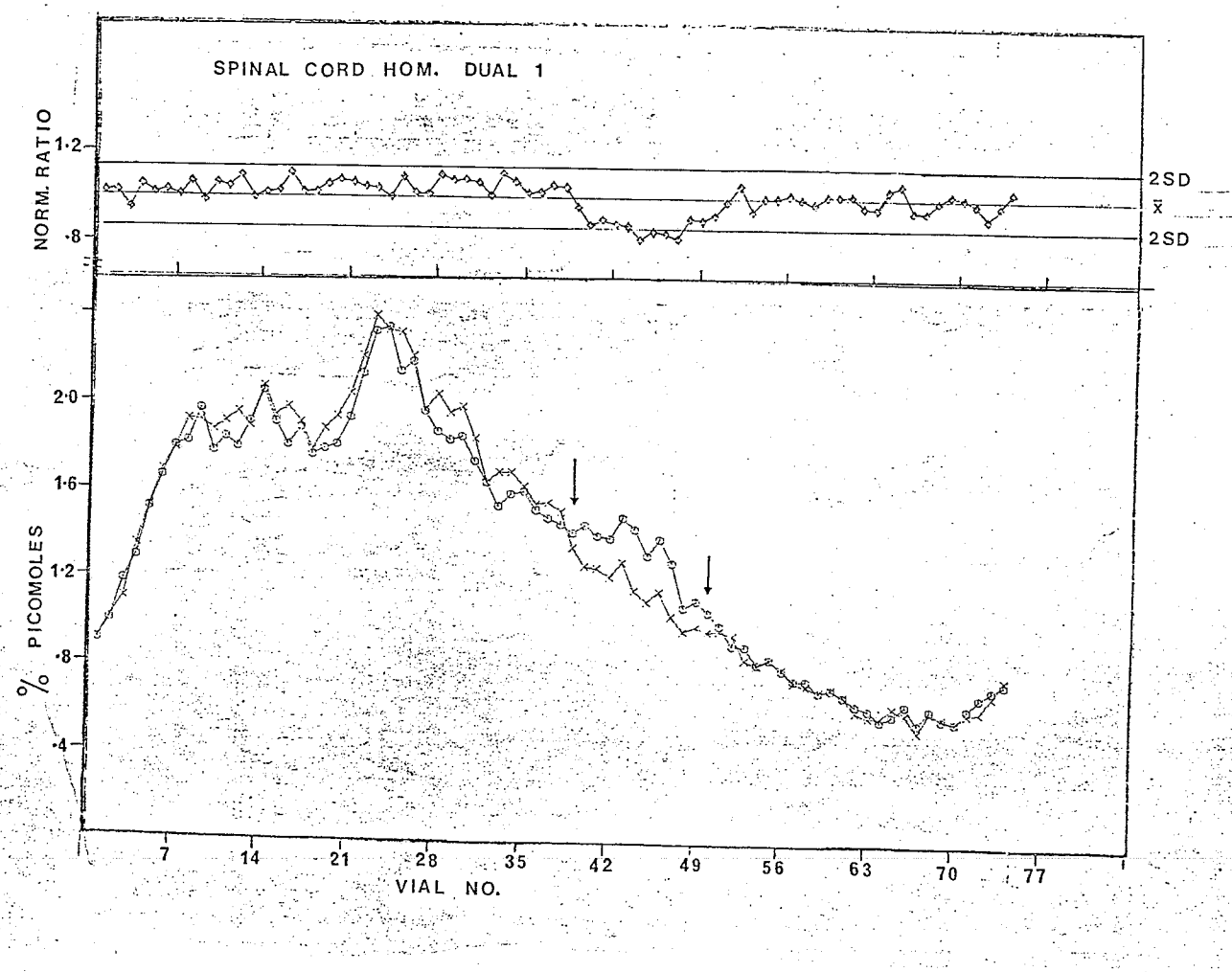

SPINAL CORD HOM. DUAL 1
NORM. RATIO
1·2
·8
2SD
x̄
2SD
2·0
1·6
1·2
·8
·4
% PICOMOLES
7
14
21
28
35
42
49
56
63
70
77
VIAL NO.

FIGURE 26

Figure 27. Semi-logarithmic plot with Log Molecular Weight($x10^{-3}$) versus the Relative Mobility(RM) of molecular weight standards.

a) 13% SDS disc gels(Type II) cast to a height of 22cm in 0.6 x 25.5 cm tubes. ●——●

b) 13% SDS disc gels(Type II) cast to a height of 12cm in 0.6 x 16 cm tubes. ▲——▲

c) 4.5% SDS-EDTA gels(Type III) cast to a height of 12cm in 0.6 x 13 cm tubes. ○——○

Relative mobilities were calculated using the trailing edge of the tracking dye front. The protein standards used were: red blood cell membranes(600ugm) prepared by the method of Dodge(144), phosphorylase b, bovine serum albumin, ovalbumin, pepsin, and α-chymotrypsin.

Molecular weight markers(10ugm) in a maximal volume of 100 l were mixed with 20 l of 40%(w/v) sucrose and 5-10 l of tracking dye, 0.05% bromophenol blue(a & b) or pyronin Y (100mgm/ml)(c) respectively.

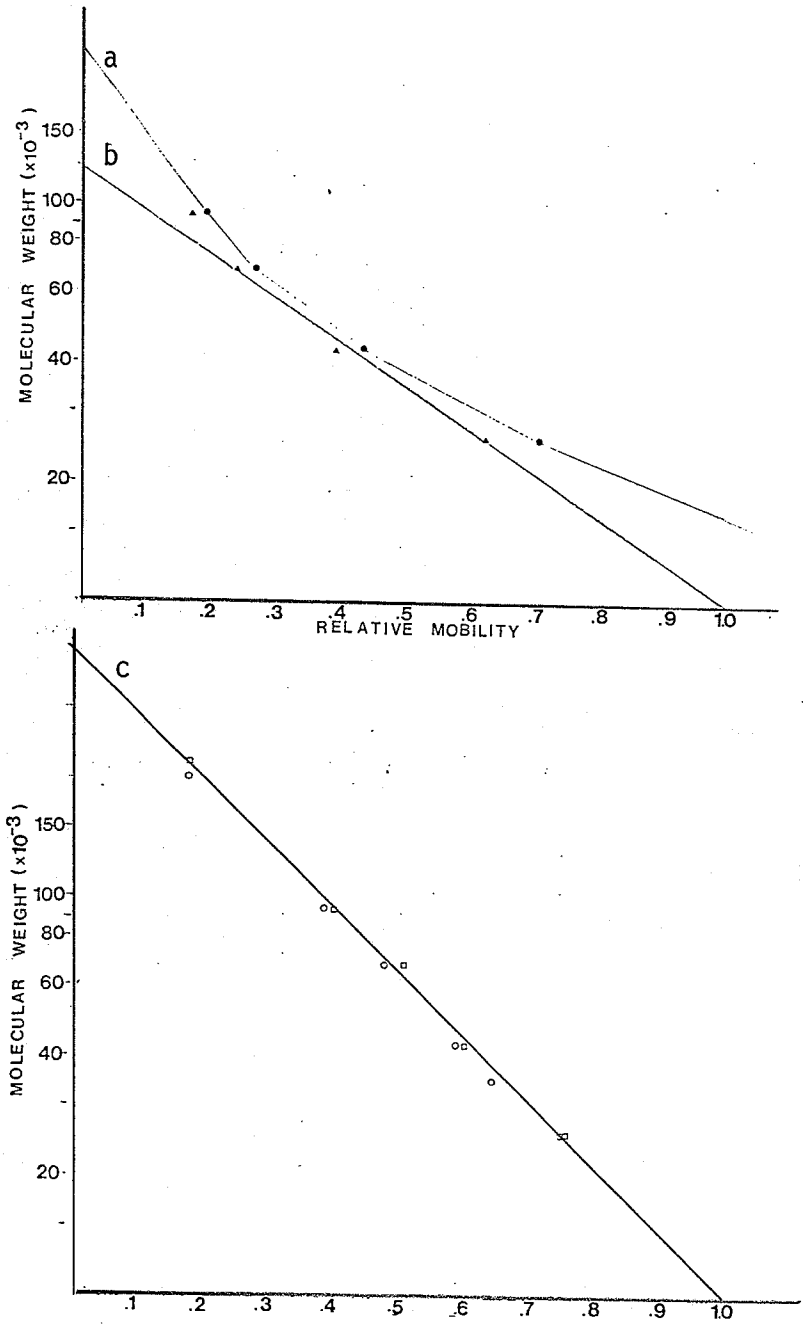

a
b
c
MOLECULAR WEIGHT (x10^{-3})
150
100
80
60
40
20
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
RELATIVE MOBILITY

FIGURE 27

repeated leaving out the data from the lipid fraction. In this revised form the normalized picomole plot and normalized ratio, show that the deviant ratios do exceed the two standard deviation limits and this can thus be classified as a significant abnormality within this fraction (Figure 26).

To check on the reproducibility of this abnormality a study of age-matched(47-day-old) normal and dystrophic mice spinal cord polypeptides was carried out(Figure 28). The abnormality was no longer detectable. A further study was undertaken to test the reversibility of the ratio's direction.

In the reverse labelling experiments on 66-day-old age-matched mice L-($U^{14}C$)leucine was injected into the normal mouse. In this spinal cord homogenate the abnormality seen in the 29-day-old mice was not seen (Figure 29). In the original analysis there seemed to be a large abnormality with the reverse direction to that found in the first experiment(Figure 30). However, on checking the recoveries of isotope applied to the gel the ^{14}C recovery was 228% while the ^{3}H recovery was 80%. Since the samples had been dialyzed to remove free leucine, contamination of the sample with ^{14}C isotope after initial preparation was the likely cause of this gross deviation in the normalized ratio. To test for(Figure 31) isotopic leucine contamination 'free' leucine isotopes both L-(4,5^{3}H)leucine and L-($U^{14}C$)leucine were electrophoresed in the discontinuous gel system on both 10% and 13% polyacrylamide gels. The free(unincorporated) leucine was found to migrate to an area consistent with the regions of abnormal $^{14}C/^{3}H$ ratios. This gave strong support to the idea of contamination in the dual labelling experiment of 66-day-old normal and dystrophic mice. Indeed, on repeating the fractionation of this same spinal cord homogenate the abnormality disappeared. The conclusion was the source of the apparent abnormality was free leucine contamination of the sample.

These results did not help in determining whether or not the abnormality in the 29-day-old dystrophic mouse was real. The ratios in this spinal cord homogenate did deviate from the mean by more than two standard deviations. The abnormal region also coincided with protein peaks in the profile of spinal cord polypeptides(39-72 days of age) separated

Figure 28. Incorporation of leucine into the spinal cord homogenate fraction(spinal roots extirpated) of age-matched(47-day-old) normal and dystrophic mice.

The normal mouse was injected with 1mCi L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (o); the dystrophic mouse with 250uCi L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation was carried out on 13% SDS disc gel(Type II), migration left to right. Gel was sliced in 2-mm slices.

