BIOCHEMICAL STUDIES ON LYSOSOMAL DISEASES USING CULTURED HUMAN SKIN FIBROBLASTS

A Thesis Presented to the Faculty of Graduate Studies of the University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of Master of Science

by

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TO DIANE

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LIST OF ABBREVIATIONS USED

1-D - one dimensional

2-D - two dimensional

4MU - 4-methylumbelliferone

alpha 4MUG- 4-methylumbelliferyl-alpha-D glucopyranoside

A.M. - acid maltase

Ci - Curie

d - dalton

DAP-I - dipeptidyl aminopeptidase I

FCS - fetal calf serum

g - unit of gravitational acceleration (approx. 10m x sec^{-2})

HCS - human cord serum

HuMc - Human McCoy's medium

IEF - isoelectric focusing

L - lysosomal subcellular fraction

µl - microlitres

M - mitochondrial subcellular fraction

ma - milliamps

ML - total mitochondrial-lysosomal subcellular fraction

ML-S - soluble portion of ML

mo - months

NEPHGE - non-equilibrium pH gradient electrophoresis

P - microsomal subcellular fraction

PAGE - polyacrylamide gel electrophoresis

PLS - post-lysosomal supernatant

PNS	- post-nuclear supernatant
rpm	- resolutions per minute
s	- seconds
S	- Svedborg unit of sedimentation
SDS	- sodium dodecyl sulfate
yr	- years
V	- volts
WC	- whole cells

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ABSTRACT

This project was initiated in response to a reported defect in a basic low molecular weight protein in fibroblasts from Pompe disease patients (Pena and Wrogemann, 1978). It was based on the observation that when Pompe and normal cell proteins were labeled with ^{3}H- or ^{14}C-leucine, combined, and then separated by their isoelectric points or molecular weights, the radioactivity incorporated by Pompe proteins was low at pI 7.9 and at 29,000d regions of the gels. After ascertaining that the original double label method was being reproduced faithfully, original and new Pompe strains were tested for the presence of the abnormality. None was seen in either isoelectric focusing (IEF) or polyacrylamide gel electrophoresis of samples in the presence of SDS (SDS-PAGE). The protein composition of several Pompe strains and their control strains was analyzed more extensively by two-dimensional (2-D) gel electrophoresis of single labeled samples, which distinguished nearly 700 cellular proteins in each strain. Examination of the gel autoradiograms failed to reveal any abnormality that could be correlated with deficiencies in either alpha glucosidase or a basic low molecular weight protein. The autoradiograms did reveal very low levels of qualitative variation between strains.

Gaucher disease, a second lysosomal disorder, revealed reproducible but different abnormalities in both one dimensional SDS-PAGE gels of double labeled samples and 2-D gels of single labeled

samples. The abnormality in 2-D gels of single label Gaucher samples was confirmed and quantitated by a double label method that was developed on DMD fibroblasts and intended for use with Pompe fibroblasts. The method involved excising select double label spots from silver stained 2-D gels and analyzing them by liquid scintillation counting. Since no spots were consistently missing in 2-D gel autoradiograms of single labeled Pompe fibroblast protein, the technique was not used in these strains.

No satisfactory explanation for the irreproducibility of the finding of Pena and Wrogemann (1978) was found.

PROLOGUE

The purpose of this investigation was to clarify the nature and role of a reported basic protein defect in the biochemical pathogenesis of Pompe disease (Pena and Wrogemann, 1978), a rare glycogen storage disease. Despite the relatively early recognition that the disease was characterized by a single enzyme deficiency, and was inherited in a simple Mendelian fashion, the clinical presentation of the disease has been quite heterogeneous. The ensuing introduction highlights the many vagaries which surround the Pompe disease, and introduces a second lysosomal storage disease, Gaucher disease, a disorder analogous in its forgone simplicity. What is known of the molecular basis of the disorder is also presented here, and proved critical in interpreting results of past observations. The concensus of facts should also serve to emphasize the necessity of pursuing novel findings with promise as missing links in poorly understood genetic disorders.

ACID MALTASE DEFICIENCY--DISEASE IN BROAD PERSPECTIVE

Acid maltase deficiency (AMD), the most appropriate name of several synonyms (eg. glycogen storage disease, typeII; glycogenosis type II; Pompe disease), is a clinically and genetically heterogeneous disorder in which the variable forms have in common the absence or reduction of the enzyme acid alpha-glucosidase (E.C. 3.2.1.20, also

acid maltase). Classically, it is presented as a vacuolar myopathy with lysosomal accumulation of glycogen, a natural substrate of acid maltase. At one time, this statement could be generalized to all forms of the disease, despite wide variation in age of onset, clinical severity, and distribution of glycogen deposits and acid maltase activity. Recent observations however demonstrate that glycogenosis type II can arise where acid maltase activity is normal, and conversely, glycogen levels are often normal where acid maltase activity is reduced. Because the severity of the disease generally correlates inversely with age of onset, it is most convenient to consider infantile, childhood or juvenile, and adult forms separately. Original case studies have been adequately reviewed by Loonen (1979) and Pena (1977). I will discuss only the most relevant features, including exceptional cases and recent findings.

CLINICAL FORMS

Infantile Form

A post mortem diagnosis on a seven-month old female provided the first description of glycogen storage disease II, which in the early 1930's became known as Pompe's disease, after the attending pathologist (Hers and de Barsy, 1973). This eponym is most often equated with the infantile form, the most severe and homogeneous of the three, demonstrating glycogen accumulation in all tissues, especially the heart, skeletal muscle, and central nervous system. Symptoms

include hepatosplenomegaly, hypotonia, cardiomegaly and other cardiac abnormalities, which contribute to poor growth, respiratory tract infections and death due to cardiorespiratory failure by one year of age. Only recently has the absence of acid maltase been demonstrated in one of several exceptional individuals not showing cardiomegaly concurrent with excessive glycogen storage in muscles (Hers, 1963).

Crossreactive material to anti-human fibroblast alpha-glucosidases has been found both present (Beratis et al. 1978) and absent (Reuser et al. 1978) in fibroblast homogenates from patients with the infantile form of the disease.

Childhood or Juvenile Form

Beyond childhood, the severity of acid maltase deficiency is dramatically reduced, usually to the skeletal myopathy, although the degree of involvement is variable (e.g. Tanaka et al. 1979). Electron microscopy reveals membrane-bound glycogen accumulation in skeletal muscles (Zellweger et al., 1965). Two more severe cases were recently reported by Danon et al. (1981) in boys who died at age seventeen, even with normal levels of muscle and urinary acid maltase activity. Both cases were similar in age at onset, and progression of proximal myopathy and cardiac abnormalities. Cardiomegaly and mental retardation were the only symptoms unusual to post-infantile forms of glycogenosis II, but these cases could have represented a novel glycogen storage defect.

Adult Form

The adult form of AMD is the most variable in age of onset, severity of weakness and degree of glycogen accumulation, although Broadhead and Butterworth (1978) have found an inverse correlation between acid maltase activity and severity of the disease among adult patients. The clinical picture has been confused with polymyositis or limb-girdle dystrophy. Adult patients demonstrate glycogen accumulation both lysosomally and in extralysosomal spaces, and show an abnormal electrophoretic profile for muscle neutral maltase (Bertagnolio et al., 1978). Both acid and neutral isoenzymes (cf. Role of Isoenzymes) appear to have normal activity in leukocytes, but the deficient acid maltase is compensated for by a third isoenzyme unique to granulocytes and kidney (see below). Acid maltase activity is reduced in the heart of individuals with the adult form, but no glycogen accumulates there (Martin et al., 1976a; Di Mauro et al., 1978).

EXPLANATIONS FOR CLINICAL AND PATHOLOGICAL HETEROGENEITY

The degree of clinical heterogeneity seen in the various forms has led some to question whether acid maltase deficiency is truly a single disease (eg. Loonen 1979). Several explanations for the variability have been put forward, and these are discussed below.

Genetic Heterogeneity

Both generalized and muscular forms of acid maltase deficiency display classic Mendelian autosomal recessive inheritance with a slight preponderance of affected males (Huijing, 1975). The possibility that the various forms resulted from different gene mutations has been investigated in several ways. Reuser et al. (1978) attempted to correct for acid maltase deficiency by somatic cell hybridization in twenty different combinations of acid maltase deficient cell strains, including infantile, juvenile, and adult forms. No complementation was observed, but this finding, of course, was not absolute evidence for identity of gene defects. Beratis et al. (1978) found that adult form AMD fibroblasts contain low levels of a catalytically normal enzyme, but that infantile form fibroblasts contain inactive enzyme that also crossreacts with anti-acid maltase antibodies. This result is partly in contrast to the reported absence of crossreactive material in fibroblasts from patients with the infantile form of the disease (Reuser et al., 1978), but the specificity of the antibodies may have differed, since the latter were prepared against liver instead of placental acid maltase. Nevertheless, mutations which alter the structure of acid maltase can have a profoundly different effect from those that affect the regulation of cellular acid maltase levels, and these studies need clarification.

Role of Isoenzymes

A variant isoenzyme of acid maltase from a heterozygous placenta was recently found to differ from the common isoenzyme in its kinetics for the substrate glycogen only, showing an S-shaped saturation curve for this substrate (Beratis et al., 1980). The isoenzyme's activity toward glycogen may be only slightly greater than that seen in adult form AMD, such that homozygotes for the variant allele could develop glycogen storage disease late in life. The authors caution that such a difference cannot be detected with maltose or 4-methylumbelliferyl-alpha-D-glucopyranoside (alpha-4MUG), since the variant displays normal kinetics on these substrates.