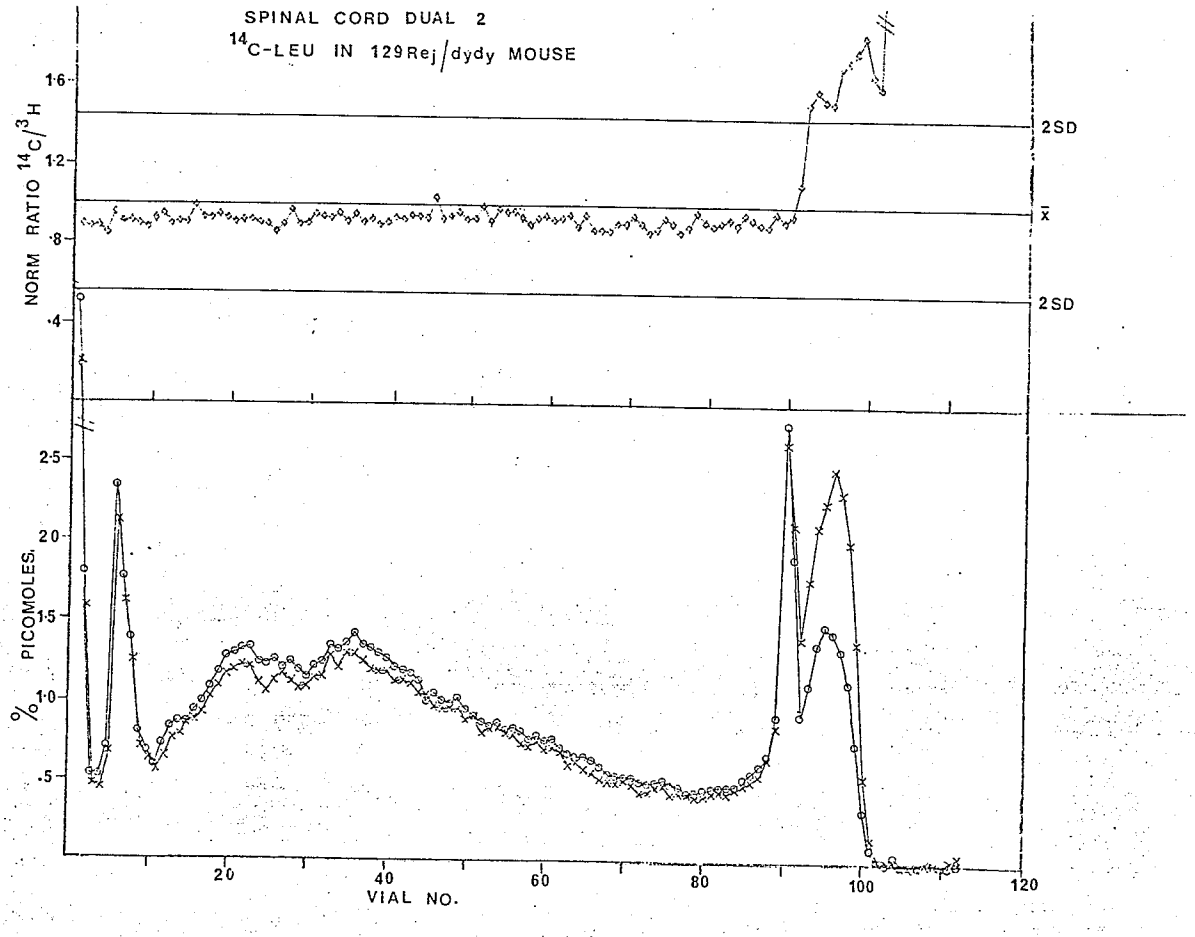

SPINAL CORD DUAL 2
^{14}C-LEU IN 129Rej/dydy MOUSE
NORM RATIO $^{14}C/^{3}H$
1·6
1·2
·8
·4
2SD
$\bar{x}$
2SD
PICOMOLES
2·5
2·0
1·5
1·0
·5
20
40
60
80
100
120
VIAL NO.

FIGURE 28

Figure 29. Incorporation of leucine into the SDS-soluble spinal cord homogenate fraction(spinal roots extirpated) of age-matched (66-day-old) normal and dystrophic mice.

The normal mouse was injected with 250uCi L-($U^{14}C$)leucine (spec. act. 330mCi/mmol) (x); the dystrophic with 1mCi L-($U^{14}C$) leucine(spec. act. 1.23Ci/mmol) (o).

Separation was on 13% SDS disc gel(Type II), migration left to right. Gel was sliced in 2-mm slices.

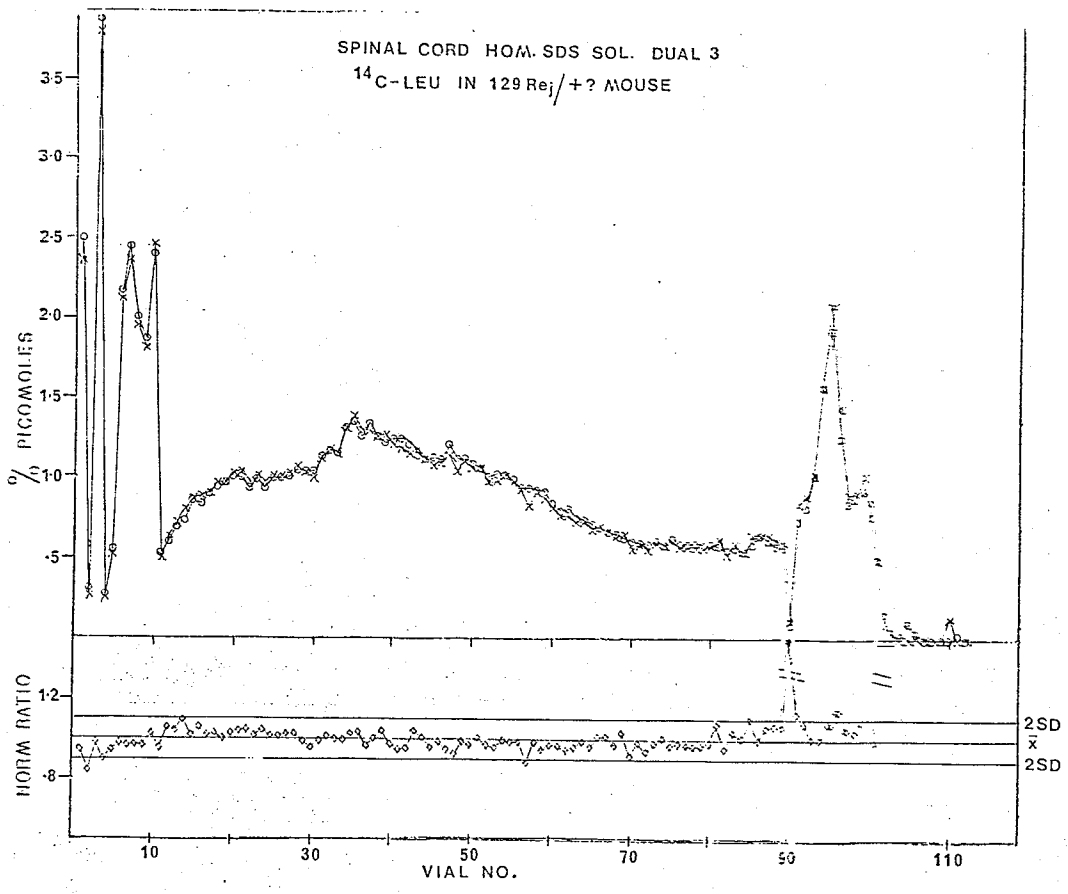

SPINAL CORD HOM. SDS SOL. DUAL 3
^{14}C-LEU IN 129 Rej/+? MOUSE
% PICOMOLES
3·5
3·0
2·5
2·0
1·5
1·0
·5
NORM RATIO
1·2
·8
2SD
x̄
2SD
10
30
50
70
90
110
VIAL NO.

FIGURE 29

Figure 30. Incorporation of leucine into the SDS-soluble spinal cord homogenate fraction(spinal roots extirpated) of age-matched (66-day-old) normal and dystrophic mice.

Separation on 13% SDS disc gel(Type II), migration left to right. Gel fractionated in 2-mm slices. Arrows indicate a 'defect' detected in this fraction due to contamination of the sample(see RESULTS AND DISCUSSION) The injections were as in Figure 29.

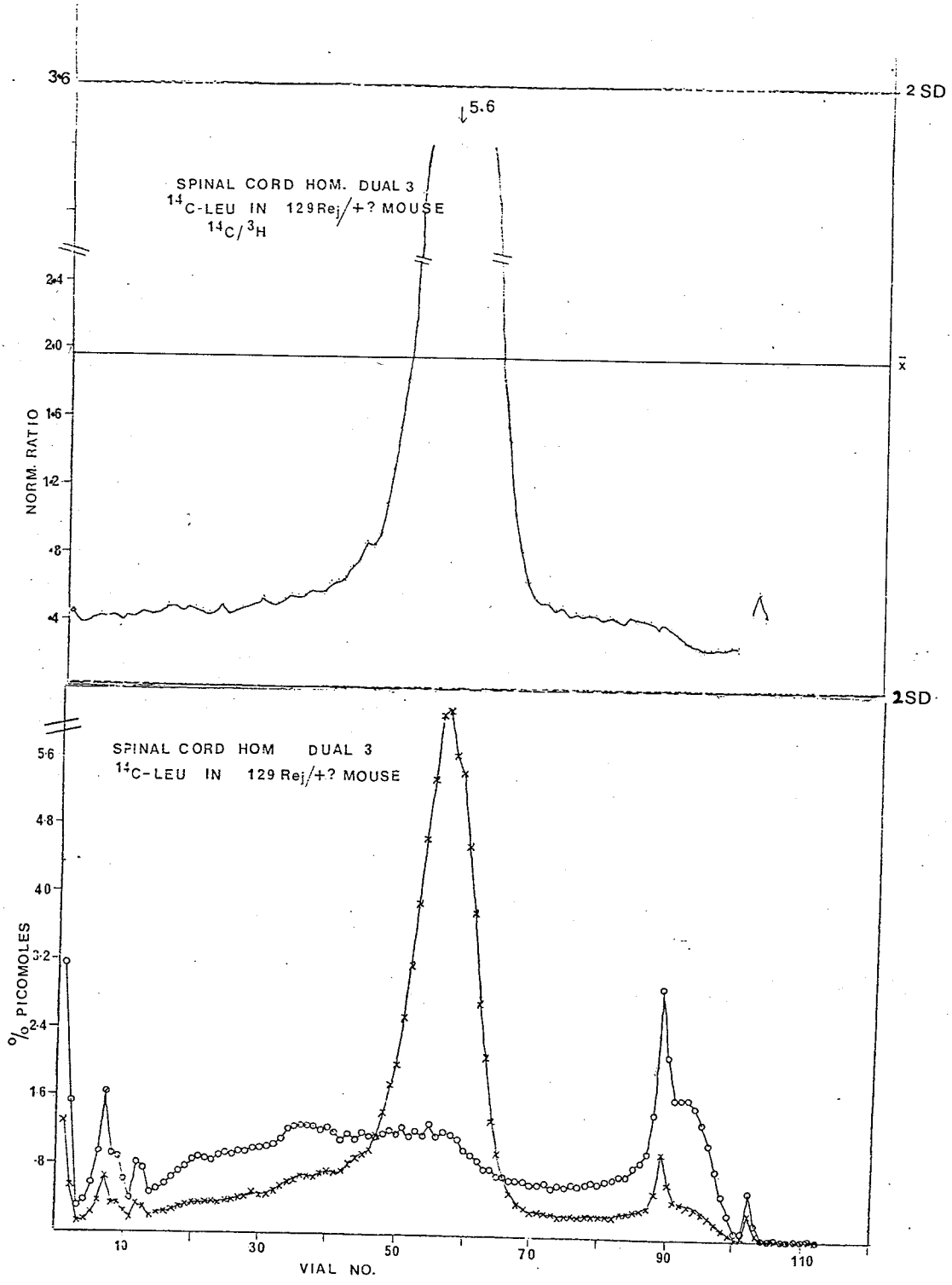

3·6
2 SD
5.6
SPINAL CORD HOM. DUAL 3
14C-LEU IN 129Rej/+? MOUSE
14C/3H
2·4
2·0
x̄
1·6
1·2
·8
·4
NORM. RATIO
2SD
SPINAL CORD HOM DUAL 3
14C-LEU IN 129 Rej/+? MOUSE
5·6
4·8
4·0
3·2
2·4
1·6
·8
% PICOMOLES
10
30
50
70
90
110
VIAL NO.

FIGURE 30

Figure 31. 'Free' leucine electrophoresed on 13% SDS disc gel(Type II).

L-(4,5^{3}H)leucine represented by (o); L-(U^{14}C)leucine represented by (x). For recoveries see APPENDIX.

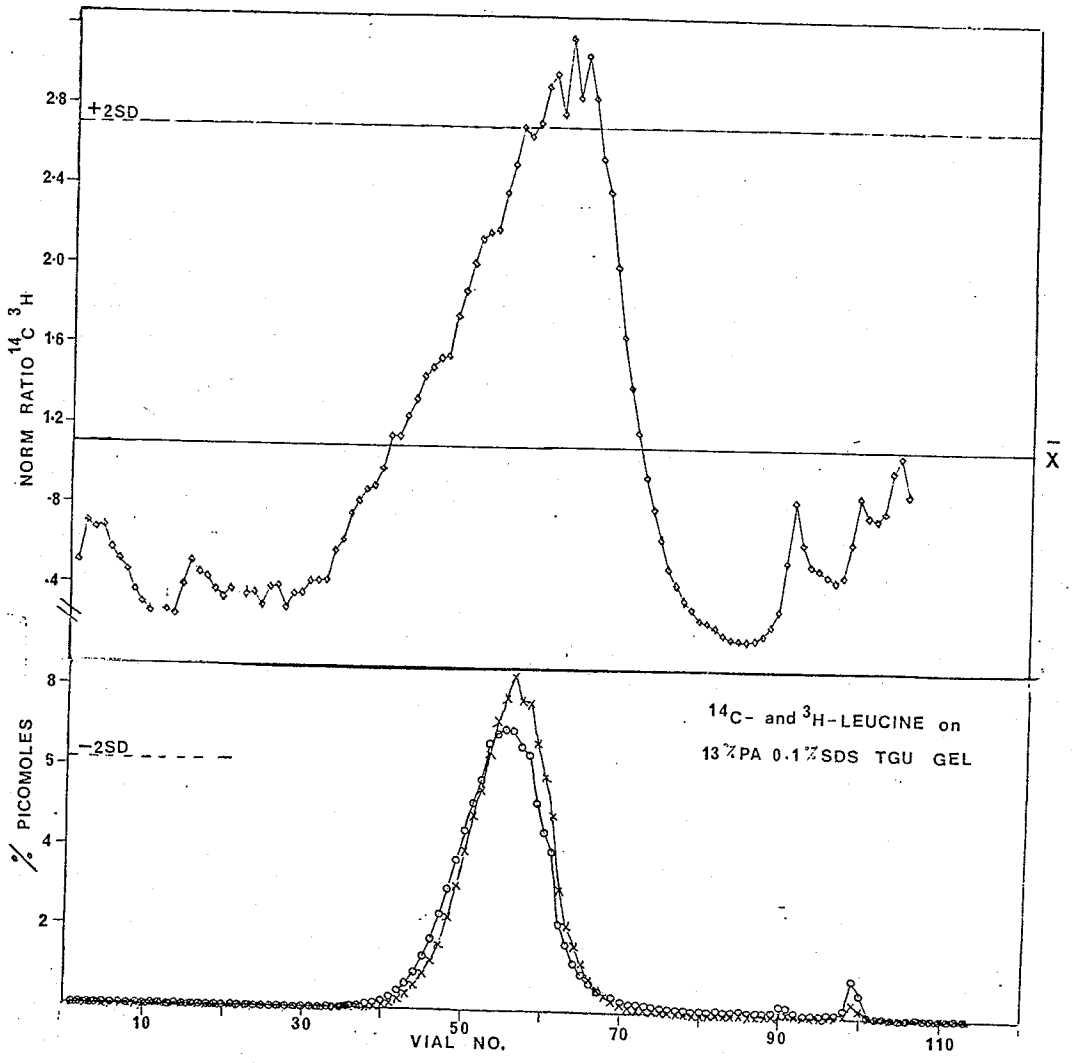

+2SD
2·8
2·4
2·0
1·6
1·2
·8
·4
NORM RATIO ^{14}C ^{3}H
$\bar{X}$
−2SD
8
5
4
2
% PICOMOLES
^{14}C- and ^{3}H-LEUCINE on
13%PA 0.1%SDS TGU GEL
10
30
50
70
90
110
VIAL NO.

FIGURE 31

on SDS-PAGE. However, the abnormality was not seen in other animal pairs and on reversal of the labels the deviant ratios did not change direction, in fact they were not seen at all. From the dual labelling experiments no clear conclusion could be drawn from the study of spinal cord polypeptides on SDS-PAGE of 29, 47 and 66-day-old normal and dystrophic mice pairs. There are several possible explanations for the above inconsistency of results in these homogenate fractions:

1. the genotype of the animals
2. 'turned on genes'
3. differences in animal maturation
4. animal starvation.

1. The problem of the homozygous or heterozygous nature of the control littermate in this strain of mouse 129Rej was outlined earlier. If the control animal had partial expression of the dystrophic gene(not otherwise expressed in enzyme or histological studies) a large difference in protein synthesis between it and the homozygous dystrophic animal may not be present. It is possible that the 29-day-old mice were each homozygous for their respective alleles and thus an abnormality was detected in this animal pair, whereas, in the reversed label experiment the control may have been heterozygous. Further experiments with 3-4 week old mice of strain 129Rej could be undertaken. If strain C57BL/6J $dy^{2J}dy^{2J}$ were used earlier studies could be undertaken and the problem of the heterozygous normal animal would be removed. This is because a homozygous normal animal could be selected as a control mouse genotype and because the C57BL/6J$dy^{2J}dy^{2J}$ mice can breed and produce all dystrophic offspring earlier abnormalities in dystrophy could be studied.

2. The abnormality may be detectable only when the gene is 'turned on' and this maybe responsible for the difference in results with mice of 29-days-of age from those of 47 and 66 days of age. The human beta, gamma and delta chains of hemoglobin and their rates of synthesis in the foetal and adult states are an example of this, similarly, the embryonic chain of hemoglobin is produced only within the first three months of gestation (19). Because the dual labelling technique measures the amount of label incorporated into polypeptides, if the protein is not being synthesized or there is a relatively slow turnover of the protein little label may be

incorporated into the polypeptide sequence during the time of the experiment. This may be especially true with a three hour labelling time in animals 4-8 weeks old. Platzer and Powell have found dystrophic muscle pathology in embryos of strain 129Rej/dydy at 19 days of age (65). Dystrophic factors detectable using our separation techniques may not be in evidence in older animals.

In murine dystrophy one of the major abnormalities is the absence of normal myelin sheaths (14,16,29,30). If the dual labelling technique is used to detect these abnormalities the animal must be undergoing myelinogenesis. According to Druse and coworkers, in the rat, where myelinogenesis is lifelong, there is still a very active synthesis of myelin components at least until 35 days of age (136). If the mice in our experiments are not undergoing active myelination little label would be incorporated into the myelin protein components and we would not be able to detect any differences in the myelin production between normal and dystrophic mice. The 29-day-old mice were likely producing myelin while the older 47 and 66-day-old mice, if producing myelin, may have had greatly reduced rates of myelinogenesis. The similar pattern of isotope incorporation into the spinal cord proteins of all three sets of mice suggests that all mice were synthesizing similar protein components(see Figures 25,28 & 29).

A second consideration to be faced here is that of the turnover times of the myelin proteins or other proteins of the nervous system. Fischer and Morell showed that myelin basic and proteolipid proteins in the mouse brain were relatively stable having a half life of 100 days (137),a result qualitatively supported by other workers (138). These experiments suggest that earlier and perhaps longer incubation periods with the label may be required to label nervous system proteins like myelin.

3. Age differences in the animals may lead to the comparison of very different protein components within the nervous system. This may be seen from studies on the maturation of nervous tissues resulting in very different profiles of lipid and protein moieties. For example, in the study of the rat optic nerve the high molecular weight proteins were present in 9-day-old rats, myelin basic proteins in 10-day-old rats and the proteolipid protein U in 12-day-old rats (139). The studying of different ages of

animals may help to elaborate on the function of the various components in the nervous system but it may also add to the confusion of what seem to be inconsistent results. These maturation differences mentioned above may be the source of the inconsistent results seen in the spinal cord homogenates in the dual labelling of mice aged 29,47 and 66 days of age. The abnormality occurring only in the 29-day-old dystrophic mouse was in a molecular weight region of the gel which was approximately that of the proteolipid protein of myelin. This may be a true defect, that is, one which is due to the muscular dystrophy or at least associated with a nervous system abnormality. The older animals may not show this abnormality because myelin is not being synthesized.

4. The dystrophic mice, strain 129Rej/dydy have a hind limb paralysis, which at an early age, may affect the effective rate of protein synthesis since this paralysis can lead to their starvation. Starvation may result in an amino acid imbalance which has been shown to alter transport of amino acids into the brain and result in an overall decrease in protein synthesis (140). This may be an even more specific decrease in myelin synthesis (147). Since hind limb paralysis in this strain of dystrophic mouse is not that apparent until 3 weeks of age, the older animals may be suffering more from the affects of starvation and may have altered protein synthesis occurring in the nervous tissues. Therefore one might detect abnormalities related to starvation in older mice and not in younger mice.

In summary then the inconsistency in the results from the homogenates of spinal cords in normal and dystrophic mice using the dual labelling procedure could have several origins. The labelling time of three hours may have been insufficient to label the myelin and the Schwann cell components of the experimental mice; the age and physical state of the mice may have caused these results because myelinogenesis was not going on. One could not rule out the possibility that the abnormality was simply the result of free leucine contamination. However, care was taken to avoid this by extensive dialysis prior to electrophoresis of the sample. Whether the abnormality seen in the 29-day-old dystrophic mouse was due to an abnormality in myelin proteins, or associated with the

the decrease in Schwann cell number or a result of abnormalities in oligodendroglial cells which give rise to the SC or another unknown factor involved in muscular dystrophy has not been determined.

ii) Spinal cord proteins separated by isoelectricfocusing

Isoelectricfocusing was also carried out on the spinal cord homogenates(lubrol solubilized fractions) in 5.1% polyacrylamide gels with an ampholyte gradient from pH 3.5-10.0 according to Wrigley (126).

The dual labelling experiment on 29-day-old mice indicated an apparent increased synthesis of proteins,by the dystrophic mouse, focusing in the acidic portion of the gel; this resulted in a deviant ratio increased from unity(Figure 32). The number of points lying outside this interval throughout the entire gel is precisely 5%. Secondly the result was not reproducible or reversible in direction in the later preparations of spinal cord homogenates. The repetition of this experiment on other similarly aged mice pairs, normal and dystrophic, and the reverse labelling experiment with the L-(U^{14}C)leucine into the 129Rej/+? mouse, of the same age, would be more precise controls, as discussed in the last section.