A first supposition to explain the variable severity in glycogen accumulation was that only affected tissues show decreased acid maltase. It is clear that nearly all tissues in both generalized and muscular forms of the disease have below normal acid maltase activity (Loonen 1979). An exception to this rule is in kidney and leukocytes, which generally show normal acid maltase activity in affected patients. This observation could not be explained until the discovery of "renal" maltase, present only in kidney and granulocytes, which differs kinetically and antigenically from normal acid maltase (Dreyfus and Poenaru, 1980). The renal enzyme is solubilized by vigorous homogenization (Potter et al., 1980) or in detergent treated homogenates (Dreyfus and Poenaru, 1980), so avoidance of these treatments permits separation of the two activities. Alternatively,

the lymphocytes when separated from granulocytes in leukcocyte preprations, are more reliable indicators of acid maltase activity (Taniguchi et al., 1978). In fact, isolated lymphocytes do have glycogen filled lysosomes in infantile patients (Bassewitz et al., 1977). The percentage of contaminating renal maltase in a mixture with acid maltase can be determined by the response to inhibitors such as turanose, maltose, and citrate, or by isoelectric precipitation of the renal maltase at pH 5.0 (Broadhead and Butterworth, 1978).

Neutral maltase, a cytosolic and microsomal isoenzyme of acid maltase, shares substrate specificity with acid maltase, but has a neutral pH optimum with low activity at acid pH (Fujimoto and Fluharty, 1978). Activity of neutral maltase is generally not altered (Reuser et al., 1978), but cases of neutral maltase reduction in both infantile and adult form AMD have been reported (Bertagnolio et al., 1978; Koster et al., 1978). Because neutral maltase is very thermolabile (Rosenfeld, 1965), it may degrade before post mortem analysis. This explanation seems unlikely in cases reported so far.

MOLECULAR BIOCHEMISTRY OF ACID MALTASE

Molecular Structure

The isolation and characterization of acid maltase has been facilitated by its affinity for polysaccharides such as dextran in

Sephadex G-100 (Auricchio and Sica, 1967). The intact enzyme from rat liver appears to have a molecular weight of 80,000d (Auricchio et al., 1968) or 114,000d (Jeffrey et al., 1970a), and that from bovine liver of 107,000d (Bruni et al., 1969). In humans, acid maltase has an estimated molecular weight of 97,000d in kidney (Auricchio et al., 1968) and 103,000d in placenta (Swallow et al., 1975). The bovine liver enzyme, which was stable in 6M urea, dissociated only upon treatment with 5M guanidine HCl into three subunits of different molecular weight and different N-terminal residues (Belenky et al., 1975). This contrasts to an earlier finding that guanidine-HCl treatment reduced the 107,000d liver acid maltase homogeneously into 26,000d species (Bruni et al., 1970). The subunit structure of acid maltase is still uncertain. Four free thiol groups and eight half-cystine residues exist in the bovine liver enzyme, but the number of disulfide bridges was not determined (Bruni et al., 1969). The stability of the enzyme at pH 7 is enhanced by sulfhydryl group reagents.

Enzyme Activity

Acid maltase is optimally active near pH 4.5, depending on cation concentration and the substrate used (Belenky and Rosenfeld, 1975; Swallow et al., 1975). The enzyme is specific for alpha-glycosidic bonds, and is conveniently assayed on its natural substrates, the polysaccharide glycogen, and the disaccharide maltose.

Alternatively, hydrolysis of 4-nitrophenyl- or 4-methylumbelliferyl--glucopyranosides provide for simple colorimetric and fluorometric determinations of activity.

Sodium, potassium, and other monovalent and divalent cations stimulate the hydrolysis of glycogen by acid maltase (Jeffrey et al., 1970a,b; Palmer, 1971a). Many monosaccharides, oligosaccharides, glycosides, and polyols are substrates and show substrate inhibition with acid maltase (Bruni et al., 1969; Jeffrey et al., 1970a; Palmer, 1971a). Antiglucosidase antibodies inhibit glycogen hydrolysis more strongly than they inhibit the splitting of maltose (de Barsy et al., 1972; Koster et al., 1976). Turanose inhibits lysosomal but not neutral alpha-glucosidase, and is used to distinguish the two in mixtures (Broadhead and Butterworth, 1978). Alternatively, KCl can be used to stimulate the lysosomal enzyme and inhibit the neutral enzyme at pH 4.0 (Soyama et al., 1977a). Other common inhibitors are Tris, erythreitol (Auricchio et al., 1968), and the cations Hg^{2+} and Cu^{2+} (Bruni et al., 1969; Soyama et al., 1977b).

The kinetic differences of acid maltase on maltose and other oligosaccharides versus polysaccharides such as glycogen has led Palmer (1971b) to suggest that two different active sites or subunits may exist in the acid maltase molecule, and that structural defects in either one might be the source of heterogeneity in acid maltase deficiency. Since glycogen breakdown is certainly abnormal in generalized acid maltase deficiency, the muscular forms of AMD would logically be deficient in oligosaccharide hydrolysis. However, there

is neither direct evidence nor an adequate basis for this prediction. Perhaps another function of acid maltase, the transfer of alpha-glucosyl residues between maltose molecules or from maltose to glycogen (transglucosylation) should be examined more closely in the event that such a pathway is important for in vivo glycogen metabolism (Palmer, 1971b).

CLINICAL AND PATHOLOGICAL HETEROGENEITY IN GAUCHER DISEASE

Analogous to the situation with acid maltase deficiency, Gaucher disease comprises a group of autosomal recessive disorders that are expressed in a variety of clinical forms. The common causitive factor among these forms is a deficiency in beta-glucosidase (E.C. 3.2.1.20), the enzyme responsible for hydrolyzing beta-glycosidic linkages in natural glycolipid substrates. Although many cases cannot be categorized (eg. Turner and Hirschhorn, 1978), three classes are presently recognized, based on the severity of the disease process (Brady, 1978).

CLINICAL FORMS

Chronic Nonneuronopathic Form (Type 1)

As the name implies, Type 1 is the least severe, sparing neurological involvement, with the first symptoms occurring anytime between infancy and late adulthood. Type 1 patients display

hypersplenism, anemia, thrombocytopenia, and bone lesions, but may also experience orthopedic difficulties and show abnormal skin pigmentation. This most common form occurs more frequently in individuals of Ashkenazi Jewish Ancestry than in non-Jews (Wenger and Olsen, 1981) .

Acute Neuronopathic Form (Type 2)

This most severe form is recognized a few months after birth, and death generally ensues by age two. Neurological problems include pseudobulbar palsy, opisthotonos, and strabismus. Affected children also show slow development, hepatosplenomegaly, laryngeal spasms and swallowing difficulty, and chronic bronchopneumonia (Wenger and Olsen, 1981).

Subacute Neuronopathic (Type 3)

Type 3 Gaucher disease is the least well defined, but mild hepatosplenomegaly often accompanies the neurological problems that develop before adolescence. These include dementia, seizures, and extrapyramidal and cerebellar involvement. The form is also known as the Norrbottnian type of Gaucher disease, after the Swedish province in which it may have originated (Dreborg et al., 1980). Other Gaucher patients may exhibit severe lung, liver or bone and joint problems in the absence of neurological problems, late onset epilepsy and intellectual deterioration, or symptoms intermediate between form 2 and 3 (Wenger and Olsen, 1981).

DIAGNOSIS OF BETA-GLUCOSIDASE DEFICIENCY

Much of the difficulty in the diagnosis of beta-glucosidase deficiency stems from the presence of multiple forms of beta-glucosidase activity on artificial and natural substrates. The enzyme exists in both membrane-bound lysosomal and soluble cytosolic forms that are unequally distributed in different tissues and species. Beta-glucosidase is mostly particulate in human spleen and placenta (Peters et al., 1976), but the cytosol form is unique in rat liver (Robinson and Abrahams, 1967; Mellor et al., 1973), and is predominant in porcine kidney (Kanfer et al., 1977). The lysosomal enzyme has beta-xylosidase (Wenger and Olsen, 1981) and beta-glucosylceramidase activities (E.C. 3.2.1.45; Kanfer et al., 1974, Peters et al., 1976) based on its ability to cleave their respective substrates. The cytosolic form does not hydrolyze glucosylceramide, but will cleave beta-galactosides, beta-xyloside, beta-D-fucosides, and L-arabinosides (Glew et al., 1976) as well as the synthetic beta-glucosides. Lysosomal beta-glucosidase also transfers beta-glucosyl residues from beta-4MUG (Raghavan et al., 1974; Kanfer et al., 1975b) or glucosylceramide to ceramide (Kanfer et al., 1977).

The most consistent abnormality in Gaucher patients is a deficiency in particulate glucosylceramidase activity. Many assays on synthetic substrates, including beta-4MUG and 4-nitrophenylglucoside reveal residual activity that is neither seen in other tissues of the

same Gaucher patient nor in the same tissue with the natural substrate (Ben-Yoseph and Nadler, 1978; Butterworth and Broadhead, 1978; Chiao et al., 1980; Choy and Davidson, 1980b; Hultberg and Ockerman 1970; Wenger and Olsen 1981). Since the natural substrate is far more complex in structure than the artificial substrate, the former may be a much better agent in detecting structural alterations in glucosylceramidase. Choy and Davidson (1980b) did find that Gaucher fibroblast glucosylceramidase is less effective in hydrolyzing glucocerebrosides with longer fatty acid chains, supporting evidence for a structural alteration in the Gaucher enzyme (Pentchev et al., 1978). Convenient assays on artificial substrates, which avoid the costly preparation and handling of ^{14}C-glucosylceramide, have been modified to include high purity taurocholate (Wenger et al., 1978a) and Triton X-100, which enhance lysosomal glucosylceramidase while inhibiting other non-specific beta-glucosidases (Raghavan et al., 1980). The modified method has so far given comparable results to those using radiolabeled natural substrate, but may be succeeded by a new method employing nonradiolabeled natural substrate (Choy and Davidson,1980b).