The next dual labelling experiments on 47 and 66-day-old mice gave indications that abnormalities existed between normal and dystrophic spinal cord proteins which were lubrol soluble. An abnormality(deviant ratio outside 2 standard deviations) was apparent in the acidic region of the isoelectricfocusing gel and one was apparent in the basic region of the gel(Figures 33 & 34). The normalized ratios showed that a decrease in the quantity of proteins synthesized occurring in the dystrophic mice. The gels were not sliced in exactly the same manner(2mm and 1mm slices) and so the comparison of peak positions in Figures 33 and 34 is approximate but seemingly more than coincidental. With ^{14}C-leucine injected into the dystrophic mouse, fractions #9 and #63(acidic and basic respectively) showed the dystrophic mouse incorporating a lesser amount of labelled precursor compared to the normal mouse. In a reversibility test with the ^{14}C-leucine injected into the normal mouse fractions, #21 and #119 (acidic and basic respectively), the dystrophic mouse again had a lesser incorporation of the labelled precursor resulting in an increase in the normalized ratio(^{14}C/^{3}H) from unity. The reversal of the direction of labelling gives support to the hypothesis that these 'defects' are

Figure 32. Incorporation of leucine into the spinal cord homogenate (spinal roots extirpated) of age-matched(29-day-old) normal and dystrophic mice.

The normal mouse was injected with 1mCi of L-(4,5^{3}H)leucine(spec. act. 1.29Ci/mmol) (o); the dystrophic mouse with 250uCi of L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (x).

Separation was on 5.1% polyacrylamide isoelectricfocusing gel with pH gradient from 3.5-10.0(left to right respectively). Gel was fractionated in 2-mm slices. Only the lubrol solubilized fraction was used for sample electrophoresis.

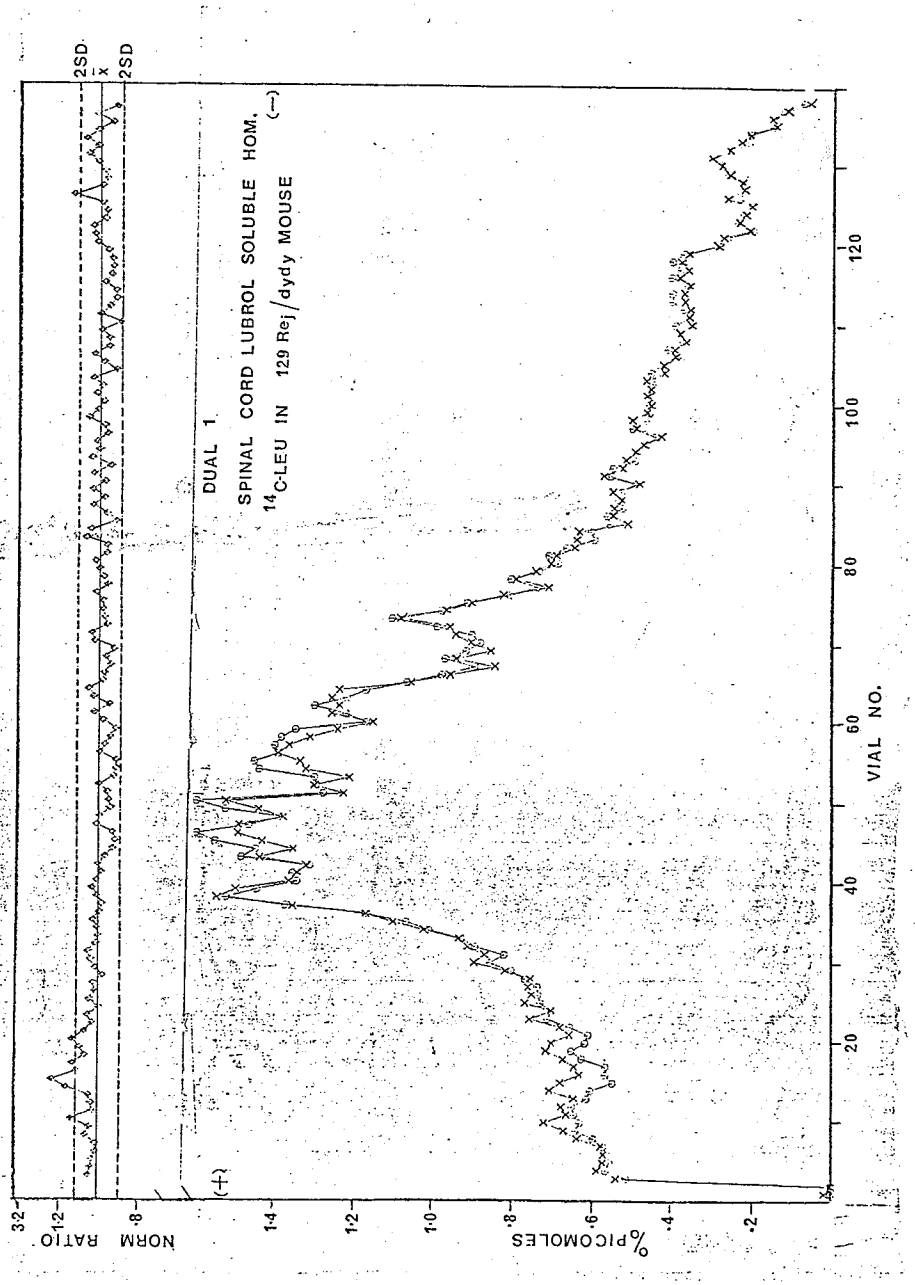

2SD
$\bar{x}$
2SD
DUAL 1
SPINAL CORD LUBROL SOLUBLE HOM. (—)
^{14}C-LEU IN 129 Rej/dydy MOUSE
(+)
NORM RATIO
%PICOMOLES
VIAL NO.
32
1·2
·8
1·4
1·2
1·0
·8
·6
·4
·2
20
40
60
80
100
120

FIGURE 32

Figure 33. Incorporation of leucine into the spinal cord homogenate (spinal roots extirpated) of age-matched(47-day-old) normal and dystrophic mice.

The normal mouse was injected with 1mCi of L-(4,5^{3}H)leucine (spec. act. 1.29Ci/mmol) (⊙); the dystrophic mouse with 250uCi of L-(U^{14}C)leucine(spec. act. 324mCi/mmol) (⊗).

Separation was on 5.1% polyacrylamide isoelectricfocusing gel(Type IV), with a pH gradient from 3.5-10.0(left to right respectively). Gel was fractionated in 2-mm slices. Only the lubrol solubilized fraction of the spinal cord homogenate was electrophoresed. Arrows indicate regions or fractions in which the dystrophic(x) samples show decreased leucine incorporation compared to normal(↓)(% picomole plot).

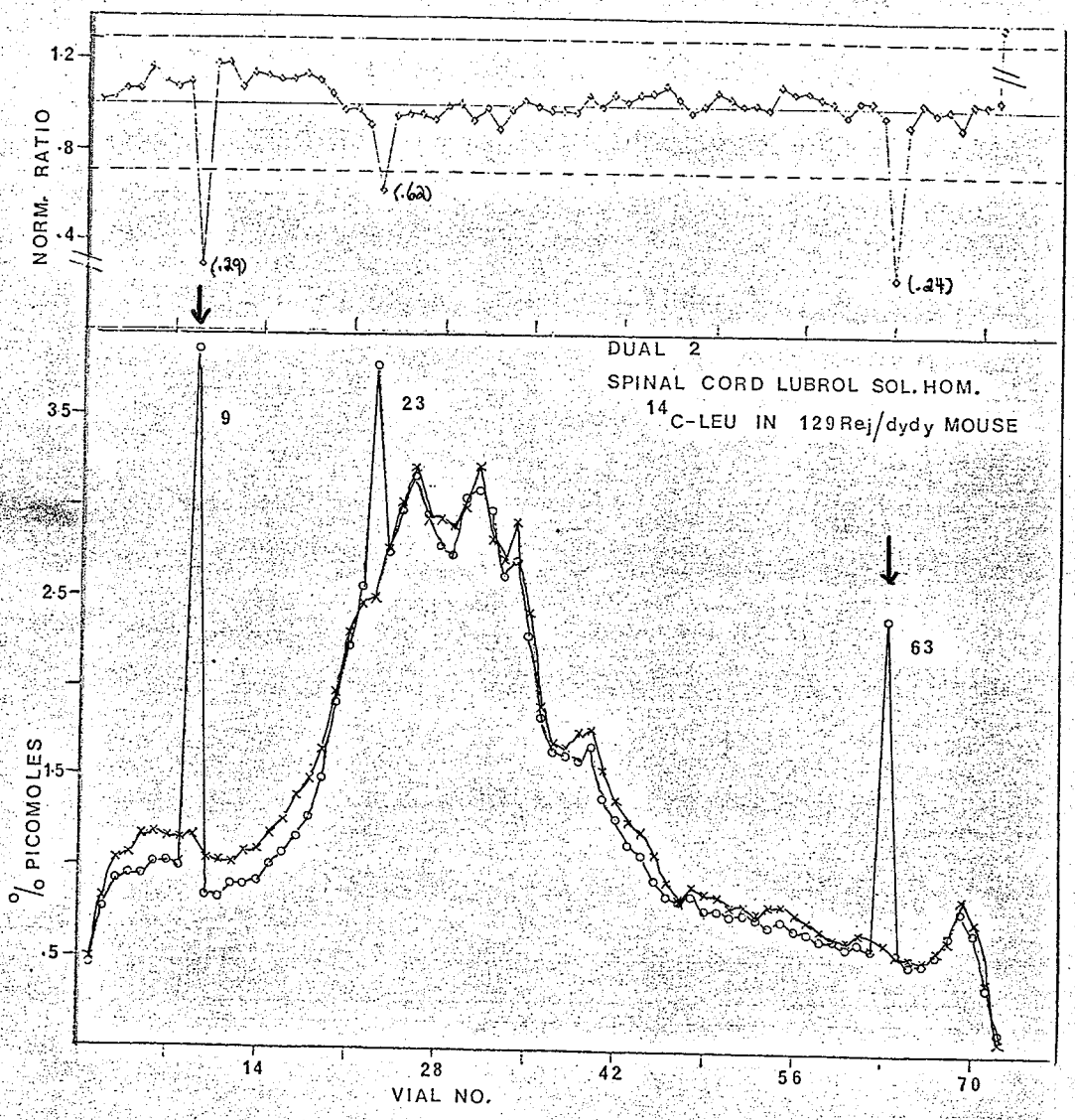

NORM. RATIO
1·2
·8
·4
(.39)
(.62)
(.24)
DUAL 2
SPINAL CORD LUBROL SOL. HOM.
14C-LEU IN 129Rej/dydy MOUSE
% PICOMOLES
3·5
2·5
1·5
·5
9
23
63
14
28
42
56
70
VIAL NO.

FIGURE 33

Figure 34. Incorporation of leucine into the spinal cord homogenate (spinal roots extirpated) of age-matched(66-day-old) normal and dystrophic mice.

The normal animal was injected with 250uCi of L-(U^{14}C)leucine(spec. act. 330mCi/mmol) (x); the dystrophic animal with 1mCi of L-(4,5^{3}H)leucine(spec. act. 1.23Ci/mmol) (o).