At present, no qualitative or quantitive enzyme alteration reliably distinguishes the different forms of the disease or predicts the clinical course (Wenger and Olsen, 1981). Mumford et al.(1976) ruled out the possibility that differences in severity and organ involvement were due to dissimilarity of brain and spleen glucosylceramidases. Further, in the Gaucher mouse, brain glucosylceramide accumulates more rapidly and with less

glucosylceramidase inhibition than in liver or spleen (Stephens et al., 1978). This finding suggests that neuronal complications would appear before other symptoms if the clinical heterogeneity was due to quantitative variation. However, using any of five different enzyme assays which employed either artifical or natural substrates, Haakanson and coworkers (1980) were able to distinguish type 3 Gaucher fibroblasts from normal control and carrier cell strains. Carrier strains could not always be distinguished from normal strains, with some overlap between them in all five tests, but the mean activity of carriers was lower than the mean control activity in all cases.

MOLECULAR BIOCHEMISTRY OF BETA-GLUCOSIDASE

General Characteristics

In rat kidney, cytosol beta-glucosidase has a molecular weight of 50,000d (Glew et al., 1976), but the lysosomal form isolated from human placenta appears to be a tetramer of 60,000d subunits (Pentchev et al., 1973). When gel filtration is performed under isotonic conditions, the fibroblast enzyme from both normal and Gaucher patients exists in only one form, with a molecular weight of approximately 150,000d (Mueller and Rosenberg, 1977). The enzyme is equivalent in size, apparent K_m and activity to the membrane bound isoenzyme of spleen, with a pH optimum of 4.2. Human leukocyte acid beta-glucosidase has two optima at pH 4.0 and pH 5.3, but the membrane-bound enzyme of placenta is most active in the higher range of

pH 6.0-6.6 (Pentchev et al., 1973). The cytosolic enzyme, often referred to as neutral beta-glucosidase, has a pH optimum between 6.0 and 6.7 (Glew et al., 1976). Similar to the human situation, the acid component of beta-glucosidase activity is deficient in a canine model of Gaucher disease (glucocerebrosidosis; van de Water et al., 1979).

Conduritol beta-epoxide effectively inhibits both beta-xylosidase and beta-glucosidase, causing glucosylceramide accumulation in tissues of the Gaucher mouse (Kanfer et al., 1975a; Stephens et al., 1979). The difference in kinetics of inhibition for beta-glucosidase and beta-xylosidase activities suggested the existence of two separate binding sites on the molecule (Stephens et al., 1979).

Treatment of samples with pure taurocholate inhibits the neutral enzyme, stimulates the acid enzyme, and shifts the pH optimum of the activated leukocyte beta-glucosidase from 4.0 to 5.0 (Raghavan et al., 1980). Incubation of leukocyte samples at pH 4.0 in the presence of NaCl also removes the interfering particulate activity at pH 4.8, which is unaltered in Gaucher patients (Butterworth and Broadhead, 1978). Steroid beta-glucosidase activity is also deficient in particulate fractions of infantile and adult form patients, but the soluble activity is reduced only in brain and spleen of infantile patients (Kanfer et al., 1975c).

Anionic detergents activate lysosomal beta-glucosidase and inhibit cytosol beta-glucosidase (Peters et al., 1976). Other activators and inhibitors of the lysosomal enzyme include phospholipids (Peters et al., 1976) and cationic detergents (Blonder et al., 1976)

respectively. Cytosol beta-glucosidase is competitively inhibited by glucono-(1--5)-lactone, and noncompetitively by N-ethylmaleimide, p-chloromercuribenzoate, 5-5'dithio-bis(2-nitrobenzoic acid) and iodoacetic acid (Glew et al., 1976). Enzyme activity is not dependent on the presence of divalent cations (Mueller and Rosenberg), 1977).

The Beta-Glucosidase Activator

Since the first report of a putative beta-glucosidase activator-protein in spleens of Gaucher patients (Ho and O'Brien, 1971), much attention has been paid to the nature of the lysosomal beta-glucosidase enzyme. The glycoprotein activator has been attributed a stabilizing function at acid pH, but this alone could not account for all beta-glucosidase activation (Pentchev and Brady, 1973). Mueller and Rosenberg (1977) did not believe the lower thermostability of lysosomal beta-glucosidase from Gaucher fibroblasts was indicative of a structural mutation, since other kinetic parameters which should have been affected were not. The simple diminution in enzyme quantity (lower V_{max}) among Gaucher patients was regarded as a regulatory dysfunction involving either synthesis or activation of beta-glucosidase. However, the in vivo importance of such an activator has been questioned (Peters et al., 1976; Wenger and Olsen, 1981), and more recent evidence shows that at least some Gaucher patients have structurally altered glucosylceramidase (Choy and Davidson, 1978, 1980a,b; Pentchev et al., 1978; Turner and Hirschhorn, 1978).

PATHOPHYSIOLOGY

Tissues of patients with Gaucher disease demonstrate large glucosylceramide-laden lysosomes in reticuloendothelial cells, particularly in spleen, liver and bone marrow, which lead to the secondary organ manifestations mentioned earlier. Because the enzyme from kidney is localized in the cytosol of proximal convoluted tubules, it is suspected to play a role in monosaccharide reabsorption, but its true physiologic role is uncertain (Glew et al., 1976; Hatakeyama et al., 1980). The importance of beta-glucosidase in neurological development is suggested by age related activities in brains of developing human fetuses (Sinha and Sinha, 1980). Further, rat kidney beta-glucosidase activity rises between 4 and 52 weeks after conception, and is markedly higher in males (Hatakeyama et al., 1980). The activity in rat kidney was found to be regulated by both hydrocortisone and testosterone, but neither of these affected mouse kidney beta-glucosidase, which may serve a different function in this species. Failure to metabolize glucosylceramide seems to be the most critical defect in Gaucher disease, but the high degree of enzyme deficiency required for sphingolipid accumulation (Stephens et al., 1978) may mean that other beta-glucosidase specificities are critical in milder forms of Gaucher disease.

MODULATION OF ENZYME ACTIVITY BY NON-ENZYMATIC ACTIVATOR PROTEINS

From the previous discussion, it is clear that a large gap exists between our knowledge of the biochemistry of certain single gene disorders, and the mechanism by which these defects are pathogenic in diseased individuals. Part of the difficulty certainly lies in the poor understanding of the basic systems, i.e. neither the molecular nor the physiological basis of the gene defects are well enough understood that a connection can be drawn between them. In practical terms, it is more feasible to characterize a molecular defect than to extricate a causative factor from a plethora of complex pathological responses.

Both Pompe disease and Gaucher disease are well suited for such an investigation for several reasons. The genetics of both follows simple Mendelian patterns, suggesting that the molecular defect is a single defective protein, at least within similar disease forms. Because both diseases have long been associated with enzyme deficiencies, the biochemistry has been worked out enough to reduce the investigation to a limited group of protein characters, whether activity, size, or cellular location. Finally, several recent investigations have pointed to novel molecular activator systems which could explain the complexity of cell dysfunction in such diseases. A short review of these systems is therefore given in this section.

Sulfatase Activator

Sulfatase A, the enzyme deficient in metachromatic leukodystrophy, was the first lysosomal enzyme to display activation by a non-enzymatic protein (Mehl and Jatzkewitz, 1964; Fischer and Jatzkewitz, 1978). The soluble activator isolated from lysosomes has a molecular weight of 23,000d and an isoelectric point of 4.3, but is not constituitive for sulfatide hydrolysis (Stinhoff and Jatzkewitz, 1975). Heterogeneous preparations from secondary lysosomes contain two components of different molecular weight that together stimulate cerebroside sulfatase, cerebroside galactosidase, cerebroside glucosidase, N-acetylgalactosaminidase, and sphingomyelinase (Mraz et al., 1976). In contrast to a single activator for all sphingolipid hydrolysis, contaminating proteins in the above prepration might have activated individual enzymes. Stevens et al. (1979) have described a patient with metachromatic leukodystrophy that may be deficient in cerebroside sulfatase activator.

Gangliosidase Activators

Variant forms of G_{M1}-gangliosidosis may be divided into one of two intergenic complementation groups: group A, in which G_{M1} beta-galactosidase (beta-gal) deficiency results from a mutation in the structural gene for the enzyme, and group B cells, which are deficient

in a cytoplasmic modification factor (Bootsma and Galjaard, 1979). Group B cell nuclei express beta-gal activity when fused with enucleated group A cells. The genetic and clinical heterogeneity of beta-gal deficiency, despite the finding of two structural loci for the enzyme, remains unexplained (Hoeksma et al., 1980). The milder adult form of the disease has in two instances been associated with neuraminidase deficiency, although the relationship is not understood (Hoeksma et al., 1980; Wenger et al., 1978b).

The importance of activators in the degradation of G_{M2}-ganglioside followed the recognition that in vitro assays of lysosomal hexosaminidase were not representative of in vivo reactions when artificial substrates, bile salts, or detergents were used to emulsify the natural substrates ganglioside G_{M2} or glycolipid G_{A2} (Sandhoff and Christomanou, 1979). In the absence of such agents in lysosomes, the carbohydrate chains on the natural substrates would be no more accessible in vivo, since lipid components are membrane incorporated and tightly packed (Sandhoff and Conzelmann, 1979a). Hechtman and LeBlanc (1977) found an activator protein in human liver extracts which enhanced the hydrolysis of N-acetylgalactosaminyl bonds of G_{M2}-ganglioside, and tentatively postulated an enzyme-activator complex based on kinetic data. Failure to activate degradation of artificial substrate (Sandhoff and Conzelmann, 1979b) and other physicochemical evidence (Hechtman and Kochra, 1980), however, suggest that this protein activator stimulates G_{M2}-ganglioside hydrolysis by the formation of mixed micelles containing activator and substrate.

Conzelmann and Sandhoff (1978) demonstrates alpha- and beta- components of hexosaminidase activator which together specifically enhance degradation of glycolipids G_{M2} and G_{A2} by the A isozyme. Infants with variant AB of Tay-Sachs disease possess normal levels of hexosaminidases A and B, but lack the beta-activator component and fail to degrade G_{M2} gangliosides.

Glucosylceramidase Activator

The identification of glucosylceramidase with beta-glucosidase activity on synthetic substrates was aided by the presence of an activator system for these enzymes. Activity on both natural or synthetic substrate was dependent on the interaction between two inactive components, one isolated from the spleen of a Gaucher patient (factor P), and the other from a control spleen (factor C; Ho and O'Brien, 1971; Ho et al., 1973). Factor C, presumably the glucocerebrosidase molecule, was a thermolabile particulate factor present only in control patients. Factor P was a thermostable soluble protein which accumulated in much higher concentrations in Gaucher patients (Ho and O'Brien, 1971; Ho 1973). The particulate factor C could be activated either by factor P or, less so, by sodium taurocholate, each with different kinetics (Ho, 1973). When solubilized, however, factor C was preferentially activated by sodium taurocholate. The binding of the enzyme to membrane thus appeared important, and further study showed that phosphatidic acid,

phosphatidylserine, phosphatidylinositol, and total membrane lipid enhanced the activation of factor C (Ho and Light, 1973). The true mechanism of activation is unknown, but the activators in this system may exert their effects by solubilizing or interacting with the enzyme, the natural substrate, or both. The enhanced sensitivity of metabolic control achieved by the involvement of multiple molecular components may suffer the consequences of multiple sites for mutation (Ho, 1973). The duplicated alpha globin gene locus similarly provides multiple targets for mutation giving rise to the alpha thalassemias, the severity of which is partly determined by the number of loci affected (Harris, 1980).

The physiological importance of the glucosylceramidase activator system only came into doubt after the purification of both Gaucher and control spleen activator proteins. The normal protein is different from the Gaucher activator in three ways: 1) it has a much reduced ability to activate glucosylceramidase (only 6% the degree of activation of the Gaucher protein), 2) it has a different amino acid composition, and 3) it is not a glycoprotein (Peters et al., 1977). Further, glucosylceramidase activity is stimulated more by sodium taurocholate or phosphatidylinositol alone than by control activator. Recent evidence in favour of simple structural alterations in the Gaucher enzyme (eg. Choy and Davidson, 1980b) challenges the necessity of activator involvement, but the nature of the Gaucher activator glycoprotein warrants further investigation.

Beta-Glucuronidase Activator

A unique posttranslational processing system functions in the intracellular localization of beta-glucuronidase and its multiple forms. In some tissues, substantial amounts of the enzyme are found associated with microsomes (M form) as well as lysosomes (L form), both arising from a single gene (Paigen et al., 1975). The molecular weight relationships suggest that cells assemble both lysosomal (L) and microsomal (X) tetramers, and the microsomal isoenzymes arise by successive addition of up to four units of the catalytically inactive protein egasyn. Egasyn (about 64,000d) probably anchors the hydrophilic beta-glucuronidase to the endoplasmic reticulum through its own hydrophobic character. Several strains of mice with allelic mutations for egasyn deficiency lack M form glucuronidase and have only trace amounts of the X form (Paigen 1979). Turnover studies indicate that synthesis of both lysosomal and microsomal enzymes is normal, but the microsomal form cannot maintain an association with the membrane in the absence of stabilizer protein, and is degraded more rapidly (Smith and Ganschow, 1978). Egasyn could localize other enzymes to membranes, but none have yet been identified (Lusis et al., 1977).

Likelihood of Activators for Alpha- and Beta-Glucosidases

If activator systems do exist for alpha- and beta-glucosidases, they are unlikely to function in the same fashion as

those just described. Because of the marked difference in amino acid composition between control and Gaucher glucosylceramidase activators, the simple absence of carbohydrate moiety in the former is probably not analagous to the absence of egasyn in beta-glucuronidase mutants. An activator for alpha-glucosidase would be different from other enzyme activators because the enzyme has no known lipid containing substrates, and therefore would not benefit from detergent-like activator-substrate interaction. It is conceivable that an activator could interact directly with the enzyme (perhaps by modifying it), or that in vivo the reactive complex is associated with lipid membranes.

DEVELOPMENT OF THE RESEARCH PROPOSAL

When two differently radiolabeled species of proteins are coelectrophoresed, any differences in label incorporation between the species should be reflected in an unusual ratio of the two isotopes at a particular gel position. When normal fibroblast cells were labeled with ^{3}H-leucine and Pompe fibroblast cells were labeled with ^{14}C-leucine, an abnormally low $^{14}C/^{3}H$ ratio was seen corresponding to a 29,000d species of soluble "mitochondrial/lysosomal" protein (Pena and Wrogemann, 1978). This species is similar in size to the 26,000d subunit of liver acid maltase (Bruni et al., 1970). When the same sample of protein was submitted to IEF, however, the deficiency in Pompe cells was at a pI of 7.9, which is much higher than the isoelectric point of the native enzyme (4.7). The same defect was seen

in fibroblasts of adult form acid maltase deficiency, but the difference between these cells and normal strains was less marked. Four hypotheses regarding the correlation of acid maltase deficiency and the deficient basic protein were proposed (Pena and Wrogemann, 1978):

(a) The defective basic protein is equivalent to acid maltase. In this instance, the basic protein could be a subunit of acid maltase. This is unlikely, since the intact liver enzyme has such a low pI and does not dissociate except under highly chaotropic conditions. The fibroblast enzyme however may have different properties, and the electrophoretic behaviour of purified fibroblast acid maltase should be tested. Alternatively, anti-fibroblast acid maltase antibodies might be tested for crossreactivity with the basic species.

(b) A third alteration secondarily led to deficiencies in both acid maltase activity and the basic protein. This might arise if the primary gene product is involved in a metabolic pathway common to both proteins. Theoretically, this could be a regulatory mutation or a posttranslational defect. A similar finding could be explained by an alteration in sedimentation characteristics of organelles from Pompe fibroblasts, such that slightly different organelle populations were being examined.

(c) Acid maltase deficiency caused the basic protein defect. It is possible that the novel defect is a metabolic or pathogenic

consequence of acid maltase deficiency. Many lysosomal storage diseases affect a large group of enzymes (eg. mucolipidosis II), presumably due to lysosomal engorgement and compensatory proliferation. The metabolic consequence is more likely than the pathogenic one, however, since fibroblasts deficient in acid maltase activity do not display the degree of glycogen accumulation seen in other affected tissues.

(d) Acid maltase deficiency is the result of the basic protein defect. This simplest explanation for this relationship is that the basic protein is an activator of acid maltase. Protein activators have recently been described for several lysosomal enzymes, and the small size of activator molecules made them attractive candidates.

EXPERIMENTS PERFORMED

The following is a summary of the experiments performed in the completion of this project.

A. The quality of executing both isoelectric focusing (IEF) and SDS polyacrylamide gel electrophoretic technique was verified by standard methods before use in experimentation.

B. The reproducibility of the cellular fractionation procedure was tested.

C. The nature of the subcellular fraction ML was established, and its quality compared in normal and Pompe fibroblast strains.

D. Several Pompe disese fibroblast strains (including original strains tested) and their control strains were double labeled, fractionated, and submitted to IEF. Gel fractions were then analyzed by liquid scintillation counting.

E. Several Pompe disease fibroblast strains (including original strains tested) and their control strains were double labeled, fractionated, and submitted to SDS-PAGE. Gel fractions were then analyzed by liquid scintillation counting.

F. Two Gaucher disease strains and their control strains were double labeled, fractionated, and submitted to SDS-PAGE. Gel fractions were then analyzed by liquid scintillation counting.

G. Several strains of Pompe disease, Gaucher disease, and control fibroblasts were single labeled and submitted to 2-D gel electrophoresis. The patterns were visualized by autoradiography.

H. A Duchenne muscular dystrophy fibroblast strain and a Gaucher fibroblast strain were each double labeled with their control strains and submitted to 2-D gel electrophoresis. Select spots were excised and analyzed by liquid scintillation counting.

SUMMARY OF RESULTS

The quality and reproducibility of all methods employed were judged good, and experimentation began with little hesitation. The ML fraction proved to be lysosomal in nature and differed little between a Pompe strain and its control strain. The ML-S fraction from Pompe strains, however, showed no consistent protein deficiencies in IEF or SDS-PAGE gels containing double labeled samples. Further, no consistent differences were observed between Pompe strains and normal strains, or among Pompe strains, in 2-D gels of ^{35}S-methionine labeled total cellular protein.

On the contrary, Gaucher disease strains showed abnormalities by both techniques. The total ML fraction of a double sample displayed a deficiency in a Gaucher protein of approximately 40,000d. Although this deficiency was not seen in autoradiograms of 2-D gels, a 50,000d spot was observed missing in the Gaucher strain. When the corresponding 50,000d protein was excised from a silver stained gel that contained a double labeled sample, the Gaucher strain was found to have incorporated 2.5 fold less radioactivity than the normal strain. A second Gaucher protein spot, which was not clearly missing in 2-D gel autoradiograms, displayed the same degree of deficiency.

Thus, employing original methods and new techniques that were successful in identifying abnormalities in fibroblasts of two other genetic disorders, I did not observe a basic protein deficiency in

Pompe disease fibroblasts.

CHEMICALS AND THEIR COMMERCIAL SOURCES

The following chemicals were obtained from SIGMA Chemical Company:

2-mercaptoethanol 4-methylumbelliferyl-alpha-D-glucopyranoside glycyl-L-phenylalanine-beta-napthylamide beta-napthylamine Tris glycine silver nitrate dimethylsulfoxide (DMSO) bromophenol blue dithiothreitol

The following chemicals were obtained from BIORAD Laboratories

sodium dodecylsulfate (SDS) ammonium persulfate glycine acrylamide

Coomassie brilliant blue N,N'-methylenebisacrylamide (BIS)

The following chemicals were obtained from BDH Chemical

sucrose sodium hydroxide citric acid ammonium hydroxide sodium phosphate

The following chemicals were obtained from Worthington Enzymes

deoxyribonuclese I-(DNase I) ribonuclease A (RNase A) ovomucoid bovine plasma albumin

The following chemicals were obtained from Amersham Corporation

L-[4,5]^{3}H-leucine (61Ci/mmol) NCS tissue solubilizer

L-[U-L]^{14}C-leucine (339mCi/mmol)

The following chemicals were obtained from Fisher Chemical Company

glacial acetic acid formaldehyde toluene glycerol

trichloroacetic acid disodium ethylenediaminetetracetic acid

hydrochloric acid TRITON X-100 sodium chloride

2-methyoxyethanol

The following chemicals were obtained from the companies specified:

urea (ultrapure) - Schwarz/Mann Inc.