Separation was on 5.1% polyacrylamide isoelectricfocusing gel(Type IV), with a pH gradient from 3.5-10.0(left to right respectively). Gel was fractionated in 1-mm slices. Only the lubrol solubilized fraction of the spinal cord homogenate was electrophoresed. Arrows indicate fractions in which the dystrophic (↓) samples show decreased leucine incorporation (% picomole plot). The normalized ratio shows a positive deviation from 'unity' when the ^{14}C is in the normal animal.

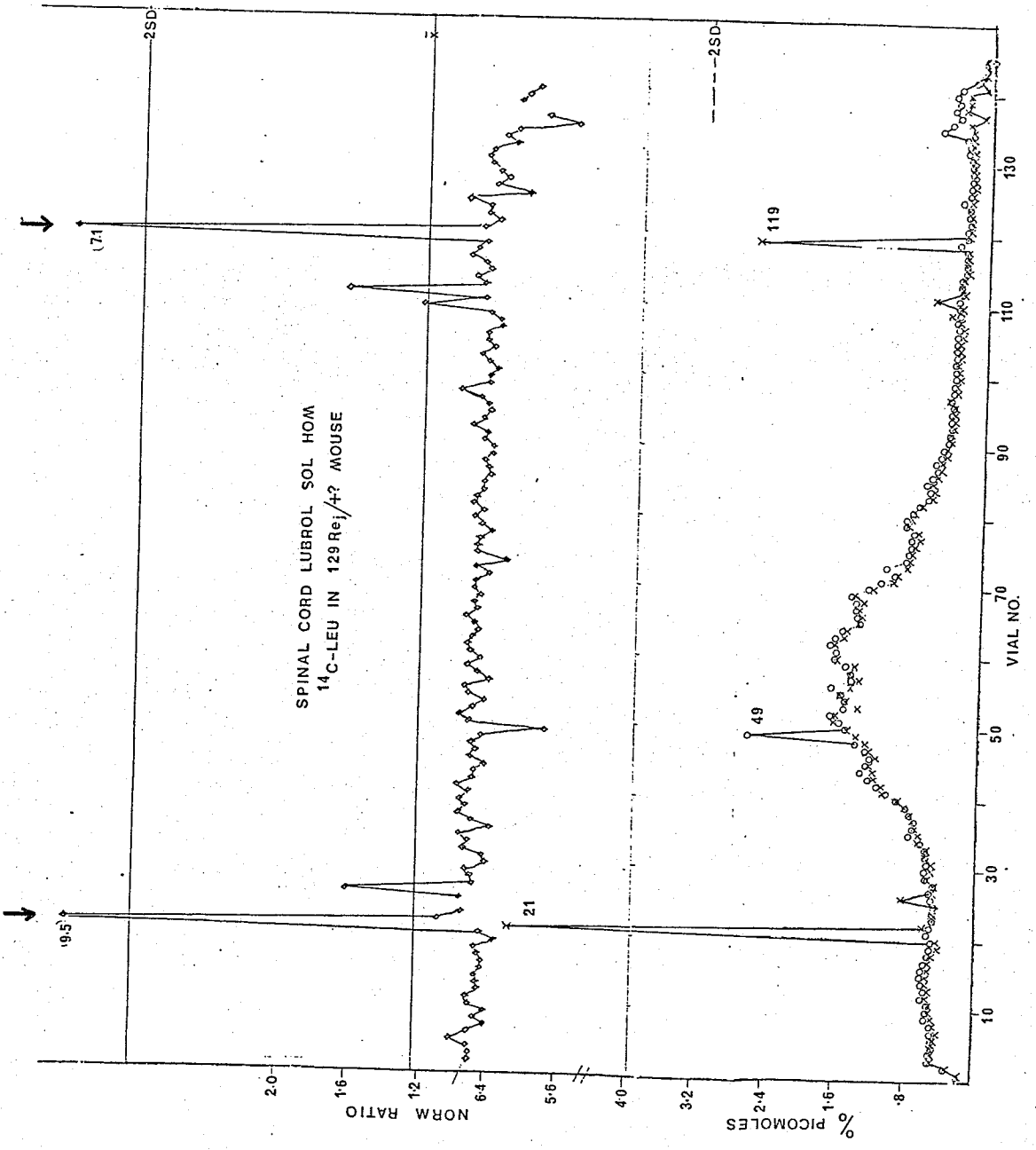

SPINAL CORD LUBROL SOL HOM
14C-LEU IN 129 Rej/+? MOUSE
2SD
2SD
NORM RATIO
% PICOMOLES
VIAL NO.
19.5
71
21
49
119

FIGURE 34

significant. These abnormalities do not appear in the younger 29-day-old dystrophic mouse. It is possible that because the animals are suffering from a much more severe hind limb paralysis than the younger animals the abnormalities here may be due to the physical state of the mice.

An abnormality also appeared in each experiment, gel fractions #23 (Figure 33) and #46 (Figure 34). This was possibly due to an artifact of some kind, for example, contamination of the vial or a dissectional error coincidently altering similar components of the system. Pooled samples (from more than one pair of animals) may help to eliminate any problem with dissections especially those involved in removing dystrophic mouse spinal roots. No problems were found in the dissection of the spinal cords.

The abnormalities seen in the spinal cord of dystrophic mice on the electrofocusing of proteins which were lubrol solubilized require substantiation in more animal pairs before the 'defects' can be attributed to the muscular dystrophy disease in the mouse.

However, these results do suggest the dual labelling technique as a sensitive tool for use in studying the abnormalities seen in histological studies in these animals. The labelling time may need to be extended (from results with SDS gels) in order to use the technique to its fullest advantage. If tissue proteins showed a high incorporation of the labelled precursor it would be helpful in studying muscular dystrophy in two cases; first, if the tissue is atrophied and second if the diseased animals are at a very early age, both cases resulting in only small amounts of tissue available for study.

2. Spinal Cord, Sciatic Nerve and Spinal Roots Separated by SDS-PAGE

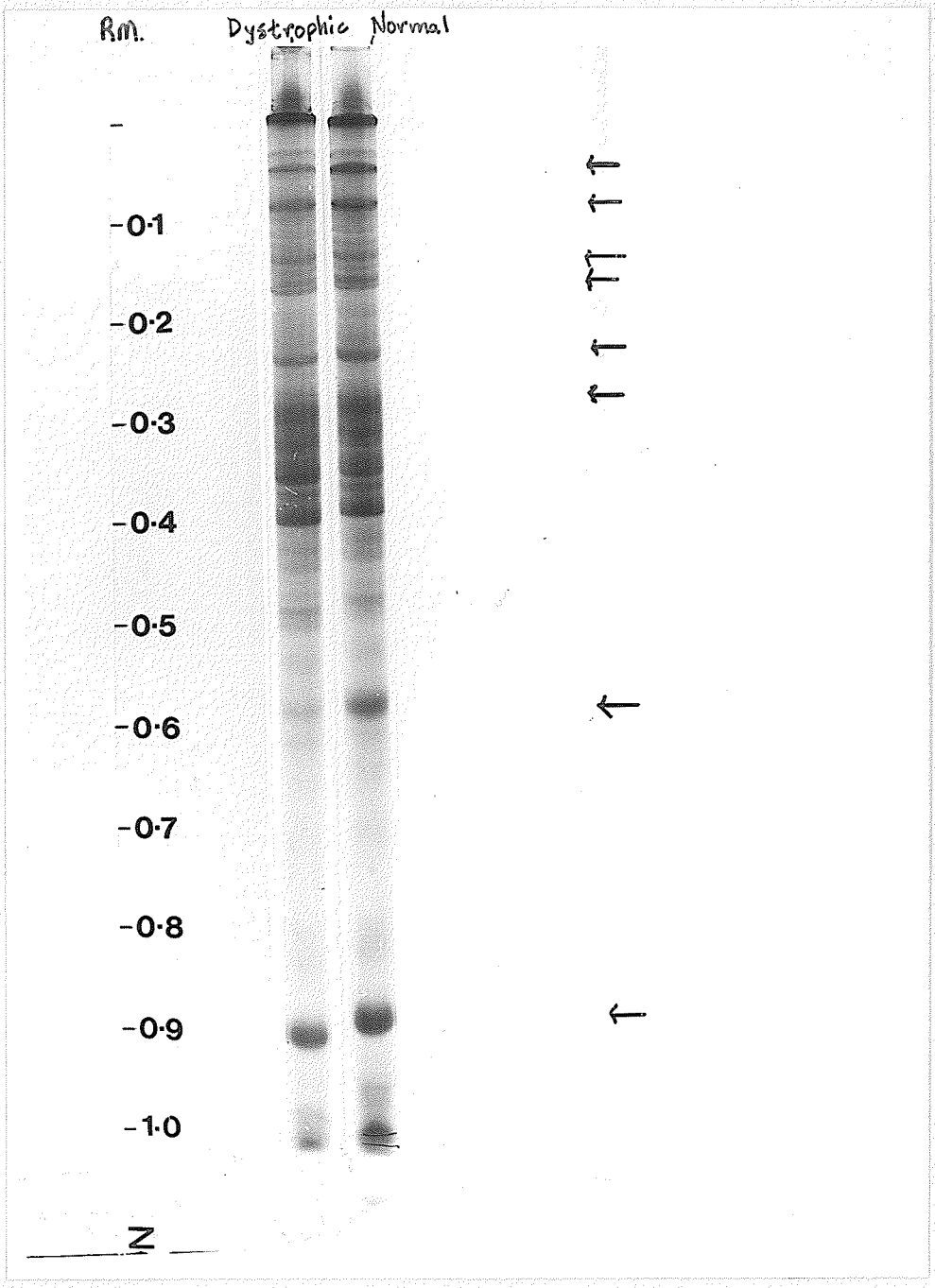
a) Spinal cord polypeptides

i) Separation on 13% SDS disc gels

Though the dual labelling studies indicated possible 'defects' in murine dystrophy spinal cord proteins they did not show the expected gross abnormalities in myelin, Schwann cell or other nervous system proteins as seen by Salafsky and Stirling (85). Because of this a study of unlabelled spinal cord proteins was carried out.

Initial studies on spinal cord homogenates(with spinal roots), after Coomassie blue staining and densitometric scannings at 620nm, showed a reduction in the protein components of the dystrophic mice between the ages of 37 and 72 days. All dystrophic spinal cord preparations were compared to normal(129Rej/+?) spinal cord preparations electrophoresed simultaneously. Spinal cord homogenates, from age-matched(72-day-old) normal and dystrophic mice, electrophoresed on 13% acrylamide SDS disc gels(Type II) showed a reduction in protein quantity in the dystrophic mouse(PICTURE I); the relative mobility of these fractions and their approximate molecular weights follow: RM 0.1-0.2(150,000-94,000); RM 0.2-0.3(94,000-62,000); RM 0.3-0.4(62,000-47,000); RM 0.5-0.6(37,000-31,000) and a RM 0.9(19,000). The molecular weights were determined from molecular weight standards electrophoresed on similar gels(Figure 27); the relative mobilities(RM) were determined using the trailing edge of the bromophenol blue tracking dye region. Several components were reduced in some of the above regions. Some of these may be myelin components but an extraction of myelin proteins was not carried out to specifically test this.