Nonidet P-40 (NP-40) - Particle Data Laboratories

Ampholytes (Ampholines) - LKB Produkter

N,N,N',N' Tetramethylethylenediamine (TEMED) - Eastman Kodak Co.

agarose - Seakem (mCi Biomedicals)

ethanol - MCB

Omnifluor -New England Nuclear

orthophosphoric acid -J.T. Baker Chemical Co.

ethylenediamine -J.T. Baker Chemical Co.

glutaraldehyde - Polysciences Inc.

CELL CULTURE TECHNIQUES

Cells

The cultured human fibroblast strains used in this study are listed in Table I.

Solutions

Human McCoy's 5a - modified (HuMcFC10, standard growth medium)

63.3 g (one envelope) of medium concentrate(See Table II)

50ml 1% streptomycin sulfate, 10^7 I.U./l Penicillin

11g sodium bicarbonate

550ml fetal calf serum

5 litres deionized water

Stored in aliquots at $4^{o}C$.

Human McCoy's leucine or methionine deficient medium

This medium was prepared as outlined in Table II.

TRYPSIN

For subcultivation:

0.5 g hog pancreas trypsin

1.0g glucose

0.13g sodium bicarbonate

890ml deionized water

50ml saline D concentrate (0.16% NaCl, 0.8% KCl, 0.09% $NaHPO_4 \cdot 7H_2O$, 0.06% KH_2PO_4, 0.0024% phenol red).

Stored in aliquots at -20°C.

For harvesting:

0.02g hog pancreas trypsin

100ml deionized distilled water

Stored at -20°C.

All but the harvesting trypsin was sterilized by Millipore filtration, tested for sterility, and stored for less than two months.

Routine Cultivation

Cells were cultivated in 100mm plastic petri dishes (FALCON) by passaging them weekly and changing medium once between passages. Each confluent dish was rinsed with 2.0ml of warm trypsin, then incubated in 1.5ml of warm trypsin for 10min at 37°C.

The cells were then released by trituration, and 0.75ml of the suspension was rapidly transferred to each of two 100mm petri plates containing 10ml of warm growth medium. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity.

Cultivation for Experiments

In early experiments designed to reproduce those of Pena (1977), cells were seeded at a density of 300,000 per 100mm dish, and the medium changed every three days. Subsequently cells were seeded at a density of 500,000 per 100mm dish, and the medium changed every two days. The time elapsed between seeding and harvesting is indicated for individual experiments. In all final ^{35}S-methionine labeling experiments, cells were seeded at a density of 20,000 per 16mm microwell, and the 0.5ml of growth medium replaced every two days.

Thawing of Cells from Storage

Each vial removed from its liquid nitrogen storage tank was rapidly warmed to 37°C, and its contents were transferred to 5ml of warm growth medium in a 60mm plastic petri dish. This medium was replaced after 2hr incubation. Upon reaching confluency, (1-2 days) the cells from each 60mm dish were suspended in 0.8ml trypsin and transferred to 10ml warm growth medium in a 100mm dish.

Freezing of Cells for Storage

Each confluent 100mm was trypsinized as above, and the suspension mixed with 5ml medium to inhibit the trypsin. This mixture was then centrifuged for 5min at 1000rpm in a sterile plastic centrifuge tube. The supernatant was discarded and the cells suspended in a sterile solution of culture medium containing either 10% glycerol or 10% DMSO. The final suspension was transferred to a plastic NUNC serum tube (Vanguard International), kept frozen at -70°C for 24hrs, and then transferred to liquid nitrogen storage tanks.

Table I: List of human fibroblasts and their origin.

The following cell strains were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J.:

Cell Strain	Condition	Donor Age	Donor Sex
GM 23	Mother of Down's	31 yr	F
GM 41	Normal	3 mo	F
GM 244	Pompe - infantile	5 mo	F
GM 248	Pompe - infantile	4 mo	M
GM 302	Normal	10 mo	M
GM 372	Gaucher - type 3	29 yr	M
GM 443	Pompe adult form	30 yr	M
GM 495	Normal	29 yr	M
GM 877	Gaucher - type 2	1 yr	M
GM 1935	Pompe - adult form	30 yr	F
GM 3329	Pompe - adult form	23 mo	M

The following cell strains were obtained from the repository for Mutant Cell Strains, Montreal, Canada.

Cell Strain	Condition	Donor Age	Donor Sex
MCH 7	Normal	3 yr	M
MCH 40	Normal	6 yr	M
WG 482	Pompe - infantile	5 mo	M

The following cell strain was obtained from the Dept. of Paediatrics, Health Sciences Centre, Winnipeg, Canada.

Cell Strain	Condition	Donor Age	Donor Sex
DC	DMD	13 yr	M

Table II. Composition of Human McCoy's 5a (modified) medium (Neuman and McCoy, 1958; McCoy et al., 1959). Deficient media contained all but leucine or methionine, depending on which amino acid was used for labeling procedures.

McCoy's 5a (modified)

mg/liter	ingredient	mg/liter	ingredient
13.4	L-Alanine	1.0	p-Aminobenzoic Acid
42.1	L-Arginine-HCl	0.5	Ascorbic Acid
45.03	L-Asparagine-H_2O	0.2	d-Biotin
20.0	L-Aspartic Acid	0.2	d-Pantothenate-Ca
35.1	L-Cysteine-HCl-H_2O	5.0	Choline Chloride
22.1	L-Glutamic Acid	10.0	Folic Acid
219.2	L-Glutamine	36.0	i-Inositol
7.5	Glycine	0.5	Nicotinic Aid
21.0	L-Histidine-HCl-H_2O	0.5	Nicotinamide
20.0	L-Hydroxyproline	0.5	Pyridoxal-HCl
39.4	L-Isoleucine	0.5	Pyridoxine-HCl
39.4	L-Leucine	0.2	Riboflavin
36.5	L-Lysine-HCl	0.2	Thiamine-HCl
14.9	L-Methionine	2.0	Vitamin B_{12}
16.5	L-Phenylalanine	600.0	Bacto-peptone
17.3	L-Proline	6,460.0	Sodium Chloride
26.3	L-Serine	400.0	Potassium Chloride
17.9	L-Threonine	4,000.0	Dextrose
3.1	L-Tryptophan	2.5	Phenol Red
18.1	L-Tyrosine	0.5	Glutathione
17.6	L-Valine		
200.0	Calcium Chloride-Anhydrous		
97.67	Magnesium Sulfate-Anhydrous		
140.0	Monobasic Sodium Phosphate-H_2O		

Cell radiolabeling

All cells were incubated with radioisotope for 48hr under standard cultivating conditions.

(a) Standard Labeling Procedure

On the day of labeling, medium from each 10cm petri dish was aspirated and replaced with 7.0ml of Human McCoy's 5A (modified) containing 10% fetal bovine serum (FBS), plus 3.0ml FBS, giving a final serum concentration of 37%. One hundred μCi (200ul) of L-[U-^{14}C]leucine was added to each of two dishes of one strain, and 50uCi (50μl) of L-[4,5-^{3}H]leucine was added to each of two dishes of the other strain. Unless specified, the specific radioactivity of stock radioisotopes was 61Ci/mmol for ^{3}H-leucine and 339mCi/mmol for ^{14}C-leucine. The specific radioactivity leucine in the medium was calculated as follows:

$$\frac{\mu\text{Ci of radioisotope added}}{\text{picomoles of leucine in (medium + serum + radioisotope)}}$$

Leucine concentration in FBS was assumed to be the same as in newborn human plasma (0.95mg/100ml; Dickinson et al., 1965). For these standard labeling conditions, the specific radioactivity was 2.316 x 10^{-5}μCi/pmole for ^{3}H and 4.5714 x 10^{-6}μCi/pmol for ^{14}C.

(b) Labeling without a serum boost

In some instances, no extra fetal bovine serum was added, and

aspirated medium was replaced by 10ml of Human McCoy's 5A (modified) containing 10% FBS and radioisotope. The specific radioactivity of leucine in this medium was 1.8032 x 10^{-5}µCi/pmole for ^{3}H and 3.5965 x 10^{-6}µCi/pmole for ^{14}C.

(c) Labeling in human cord serum

In two experiments, the labeling medium contained 10% FBS plus 5% human cord serum and radioisotope. Under the assumption that the sera contained similar concentrations of leucine, the specific radioactivities were 1.8798 x 10^{-5}µCi/pmol for ^{3}H and 3.7195 x 10^{-6}µCi/pmol for ^{14}C.

Note: Processing of data by SCINT II (cf. Detection of radioactivity in gels) required entry of the reciprocal of the specific radioactivity.

(d) Labeling with ^{35}S-methionine

In one experiment two cell strains to be examined by 2-D gel electrophoresis (method of Garrels, 1979) were independently labeled with ^{35}S-methionine. Cells were given the standard 3.0ml boost of FBS along with 7.0ml of McCoy's medium (10% FBS). This was followed by 100uCi (12µl) of ^{35}S-methionine (1042Ci/mmol) in each 100mm petri dish.

In the final 2-D gels of single label samples (methods of O'Farrell,1975,O'Farrell et al.,1977) five different cell strains were independently labeled with ^{35}S-methionine. The cells were grown in 16mm

microwells, and labeled in methionine defficient medium. This labeling medium consisted of 0.21ml of McCoys modified medium containing no methionine, 0.07ml of FBS, and 25uCi of ^{35}S-methionine (1222 Ci/mmol) per microwell.