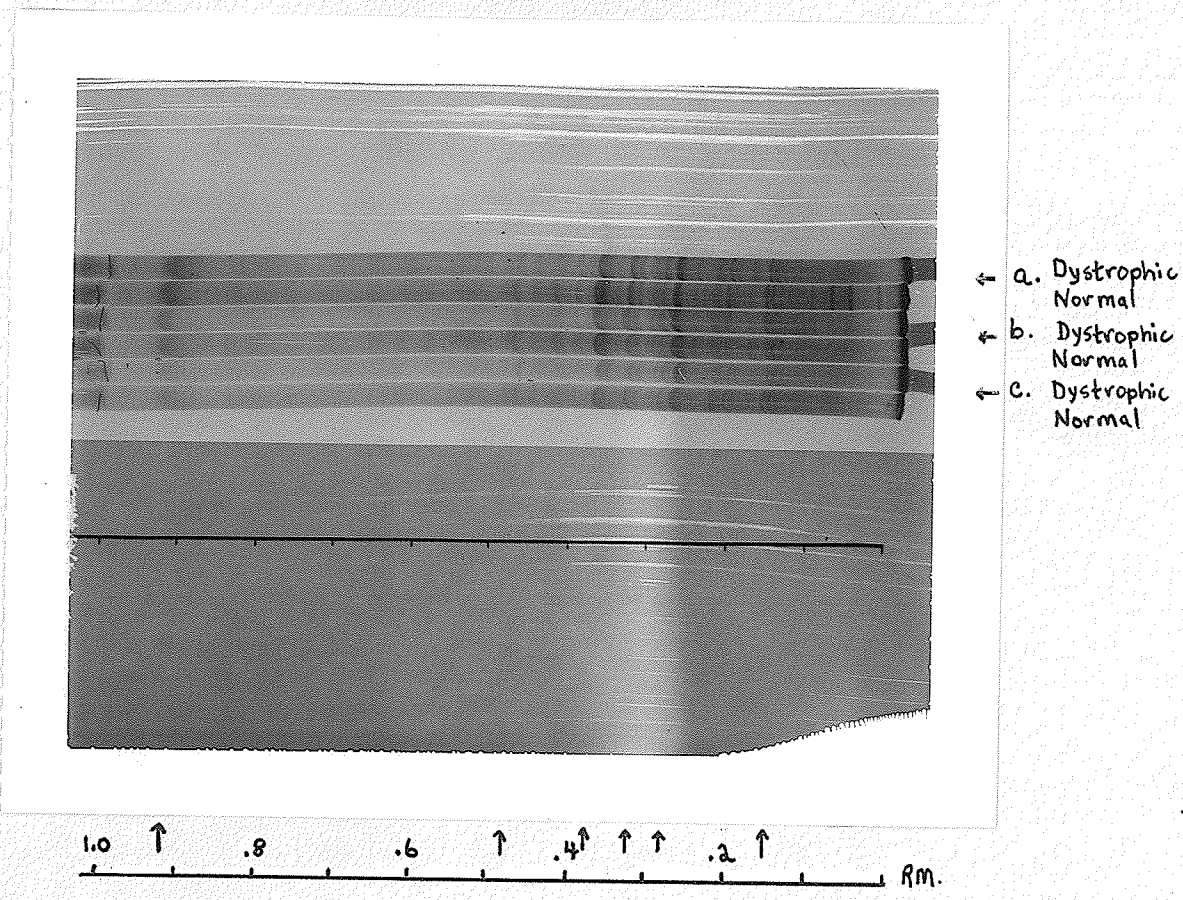
In spinal cord homogenate samples with the spinal roots extirpated areas of reduced protein quantities were still seen in the dystrophic mouse preparations compared to a normal littermate(PICTURE II). The results here indicated that areas of relative mobilities 0.2-0.3, 0.3-0.4, 0.4-0.5 and 0.9 show decreased quantities in dystrophic mouse protein. Spinal cord homogenates with and without spinal roots show abnormalities.



PICTURE I

Spinal cord homogenates(with spinal roots) of 129Rej/dydy and 129Rej/+? mice electrophoresed on 13% SDS disc gels.

Mice were a littermate pair(72-days-old). Arrows indicate areas where dystrophic sample had a lesser amount of protein(Coomassie blue staining material) compared to normal mouse sample. Only the SDS-soluble fractions of the spinal cord homogenates were layered on the gel. 1025ugm protein from the normal mouse and 1021ugm protein from the dystrophic mouse were the original samples.



PICTURE II

Spinal cord homogenates(without spinal roots) of normal and dystrophic mice electrophoresed on 13% SDS disc gels.

The mice were a)46 b)44 and c)39 days of age. Arrows indicate areas where dystrophic sample appearred to have lesser amounts of protein. Samples were originally 200ugm of protein. Only the SDS-soluble fraction of the spinal cord homogenates were layered on the gel.

ii) Separation on 4.5% SDS-EDTA gels(Type III)

Using a gel elctrophoresis system similar to Salafsky and Stirling (85) the spinal cord homogenates were studied. The spinal cord homogenates show a very distinct pattern after Coomassie blue staining and densitometric scans at 620nm(Figure 35). Here was indication that several high and low molecular weight components were reduced in quantity within 39,44 and 46-day-old dystrophic mice compared to their normal littermates. The suggested molecular weights for proteins observed in abnormal amounts were determined from molecular weight standards which were electrophoresed on similar gels(Figure 27). Those proteins decreased in dystrophic mice were 140,000; 82,500; 64,000; and 53,000. These same regions in normal mice had apparent molecular weights of 140,000;, 84,000; 66,000; and 55,000. Experimental error involved in determining the molecular weights of these components by their relative mobilities in comparison to standard marker proteins may mean the differences between dystrophic and normal proteins are not really significant. Another component of 79,000 daltons seemed to be decreased in the dystrophic mouse spinal cord as well. The molecular weights of these components indicate they are not all myelin components. The component of 64,000(129Rej/dydy) and 66,000(129Rej/+?) may be the protein X(Wolfgram protein) of myelin which is known to have a weight of 64,000 in the rat (143). These abnormalities were not singled out so strongly by Salafsky and Stirling (85).

b) Spinal Root and Sciatic Nerve polypeptides

The spinal root and sciatic nerve homogenates in the dual labelling study had not incorporated sufficient label to give significant ratios for analysis. Because of this these tissue preparations were studied by SDS gels. Earlier histological and electrophoretic evidence(16,29,31, 85,143). indicated abnormalities might be found in these tissues.

i) Separation on 13% SDS disc gels

The sciatic nerve(proximal to the sciatic notch and distal to the foot) and the spinal roots(cervical, thoracic & lumbar dorsal and ventral

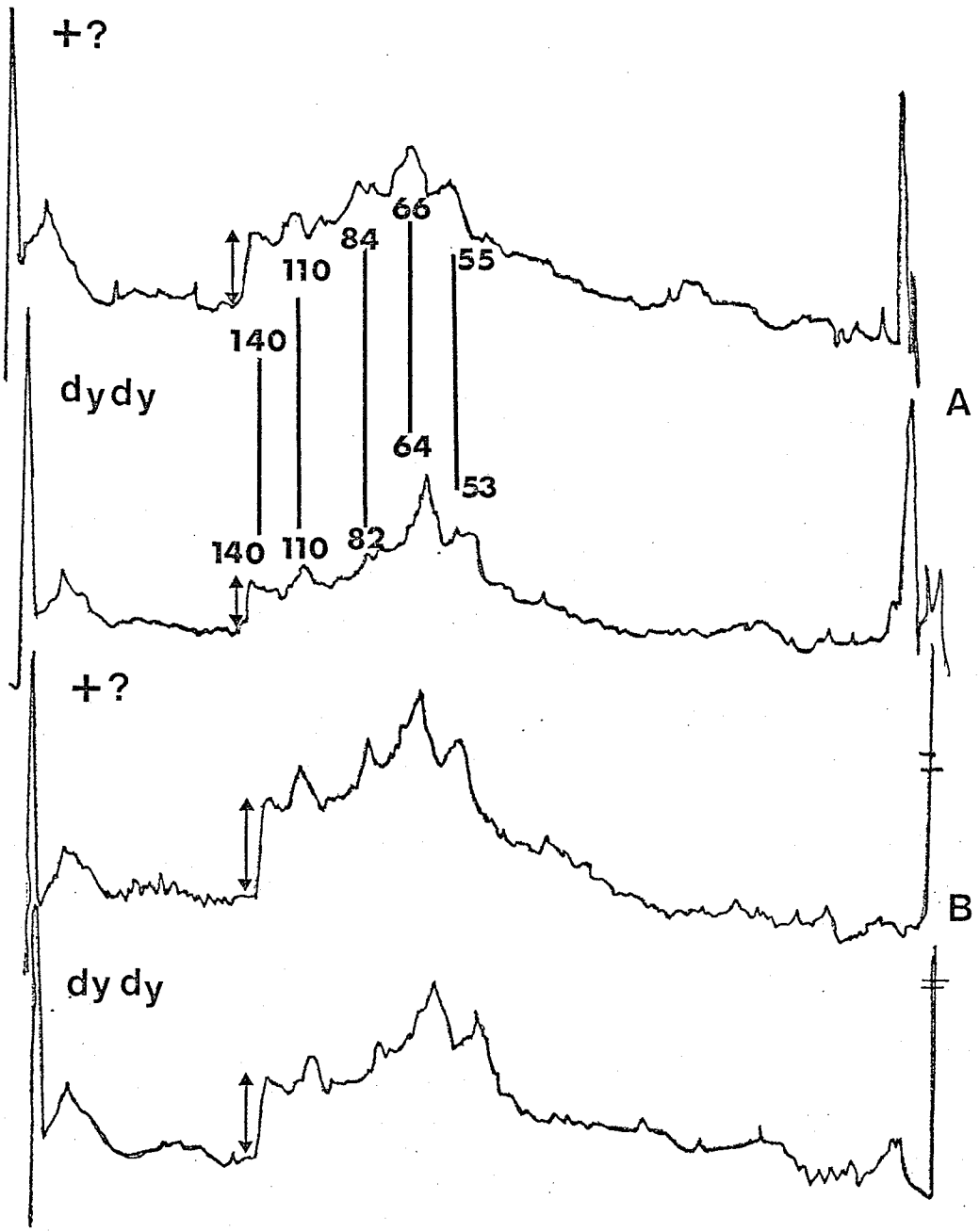


Figure 35. Densitometric scans of spinal root homogenates of 129Rej/+? and 129Rej/dydy mice. (A)44-day-old (B) 39-day-old littermate pairs. Arrows indicate areas of decreased amounts of protein in the dystrophic samples(as determined by Coomassie blue staining-Fairbanks,G. et al,1971). Molecular weight in daltons x 10^{-3} are shown for proteins with either decreases in protein concentration or altered electrophoretic mobility(RM_{av} from three electrophoretic runs).

Densitometric scans were at a wavelength of 620nm on Joyce Loebl Chromoscan.

Separation on 4.5%PA SDS Tris-EDTA gels(see methods).

roots) were homogenized and an SDS-soluble fractions were electrophoresed on 13% SDS disc gels(Type II). They both showed distinctive Coomassie blue staining protein profiles.