CELL FRACTIONATION

Fibroblasts were either scraped in 1.5ml per 10cm dish of phosphate-buffered saline, pH 7.4 (PBS), or trypsinized in 1.5ml per dish of 0.02% porcine pancreatic trypsin for 15 min at 37°C. Trypsin was inhibited with 0.3 ml of 0.6% ovomucoid, and cells released from the dish by trituration with a Pasteur pipette. From this point on, cells and fractions were kept at 4°C. Cells were washed three times in 5ml of cold PBS by suspension and centrifugation for 5 min at 1000 rpm in a Sorval SS-34 rotor (Sorval RC2-B centrifuge; Ivan Sorvall Inc., Norwalk, Connecticut). The final cell pellet was suspended in 4.0ml of 1mM EDTA, 5mM sodium phosphate, pH 7.4 and incubated for 15 min in an ice cold 7ml Dounce homogenizer. Cells were disrupted with 4 strokes of a tight-fitting glass pestle, and the homogenate restored to isotonicity with 1.3ml of 1M sucrose, 1mM EDTA, 5mM sodium phosphate, pH 7.4.

Fractionation was carried out according to Pena and Wrogemann (1978) in a Beckman L3-40 ultracentrifuge (Beckman Instr. Inc., Palo Alto, CA) equipped with an ω2t integrator accessory. Subcellular

particles were sequentially removed according to S_{min} values (sedimentation value of the minimal particle size in Svedborg units) of 90,000S, 2,300S and 40S. These centrifugations sedimented nuclei and unbroken cells, heavy and light mitochondria (including lysosomes), and microsomes respectively, leaving soluble protein in the final supernatant (Appelmans et al., 1955; de Duve et al., 1955). The integrator settings were determined from the following formula:

$$\int_0^t \omega^2 t = \frac{\ln R_{max}/R_{fluid} \times 10^{13}}{S_{min}}$$

where ω is the angular velocity in rad/s, t is the time in s, R_{max} and R_{fluid} are the radii from the axis of the centrifuge head to the bottom and top of the fluid column in the centrifuge tube respectively. It was thus possible to control for sedimentation during acceleration and deceleration, and for small variations in fluid height. For nuclei and unbroken cells, it was convenient to centrifuge the homogenate at 2,000 rpm in the Sorvall SS-34 rotor using the following conversion:

$$\text{time(min)} = \frac{\ln\left(\frac{R_{max}}{(\text{fluid height}) \sin\theta} \cdot \frac{10^{13}}{\text{min}}\right)}{\left(\frac{\pi}{30} \cdot \text{rpm}\right)^2 \cdot 60}$$

where fluid height is the vertical length of the homogenate fluid column, θ is the fixed angle of the rotor (34^{o}C for SS-34), and $\frac{\pi}{30}$ x rpm is equal to ω. Acceleration and deceleration times were negligible

at this rotational velocity.

Fluid heights were measured before each centrifugation and calculations performed, but the following values were usually obtained when four 10cm petri dishes were prepared in the above protocol:

90,000S centrifugation: 10-12 min at 2,000 rpm in Sorvall SS-34 rotor

2,300S centrifugation: 75-80 x 10^7 (running) plus 11 x 10^7 (decelerating) rad^2/s (approximately 10 minutes running time at 15,000 rpm in Beckman 65 rotor).

The solubilized mitochondrial-lysosomal protein was prepared by freezing-thawing the washed 2,300S pellet in 600ul of deionized distilled water (DDW).

ASSAY OF ACID MALTASE ACTIVITY

Cells were harvested and fractionated as described above, the samples sonicated briefly (Biosonik; Bronwill Scientific, Rochester, NY), and submitted to five cycles of freezing in an ethanol/dry ice bath and thawing. Freeze-thawed samples were centrifuged for 10 min at 100,000xg in a Beckman airfuge (Beckman Instr. Incorp., Palo Alto, CA) and supernatants withdrawn for assay. An assay using alpha-4MUG as substrate and 4MU as standard was employed (Fujimoto et al., 1976). The reaction mixture contained 50µl of sample, 200µl of a 9:1 (v:v) mixture of McIlvaine's buffer (citrate-phosphate), pH 4.0 and 60mM

alpha-4MUG in 2-methoxyethanol. The substrate was thus 4.8mM in the incubation mixture. The 30 min incubation at 37°C was terminated by adding 2.8ml of cold glycine-NaOH, pH 10.5. Fluorescence at an excitation wavelength of 366nm and an emission wavelength of 450nm was determined on an Aminco-Bowman spectrofluorometer (American Instr. Co., Silver Spring, MD). Protein concentrations were determined by the method of Lowry et al.(1951) using bovine plasma albumin as a standard. The specific activity was expressed as nanomoles of alpha-4MUG hydrolyzed per hour per mg of cell protein.

ASSAY OF DIPEPTIDYL AMINOPEPTIDASE-I ACTIVITY

Dipeptidyl aminopeptidase I (DAP-I, cathepsin C) was measured as a lysosomal marker enzyme (Barret and Heath, 1977) by the method of Gelman et al. (1980). The release of beta-napthylamine from the substrate glycyl-L-phenylalanine-beta-napthylamide was measured fluorometrically at an excitation wavelength of 365nm and an emission wavelength of 460nm on the Aminco-Bowman spectrofluorometer. The reaction mixture contained 0.1mM substrate, 1.0mM dithiothreitol (DTT), 30mM sodium chloride, 1.0mM EDTA, 0.02% Triton X-100, 50mM sodium acetate buffer, pH 5.0 and 50µl of sample in a final volume of 200µl. The reaction was terminated after 30 min at 37°C by adding 2.0ml of cold 50mM glycine-NaOH, pH 10.4, 5mM EDTA. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine plasma albumin as standard. The specific activity was

expressed as nanomoles of substrate hydrolyzed per hour per mg cell protein.

Aliquots of frozen incubation buffer stock without DTT was diluted with frozen aliquots of DTT stock on the day of the assay. Substrate stock was 8.6mM dissolved in DMSO and was good for 3 weeks in a dark bottle at 4°C. Beta-napthylamine stock was 20mM in ethanol and was good for 1mo in a dark bottle at 4°C.

GEL ELECTROPHORESES

Solutions

The composition of solutions that are not defined in the text of the described methods may be found in Appendix A.

Isoelectric Focusing (IEF)

(a) Sample preparations

Method of Pena (1977)

The supernatant of samples that were freeze-thawed in water was incorporated into the gel mixture directly.

Method of Garrels (1979)

Procedures were carried out quickly with precooled materials at 4°C.

WHOLE CELLS:

Cells were rinsed three times in cold phosphate buffered

saline and approximately 500µl/mg cell protein of Staphylococcal nuclease solution was added. The cells were scraped with a rubber policeman, and disrupted by several passages through a 1 ml disposable syringe with a 26 gauge needle. A small aliquot (approx. 25µl) was removed for protein determination, and the rest transferred to a 1.5ml Eppendorf microcentrifuge tube. This was immediately mixed with 0.12 volumes of 3% SDS, 10% 2-mercaptoethanol, followed by 0.12 volumes of DNase I-RNase A within the next 10s. Before 15s had elapsed, the sample was frozen and submitted for lyophilizing. Samples were redissolved immediately after vacuum release in sample buffer to give a final SDS concentration of 0.72% (Garrels recommends 0.3%, but a higher protein concentration is necessary for staining of unlabeled samples).

MITOCHONDRIAL/LYSOSOMAL PELLET:

The ML-pellet was suspended in 500µl/mg protein of Staphylococcal nuclease buffer (i.e. without micrococcal nuclease) and mixed with 0.12 volumes of 3% SDS, 10% 2-mercptoethanol. The sample was quickly frozen, lyophilized and resuspended in sample buffer to give a final SDS concentration of 0.3%.

Prepared samples were stored at -70°C.

Method of O'Farrell (1975)

Confluent fibroblasts were scraped directly in 250µl of lysis buffer per mg of cell protein. Samples were transferred to capped microcentrifuge tubes and stored at -70°C.

(b) Gel composition

Composition of Pena (1977)

Gels containing 1% ampholytes (pH 3.5-10.0), 5.0% acrylamide, 0.17% N,N'-methylenebisacrylamide (BIS), 5.0% sucrose, 0.005% ammonium persulfate, and aqueous sample were cast to a height of 16.0cm in 0.6 x 20.0cm glass tubes. The gels were overlayed with water, which was removed after the gel polymerized.

Modified Composition of Garrels (1979)*

Gels containing 2% ampholytes (pH 6-8), 3.8% acrylamide, 0.2% Bis, 4% NP-40, 9.5M urea, 0.001% ammonium persulfate and 0.07% N,N,N',N'-tetramethylethylenediamine (TEMED) were cast to a height of 12.0cm in 0.12 x 16.0cm glass tubes. The gels were not overlayed but unpolymerized acrylamide was removed before prefocusing.

*The acrylamide stock was modified from 28.35%T;1.35%C to 30%T;1.0%C as described by O'Farrell (1975). (T - total acrylamide, C - crosslinking acrylamide).

Composition of O'Farrell(1975)

IEF gels containing 2% ampholytes (1.6% pH 5-7,0.4% pH 3.5-10), 3.8% acrylamide, 0.2% Bis, 2% NP-40, 9.2M urea, 0.001% ammonium persulfate, 0.07% TEMED were cast to a height of 12.0cm in 0.2 x 16.0cm glass tubes. The gels were overlayed with 8M urea for 1 to 2

hours. This was replaced by 50µl of lysis buffer, which in turn was overlayed with 50 µl of water. Both lysis buffer and water were removed before prefocusing.

Composition of O'Farrell et al.(1977)

NEPHGE gels contained 2% pH 7-9 ampholytes,0.002% ammonium persulfate, 0.14% TEMED, but were identical to O'Farrell IEF gels in all other respects.

(c) Electrophoretic conditions

All gels were focused in custom designed chambers, in which electrolyte reservoirs were separated by a long cylinder for direct water cooling of tubes.