The spinal roots in both 44 and 46 day old dystrophic mice showed decreased protein in areas of relative mobility 0.2-0.3, 0.3-0.4 and 0.5-0.6(Figure 36). In areas of higher molecular weight the decreases in protein material were not as reproducible between the different age groups of mice. This problem may be due to poorer penetration of the sample into the gel(due to variable amounts of lipid and protein in the sample) or due to inconsistencies of sample components after centrifugation of a small volume of sample to get an SDS-soluble fraction. The dissection of the spinal roots may also give problems with reproducibility in these experiments. Normally the larger lumbar spinal roots are studied (16, 85). An abnormality of a protein with an approximate molecular weight of 165,000 (85) was not in evidence in these experiments. The most interesting abnormality seen in the spinal roots is that occurring with a relative mobility of .571(in the dystrophic mouse) and .557(in the normal mouse) the approximate molecular weight being 33,000(Figure 36; comparison of PICTURE I and PICTURE II).

Several abnormalities were seen in the sciatic nerve samples with decreased quantities of protein in the dystrophic mice at each of the ages studied(39,44 and 46 days of age)(PICTURE III). Densitometric scans of these same fractions also indicate the reduced protein concentration in the dystrophic mice sciatic nerves(Figure 37).

ii) Separation on 4.5% SDS-EDTA gels

The sciatic nerve and spinal root fractions run on these gels were not as well resolved as Salafsky's experimental samples(85). The dystrophic samples were not reproducibly different from normal mice samples at any of the ages studied.

Figure 36. Densitometric scans of spinal root homogenates of 129Rej/+? and 129Rej/dydy mice.

(A) 44-day-old littermate pairs
(B) 46-day-old littermate pairs

were separated on 13% SDS disc gels(Type II). Arrows indicate several areas of decreased amounts of protein in the dystrophic samples(as determined by Coomassie blue staining of protein). Gels were scanned at 620nm on Joyce Loebl Chromoscan. Gels were shrunk in 50% methanol for 1 hour before scanning.

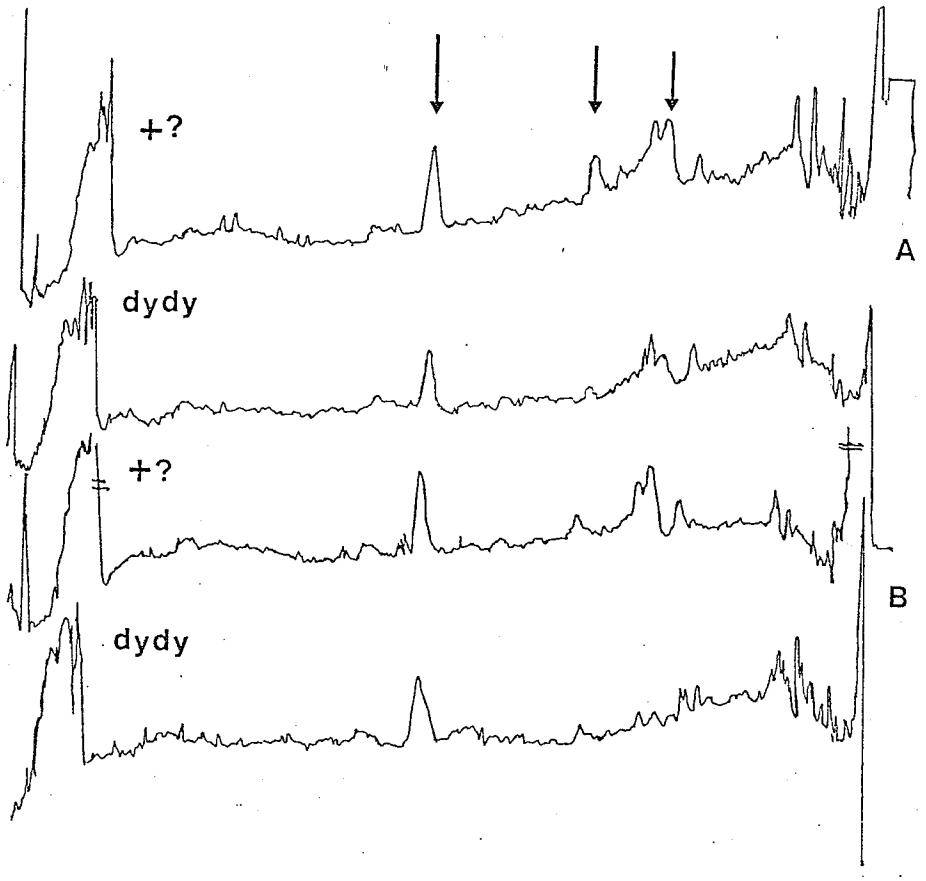
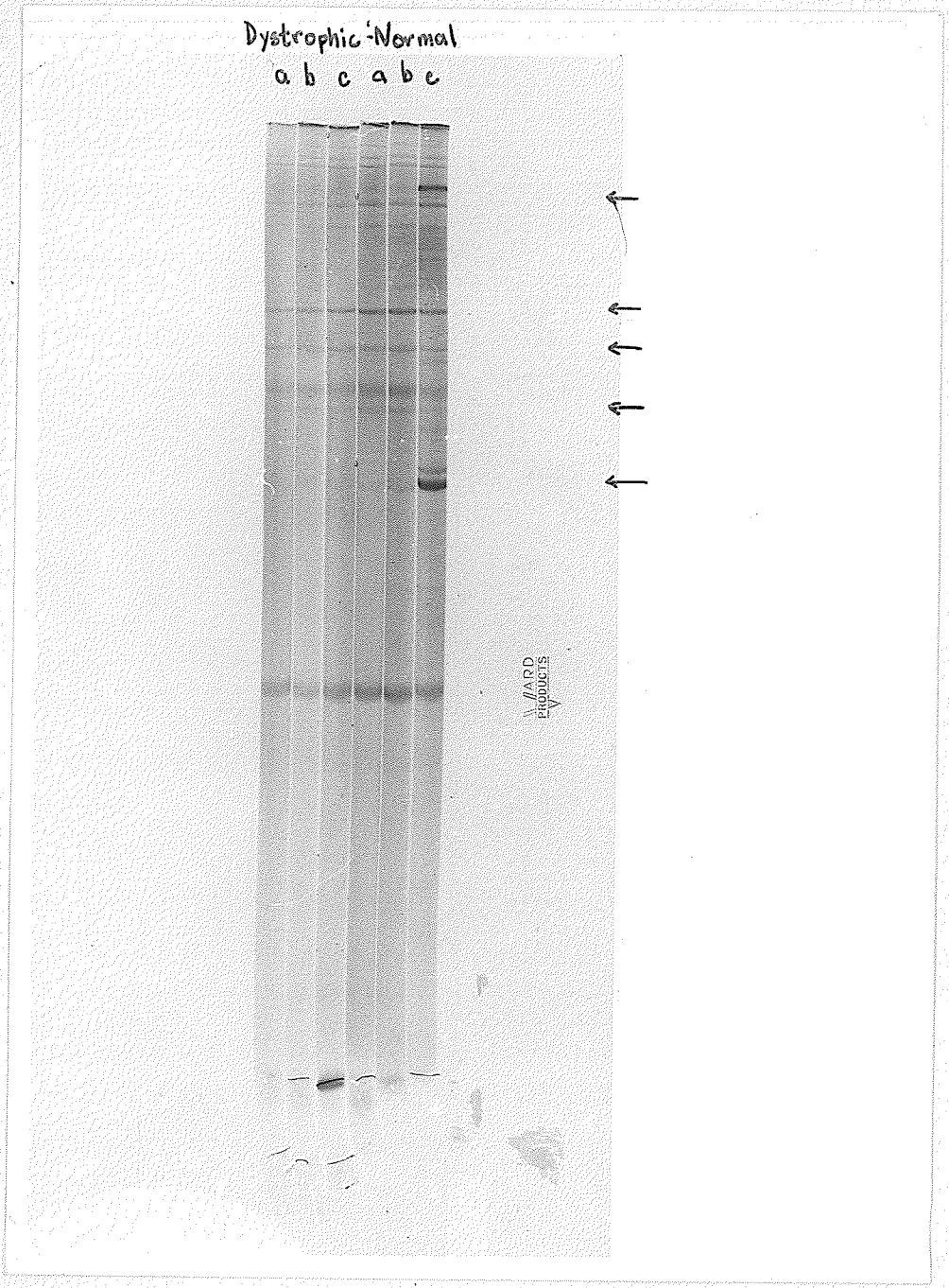


FIGURE 36



PICTURE III

Sciatic nerve homogenates of normal and dystrophic mice electrophoresed on 13% SDS disc gels.

The mice were a)39 b)44 and c)46 days of age(dystrophic and normal animals always were littermates*). Arrows indicate areas where a lesser amount of protein appear in the dystrophic samples. Samples were originally 84ugm of protein. Only the SDS-soluble fraction of the sciatic nerve homogenates were layered on the gel.

*In all studies when animals are compared normal to dystrophic the animals are littermate pairs.

Figure 37. Denitometric scans of sciatic nerve homogenates of 129Rej/+? and 129Rej/dydy mice.

(A) 129Rej/+? mice, 46,44 and 39 days of age respectively, (B) 129Rej/dydy mice, 46,44 and 39 days of age respectively, were separated on 13% SDS disc gels(Type II). Arrows indicate regions of decreased quantities of dystrophic mouse protein (as determined by Coomassie blue staining). Scanning wavelength was 620nm using Joyce Loebl Chromoscan. Gels were shrunk in 50% methanol for 1 hour before scanning.