Method of Pena (1977)

The upper reservoir contained the anolyte 5% orthophosphoric acid, and the lower reservoir contained the catholyte 5% ethylenediamine. Power was supplied by an Ortec 4100 pulsed constant power supply in the following regimen: 50V for 0.5hr, 100V for 1hr, and 150V for 16hr, all at a pulse rate of 50pps at 0.5 microfarads. To sharpen zones, the power was then raised to 300V (100pps) for 2 min and then 400V (200pps) for 2 min. A cooling temperature of 4°C was maintained throughout the procedure.

Method of Garrels (1979)

The upper reservoir contained the catholyte 0.1M sodium hydroxide and the lower reservoir contained the anolyte 0.01M orthophosphoric acid. Dialysis membrane, secured with rings of Tygon tubing prevented the gels from sliding out of glass tubes while focusing. The air bubble at the top of each tube was released while applying 10µl of IEF sample overlay buffer. Power was applied in the following regimen:

Prefocusing - 300V, current noted; voltage raised gradually until 1000V was reached (approx. 45 min), never exceeding the current recorded initially at 300V.

Sample Application - Less than 30µl of sample was then applied beneath the overlay solution.

Sample Focusing - 1000V for 19hr.

A cooling temperature of 28°C was maintained throughout the procedure.

Method of O'Farrell (1975)

The upper reservoir contained the catholyte 0.02M sodium hydroxide and the lower reservoir contained the anolyte 0.01M orthophosphoric acid. Dialysis membrane was optional for these gels. The air bubble at the top of each tube was released while applying 40µl of lysis buffer. Power was applied in the following regimen:

Prefocusing - 200V for 0.25hr, 300V for 0.50hr, 400V for 0.50hr.

Sample application - Lysis buffer and sodium hydroxide were removed from the tubes and less than 50µl of sample was applied to the gel. This was overlayed with 40µl of sample overlay

solution.

Sample focusing - 400V for 12hr, 800V for 1hr.

A cooling temperature of 20°C was maintained throughout the procedure.

Method of O'Farrell et al. (1977)

The upper resevoir contained the anolyte 0.01M orthophosphoric acid and the lower resevoir contained the catholyte 0.02M sodium hydroxide. Samples were electrophoresed from anode to cathode with power applied at 400V for 4hr without prefocusing. Conditions were otherwise identical to isoelectric focusing (O'Farrell, 1975).

SDS-POLYCRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

(a) Sample preparation

Method of Pena (1977)

Samples were suspended in 1% SDS, 62.5mM Tris-HCl, pH 6.8 and heated for two minutes at 100°C. Samples were then made 1% in 2-mercaptoethanol and heated for another minute at 100°C. This treatment was followed by the addition of 0.20 volumes of 40% sucrose and 10µl of 0.05% aqueous bromophenol blue. In all samples, the ratio of SDS to protein (w:w) was greater than three. Less than 300µg protein in no more than 150µl was loaded on each gel (Weber and Osborne, 1975).

Method of O'Farrell (1975)

SDS-PAGE as described by O'Farrell (1975) for 2-D gels was used for both 1-D and 2-D gel electrophoresis. For single dimension SDS-PAGE, samples were prepared as above, but the bromophenol blue was left out and added to the upper reservoir buffer before electrophoresis. For gels of 0.075cm thickness, less than 25µl of sample containing no more than 15µg of protein was applied to each 0.4cm wide lane in the stacking gel.

For 2-D gels, isoelectric focusing gel rods were equilibrated at room temperature for 1 to 2hr in 5ml SDS sample buffer to ease the entry of proteins into the SDS slab gel. Unequilibrated gels required a special treatment described later (cf. electrophoretic conditions).

(b) Gel composition

Composition of Pena (1977)

Separating gels containing 13% or 10% acrylamide, 0.26% or 0.20% Bis respectively, 0.1% SDS, 6M urea, 375mM Tris HCl pH 8.6, 0.04% ammonium persulfate and 0.06% TEMED were cast to a height of 20.0cm in 0.6 x 26cm glass tubes. After polymerization (approx. 1.0hr), the water overlay and unpolymerized acrylamide were removed and 2cm of a stacking gel containing 3.0% acrylamide, 0.38% Bis, 0.1% SDS, 62.5mM Tris-HCl, pH 6.8, 0.10% ammonium persulfate and 0.125% TEMED was poured and overlayed with water. The gel polymerized in about 0.5hr.

Composition of O'Farrell (1975)

Separating gels contained 13% or 10% acrylamide, 0.35% or 0.27% Bis respectively, 0.1% SDS, 375mM Tris-HCl, pH 8.8, 0.006% ammonium persulfate and 0.0006% TEMED, and were poured in slabs of 0.075 or 0.15cm thickness. Gels were overlayed with water-saturated isobutanol. After polymerization (about 1hr),the overlay was removed and a stacking gel containing 4.4% acrylamide, 0.12% Bis, 0.1% SDS, 12.5mM Tris-HCl, pH 6.8, 0.008% ammonium persulfate and 0.003% TEMED was cast to a height of 2cm or greater. For single dimension gels, teflon slot formers were inserted into the stacking gel and removed after polymerization was complete (about 0.5hr).

(c) Electrophoretic Conditions

Method of Pena (1977)

Tube gels were electrophoresed in a long chamber of the same design as that used for isoelectric focusing, with direct water cooling separating the electrophoresis buffer reservoirs. Both reservoirs were filled with 50mM Tris, 385mM glycine, 0.1% SDS, and samples were electrophoresed from the upper cathode to the lower anode at 60V until the tracking dye reached the separating gel, followed by 120V for approximately 24hr, when the tracking dye was a few cm from the bottom of the tube. A cooling temperature of 20^{o} was maintained throughout the procedure.

Method of O'Farrell (1975)

For 1-D gels and the second dimension of equilibrated isoelectric focusing gels, both electrode buffer reservoirs (BIORAD model 220 or BIORAD "Protean" vertical slab electrophoresis cells) were filled with SDS running buffer, and a cooling temperature of 15°C was applied. Two hundred and fifty µl of 0.05% bromophenol blue was added to the upper (cathodal) reservoir, and a constant current of 20ma per gel was applied until the tracking dye was a few cm from the bottom (4-6hr depending on gel thickness and length).

Unequilibrated isoelectric focusing gels, for which the elution of protein during equilibration was undesirable, were first electrophoresed for 0.5hr at 20ma per gel with high SDS (2%) running buffer in the cathodal reservoir and regular running buffer in the anodal reservoir. The upper (cathodal) buffer was replaced by regular SDS running buffer and bromophenol blue, and electrophoresis continued as above.

GEL STAINING PROCEDURES

(a) Single Dimension Tube and Slab Gels

Gels were fixed and stained for 24hr in 50% TCA, 0.1% coomassie brilliant blue R250, and destained in 25% methanol, 8% acetic acid.

(b) Two dimensional gels of O'Farrell (1975)

Method of Anderson and Anderson (1977)

Gels were fixed and stained in 47.5% ethanol, 0.1% Coomassie brilliant blue R250, 5% acetic acid and destained as follows: 40% ethanol, 3% acetic acid, 1-2hr, repeat; 30% ethanol, 3.5% acetic acid, 1-2hr.

Method of Oakley et al.(1980; Silver-staining method)

Gels were fixed for 30min in 10% glutaraldehyde, rinsed three times in DDW at 10 minute intervals, then soaked in DDW overnight. Water was drained, replaced with freshly prepared ammoniacal silver solution (1.4ml fresh ammonium hydroxide, 21ml 0.36% NaOH, 4ml 19.4% $AgNO_3$ [slowly with agitation], 73.6ml DDW), and the vessel agitated for 15min. The gel was transferred to another dish to wash in DDW for 2 min, and again into a freshly prepared solution of 0.005% citric acid, 0.019% formaldehyde until desired degree of staining is reached. Overstained gels were destained with 25% KODAK rapid fixer solution A in DDW.

PRESERVING OF GELS

Wet gels were stored in 8% acetic acid

Slab gels were soaked in 10% acetic acid, 1% glycerol for 1hr before drying on Whatman #1 filter paper in a BIORAD model 224 slab gel drying apparatus. Gels 0.075cm and 0.15cm required 1hr and 2hr of heating under vacuum respectively.

DETECTION OF RADIOACTIVITY IN GELS

Liquid scintillation analysis of single dimension gels

Gels were sliced in 2mm fractions, minced, and extruded directly into Beckman Poly-Q plastic scintillation vials by a Gilson Aliquogel fractionator. After drying overnight in an oven at 45°C each vial was prepared for counting by addition of the following reagents (a) 80µl H_2O (b) 500ul NCS tissue solubilizer, incubated for 2hr at 45°C, (c) 40µl glacial acetic acid, (d) 10ml of 0.4% Omnifluor in toluene. Vials were capped, mixed well, and counted in a Beckman LS-7500 liquid scintillation system. Variable quench of samples was corrected automatically through determinations of the Compton edge from an external gamma source (Long, 1977). Quench curves were prepared as described by Boeckx et al. (1973), and counting efficiencies were approximately 34% for ^{3}H and 76% for ^{14}C.

Liquid scintillation analysis of 2-D gels

Stained gels were photographed, and selected spots were excised by puncture with hematocrit tubes (i.d. 1mm). For large spots, the single puncture was made in the upper middle area of the darkest staining region. Punctures were also made in different blank regions of the gel, including those in proximity with the spots of interest. The gel samples were extruded into plastic scintillation vials, dried, and prepared as 1-D gel samples (see above).

Analysis and plotting of data

Liquid scintillation data were punched on paper tape and analyzed by a Control Data Corporation Cyber 171 computer system using the computer program SCINT II (Wrogemann et al., 1977). The program converted counts per minute to disintegrations per minute, calculated the picomoles of labeled amino acid incorporated through the specific radioactivity of leucine in the labeling medium, normalized the incorporation of each radioisotope, and determined the normalized ratio of ^{14}C to ^{3}H. The picomoles, % picomoles, and ratio of percent ^{14}C picomoles to percent ^{3}H picomoles were plotted as a function of vial number on a Zeta Research Industries plotter.