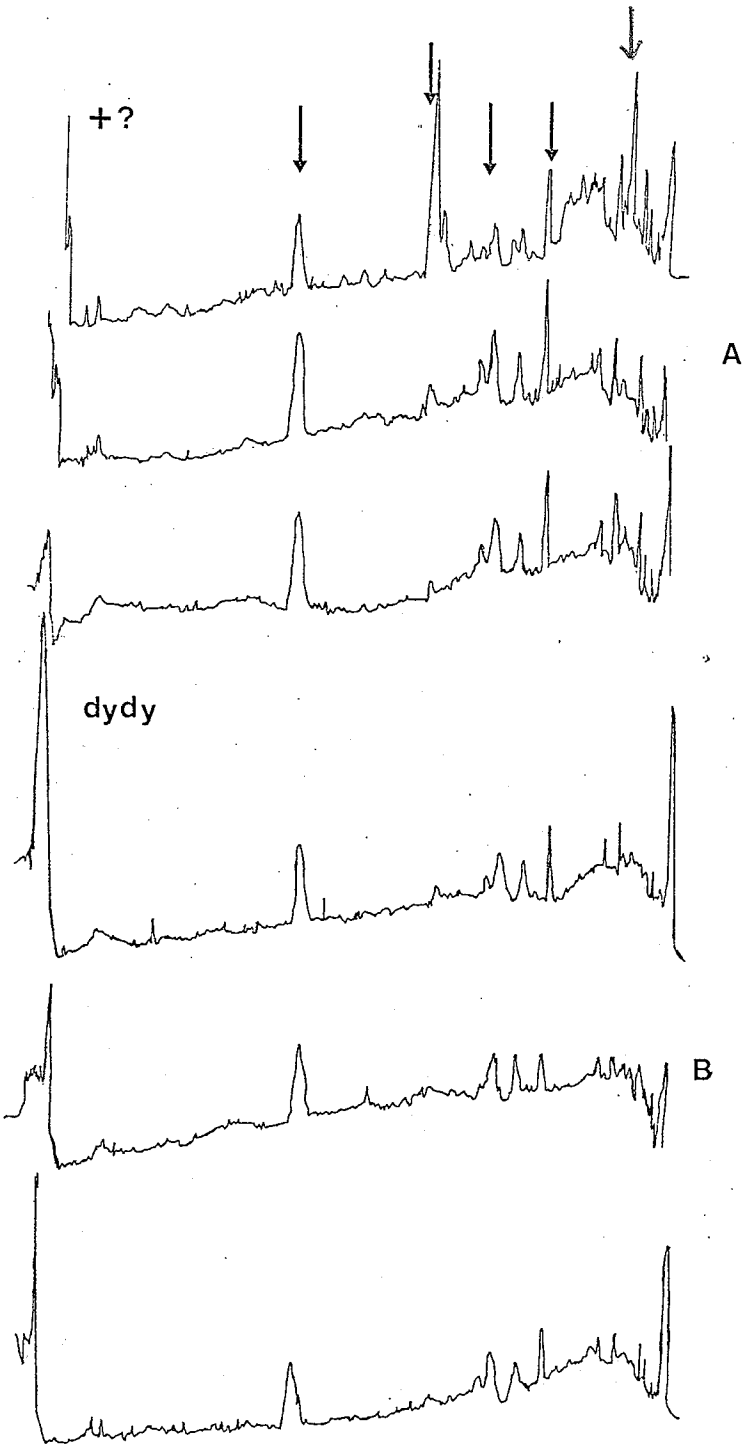


FIGURE 37

C) Discussion

The conclusions from both the SDS gels and isoelectricfocusing gel experiments were that no abnormality in individual protein fractions could be detected when comparing dystrophic and normal hamsters. The lack of resolution of the many protein components of the subcellular fractions could also be the reason for not finding an abnormality in the dystrophic hamster. If higher resolution could be achieved an abnormality may be uncovered. This may require the two-dimensional gel electrophoresis as suggested by Klose(127). Purification of the subcellular organelles or their components may also give rise to a more sensitive technique. It should be noted that the differential centrifugation process utilized in these experiments will not give an absolute separation of the nuclear, mitochondrial or microsomal fractions of a cell or tissue.

Care should be taken in the use of leucine isotopes in dual labelling studies involving nervous system particulate fractions. If the leucine isotopes are uniformly(L-(U^{14}C)-) and specifically(L-(4,5^{3}H)-) labelled they may show an apparent defect in incorporation into the lipid moieties of these tissues. This is an artifact of the labels due to a relative excess of ^{14}C-atoms in leucine degradation products which are lipid precursors. Consideration of another amino acid for the labelling of nervous tissues was considered. However, the economy and high rate of incorporation of leucine was thought to outweigh the use of another amino acid. Because the lipid moieties can be removed by acetone extraction the artifacts caused by the metabolism of the radioactive precursor can be avoided.

In the dual labelling investigation of the nervous system proteins of dystrophic and normal mice, strain 129Rej, abnormalities were detected in the spinal cord homogenates(with spinal roots) of three pairs of mice. In the 29-day-old dystrophic mouse a decreased amount of protein with an approximate molecular weight of 23,000 was found when polypeptides were separated by SDS disc gel electrophoresis. Whether this is a myelin component or another abnormally synthesized protein has not been determined. The defect in this single animal did not meet all the criteria for an abnormality as defined earlier in this paper. In older dystrophic mice,

proteins of the spinal cord in a lubrol soluble fraction on isoelectric-focusing, there was a reduction in the amount of protein in two regions. Because the large number of abnormalities seen in histological studies were not seen with the dual labelling technique suggestions for increasing the sensitivity of the technique in the study of nervous tissue could be made.

When further dual labelling studies are carried out longer labelling times and incorporation of label at the earliest stages of the clinical disease would be adviseable. Also purified membrane fractions would significantly increase the resolution of the analytical separation procedures. The selection of appropriate control animals is of major importance in verifying the presence of these abnormalities. In looking for the expression of a possible point mutation or any neural defect in these dystrophic animals one must pay attention to the genotypic nature of the control animal. A normal mouse in the heterozygous state may express the recessive mutant allele to some degree and this effectively decreases the sensitivity of the technique to abnormalities. An animal in which this would not be a problem is the allelomorphic mutant with muscular dystrophy, strain C57BL/6Jdy^{2j}dy^{2j}.

The study of these animal models and their muscular dystrophy characteristics will only be applicable to the human muscular dystrophies when they are finally understood in terms of their primary expression of the genetic lesion and in their disease process. However, the route to such understanding lies in the identification of secondary and even tertiary abnormalities. Dual labelling is a sensitive quantitative probe of such abnormalities and its own unique advantages will undoubtably help solve these basic problems.

CONCLUSIONS

The dual labelling technique removes many problems with sample handling in the study of the metabolism of normal and diseased animals and can be utilized for in vivo or in vitro experiments. Tissue samples are combined and processed throughout the homogenization, isolation and separation of cellular components. In this way random error arising due to different handling of the samples is eliminated. In addition, this technique offers the unique advantage of monitoring incorporation of the label into the live animal at any stage in its life. One can choose a labelled precursor to suit the study of either protein, carbohydrate, or lipid metabolism.

No abnormalities were detected in a study of brain subcellular fractions of Syrian hamsters(1-6-days-old). Normally, in comparing dystrophic and normal animals, there is little variability in the components of this tissue. This low noise level in the experiments on brain tissue should enhance the possibility of detecting any abnormalities, therefore the inability to detect any abnormality is taken as reliable evidence for the lack of a major defect in brain protein components.

Similarly in murine dystrophy(129Rej/dydy) no abnormalities were detected in the brain subcellular fractions. However, abnormalities were indicated in the labelling scans of spinal cord homogenates on SDS gels of 29-day-old mice and on isoelectricfocusing gels of 47- and 66-day-old mice. Further experiments with unlabelled tissue, spinal cords, sciatic nerves and spinal roots, supplied evidence for quantitative differences in the protein content of these tissues. The results using these separation techniques support the findings of histological abnormalities in murine dystrophy. Because the number of protein components in each of these fractions is large, two-dimensional or other one-dimensional separation techniques may be required to resolve one component from another. It is clear, however, that the sensitivity of dual labelling could be increased by increasing the duration of label incorporation. In order to incorporate significant amounts of label, in a reasonable time, an earlier time of labelling may be required; for

instance, during foetal development of the animal. In murine dystrophy histological evidence suggests that the abnormalities are localized in specific regions. Sensitivity of the dual labelling technique could therefore be further increased by isolating those particular regions from surrounding tissue. Tissue culture of sample tissues is a method of magnifying the abnormality which would increase the sensitivity of this technique.

Elucidation of the metabolic basis of an inherited disease such as muscular dystrophy will ultimately derive from observations of differences in the composition between dystrophic and normal animal tissue. The development of abnormalities can be followed in vivo using dual labelling, and the experiments described herein successfully illustrate the application of this technique to this important problem.

APPENDIX

This appendix contains the percentage recovery(of radioactive label) tables for the different gel systems used in this study of normal and dystrophic animal tissues with the dual labelling technique. Deviations from complete recovery, illustrated by the data, seem to represent both a systematic error and a significant random error.

The systematic error is unidentified at this time but results in a higher recovery of ^{14}C-leucine compared to ^{3}H-leucine. The random error may be associated with low numbers of counts in several of the samples or a failure of labelled components in the samples to electrophorese.

TABLE I

PERCENTAGE RECOVERY OF RADIOACTIVE LABEL
on 10% SDS GELS(TYPE I)

Age in Days	Hamster Subcellular Fraction	% ^{14}C	% ^{3}H
1 & 2	Brain Supernatant	94.0	86.4
1	Brain Supernatant	44.6[c]	44.9[c]
3[a]	Brain Supernatant	75.0	89.6
3[a]	Brain Microsomal pellet	83.4	83.6
3[b]	Brain Mitochondria	105.3	110.4
3[b]	Brain Microsomal pellet	87.9	89.4
3[b]	Brain Mitochondria	112.7	113.8

a & b
Two different age-matched pairs of RB and BIO 14.6 hamsters.

All samples were under 100ugm of protein.

c
Low label recovery due to significant random error perhaps failure of of labelled components to electrophorese.

TABLE II

PERCENTAGE RECOVERY OF RADIOACTIVE LABEL
on SDS DISC GELS(TYPE II)

Age in Days	Hamster Subcellular Fraction	% ^{14}C	% ^{3}H
6	Brain Supernatant	92.7	90.2
	Brain Mitochondria	102.6	95.8
	Brain Nuclear pellet	103.7	96.2
	Brain Microsomal pellet	99.5	95.9
3	Brain Supernatant	96.0	79.1
	Brain Mitochondria	97.2[a]	66.1[a]
	Brain Mitochondria	26.2	24.8
30	Spinal Cord Homogenate	95.3	54.8
	Spinal Cord Homogenate	90.4	76.4
6	Brain Nuclear Pellet Acetone Extracted	64.8	54.1
	Brain Nuclear Pellet Non-extracted	85.4	75.3
3	Brain Nuclear Pellet Acetone Extracted	62.4	60.4
	Brain Nuclear Pellet Non-extracted	41.6	42.7

[a] % recovery calculation (cpm(counts per minute) rather than picomoles were used as the basis for the calculation)

All disc gels were 10% SDS disc gels except for spinal cord homogenate samples where disc gels were 13% PA, 0.26% BIS with 6M urea.

TABLE III

PERCENTAGE RECOVERY OF RADIOACTIVE LABEL
on SDS DISC GELS(TYPE II)

Age in Days	Mouse Subcellular Fraction	% ^{14}C	% ^{3}H
29	Brain Supernatant	85.8	80.0
47	Brain Supernatant	110.3	102.2
66	Brain Supernatant	72.8	71.1
29	Brain Mitochondria	87.2	85.4
47	Brain Mitochondria	88.4	78.6
66	Brain Mitochondria	93.6	93.0
29	Spinal Cord homogenate	108.4	100.7
47	Spinal Cord homogenate	89.3	79.9
66	Spinal Cord homogenate	228.5[a]	79.8[a]
	Spinal Cord homogenate	141.2	142.2
	Spinal Cord homogenate (SDS-soluble fraction)	57.9	54.5
29	Sciatic Nerve homogenate	97.4	85.9
47	Sciatic Nerve homogenate	96.7	83.6
66	Sciatic Nerve homogenate	82.6	86.3
29	Spinal Root Homogenate	90.4	16.1
	Spinal Root Hom.-Gel T.D. Region	31.2	21.5
	Free Leucine: L-(4,5 ^{3}H)- and L-(U ^{14}C)-leucine on 13% SDS disc gel	85.4	72.7

Sciatic nerve and spinal root homogenates had low incorporation of labelled leucine (2000cpm/gel) giving low counts above background and were not presented in the body of the thesis.

[a] This spinal cord homogenate was contaminated with ^{14}C-leucine as discussed in RESULTS AND DISCUSSION.

TABLE IV

PERCENTAGE RECOVERY OF RADIOACTIVE LABEL
on 5.1% ISOELECTRICFOCUSING GELS

Age in Days	Subcellular Fraction	% ^{14}C	% ^{3}H
6	Hamster Brain supernatant	86.4	80.7
6	Hamster Brain mitochondria	91.3	85.2
6	Hamster Brain Nuclear pellet	98.9	87.5
29	Mouse Spinal Cord Homogenate	90.4	83.5
47	Mouse Spinal Cord Homogenate	95.2	87.0
66	Mouse Spinal Cord Homogenate	103.8	88.9

All samples were solubilized in lubrol except the brain supernatant fractions.

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