Autoradiography of 2-D Gels

Kodak X-OMAT or FUJI Rx X-ray films were placed over dried slab gels exposed in cassettes, and the films developed for 7 min in Kodak D-19 developer, fixed for 5 min in Edwall quick-fix and washed for 0.5hr in water.

RESULTS

PRELIMINARY DATA

Several preliminary experiments were performed to evaluate the quality and reproducibility of the techniques. These experiments included comparison of the separative techniques and cell fractionation procedures performed here with those originally employed by Pena (1977). As a precaution, enzyme deficiencies in all fibroblast strains from diseased individuals were confirmed. In addition, the lysosomal nature of Pena's (1977) ML fraction was verified.

Reduced Alpha-glucosidase activity in Pompe disease

Several fibroblast strains derived from normal donors and individuals with Pompe disease were assayed for alpha-glucosidase activity to ensure that they were correctly designated. All Pompe strains showed very low levels of activity on the artificial substrate alpha 4-MUG compared to control strains (Table III).

Reduced Beta-glucosidase activity in Gaucher disease

The Gaucher and control fibroblasts and used in my studies were assayed for beta-glucosidase activity by Dr. M.C. Stephens. The Gaucher strains displayed lower beta-glucosidase activity than control strains (Table IV). The assay was performed with the artificial substrate 4-methylumbelliferyl-beta-D-glucopyranoside at pH 5.0.

SDS-PAGE

Molecular weight standardization

Four major types of SDS-PAGE gels were electrophoresed: 10% and 13% tube gels, and 10% and 12% slab gels. A standard curve was prepared for all but 10% slab gels (Figs. 1-3), but in preliminary 10% slab gels, the molecular weight markers phosphorylase a (approx. 94,000d) and carbonic anhydrase (approx. 29,000d) were applied to the second dimension of 2-D gels, and used to establish reference spots for these two areas of interest. The reference spots were reliable internal markers of molecular weight,and their positions denoted by arrows in the final autoradiograms. The tube gels differed from the slab gels in that they contained urea (6M) and they were electrophoresed with an electrode buffer containing twice the concentration of glycine. The relative mobilities (R_f) of marker proteins in my gels were slightly different from those of Pena(1977), but such variation is known to occur. The reason for the non-linearity of the curves in slab gels is unknown, but these mobilities were consistent in several experiments. Low molecular weight proteins are known to deviate from the linearity of $\log R_f$ vs molecular weight, and perhaps the absence of urea enhanced the deviation in the slab gels (Weber and Osborne, 1975).

Separation of protein samples

The resolution of proteins in tube gels is displayed in Fig. 4. The better resolution of unlabeled total fibroblast protein in 1-D

slab gels (Fig. 5) led to the electrophoresis of sequential subcellular fractions from two pairs of normal versus Pompe fibroblast strains by this technique (Fig. 6). No major differences were seen among whole cell homogenates (WC) or among mitochondrial/lysosomal (ML-T) fractions. The differences seen in soluble ML fractions (ML-S) were likely due to differences in the amount of protein applied. Nevertheless, the fractionation demonstrates purification by exclusion of some protein bands in each step.

Electrofocusing - pH Gradients

Three major types of electrofocusing were performed: isoelectric focusing by the method of Pena (1977) or O'Farrell (1975), and non-equilibrium pH gradient electrophoresis (NEPHGE) by the method of O'Farrell et al. (1977). Fig. 7 represents a gel of the first type that was electrophoresed simultaneously with a gel containing double labeled sample. The gel was sliced automatically into 2mm sections and two of these were combined for each pH measurement. Deionized distilled water was degassed, 500ul was added to each fraction, and the fractions were incubated overnight at 4°C. pH was determined at 4°C with a Radiometer (Copenhagen, Denmark) pH-microelectrode. The profile is nearly identical to that of Pena (1977), covering a broad pH range which is very linear between pH 4.0 and pH 8.0. This range and linearity was maintained in subsequent gels examined this way. To cover this range in the first dimension of 2-D gels, two separate electrofocusing methods were required (IEF & NEPHGE). The pH gradient

profiles of these gels is presented in Figs. 8 and 9. The individual gel fractions were prepared as above but gels were measured at 20°C.

The fractionation procedure - Dobbs vs Pena

The subcellular distribution of protein and cpm, notably in the ML fraction, was similar in my experiments and in those of Pena (1977;Table V). It is therefore unlikely that there were any major differences in the subcellular fractions being examined, and thus, that the species of protein containing the reported defect was not being screened. Several experiments measuring the level of alpha glucosidase in different subcellular fractions showed that the ML fraction contain did the highest specific activity of this enzyme, and that the activity was enriched approximately three-fold with respect to the whole cell homogenate (Table VI). The enrichment in activity was not expected to exceed the reduction in protein content from the homogenate to the ML fraction, which was approximately six-fold. The remaining activity, which should have been in the ML-S, may have been lost to the nuclear fraction, which contains unbroken cells, and the cytosolic fraction, which contains soluble protein from lysed organelles. The high recovery indicates that the enzyme was probably not significantly degraded during the assay.

The lysosomal nature of the ML fraction

In a separate experiment, the sedimentation pattern of a second lysosomal enzyme (Barrett and Heath, 1978), dipeptidyl

aminopeptidase I (DAP-I) resembled that of alpha glucosidase. Originally, the fractionation procedures of Pena (1978) was employed, and the activity of both enzymes was determined in each fraction. The treatment, however, did not permit the recovery of enough DAP-I activity for valid comparison. As a recourse, the fractionation procedure of Gelman et al. (1980) was employed, and provided two important observations. First, the specific activity ratios of acid maltase to DAP-I in the different subcellular fractions was constant (Table VII), suggesting that the L fraction did contain both enzymes and was therefore lysosomal in nature. Second, the relative specific activities of DAP-I in subcellular fractions of both normal and Pompe strains was similar (Table VIII), suggesting that the lysosomes containing DAP-I were not much different in sedimentation characteristics between the two strains. Lysosomal size heterogeneity could still exist outside the enzymes studied, but the above result reduces the likelihood that Pena's (1977) observation was generated in such a fashion.

The above experiments provided the foundation for attempting to reproduce Pena's finding of the basic protein defect in fibroblasts from Pompe's individuals. The procedures used were readily learned and standard tests proved them to be very similar to the original method. Further, the ML fraction was demonstrated to be enriched in lysosomes, and the enrichment was similar in normal and Pompe strains. This last

observation detracts from the hypothesis that the defect was a secondary consequence of alpha glucosidase deficiency and resulted from altered lysosomal size.

Table III: Reduced alpha-glucosidase in Pompe disease patients.

Cell Strain	Donor Age	Donor Sex	Specific Activity
Normal Strains			
LMF	Fetal	M	117
GM 23	31 yr	F	200
GM 41	3 mo	F	75
GM 302	10 mo	M	72
Pompe Strains			
GM 244	5 mo	F	12
GM 248	4 mo	M	4
GM 443	30 yr	M	19
GM 1935	30 yr	F	2
GM 3329	23 mo	M	3
WG 482	5 mo	M	<1

* Activity is expressed as nmoles of 4-methylumbelliferyl-alpha-D-glucoside hydrolyzed per hour per mg cell protein.

Table IV: Reduced beta-glucosidase in Gaucher disease patients.

Cell Strain	Donor Age	Donor Sex	Specific Activity
Normal Strains			
GM 302	10 mo	M	31
GM 495	29 yr	M	166
Gaucher Strains			
GM 372	29 yr	M	12
GM 877	1 yr	M	3.5

* Activity is expressed as nanomoles of 4-methylumbelliferyl-beta-D-glucoside hydrolyzed per hour per mg cell protein.

Note: These data were communicated by Dr. M.C. Stephens.

Table V: Similarity of the fractionation procedure. Subcellular distribution of protein and cpm in radiolabeled human fibroblast cell strains (% of whole cell homogenate).

Fraction	PENA (1977)[1]			DOBBS (1982)[2]		
	^{3}H	^{14}C	Protein	^{3}H	^{14}C	Protein
N	21.9 ± 2.7	22.8 ± 2.08	20.6 ± 3.8	18.3	16.2	16.7
ML	13.7 ± 2.18	13.6 ± 1.36	9.9 ± 0.35	17.0	20.1	11.7
P	13.0 ± 1.42	12.6 ± 1.18	10.3 ± 0.76	4.7	3.7	5.4
S	47.2 ± 2.39	47.6 ± 2.14	53.0 ± 2.79	47.9	51.4	64.0

1. Data are expressed as the mean ± standard error from seven different experiments.

2. Data analyzed from one experiment only.

Table VI: Enrichment of alpha glucosidase in subcellular fractions prepared by the method of Pena (1977).

Fraction	Units[1] of Activity	Specific Activity[2]	Relative Specific Activity
Total homogenate	42.4	95.9	1.0
Pellet N (90,000S)	6.0	159.3	1.66
Pellet ML (2,300S)	13.3	343.3	3.56
S Fraction (2,300S supernatant)	17.8	22.5	0.23
Units Recovered	31.1/42.4 = 87.5%		

1. Units are expressed as nanomoles of alpha-4MUG hydrolyzed per hour.

2. Specific activity is expressed as units per mg cell protein.

Table VII: Relative activities of two lysosomal enzymes in a normal fibroblast strain (GM302). Data are expressed as ratios of specific activity of acid maltase to dipeptidyl aminopeptidase-I in different subcellular fractions.

Fraction	Ratio AM/DAP-I
WC	2.6*
N	3.7
L	3.9
PLS	3.9

* This low ratio is consistent with recovery calculations indicating that DAP-I activity was less stable during subcellular fractionation.

Table VIII: Relative specific activity of lysosomal enzymes in normal and Pompe disease fibroblast strains. Data are expressed as ratios of specific activity in fractions compared to whole cell homogenate (WC).

		DAP-I		AM
Fraction	Strain	GM248	GM302	GM302
WC		1.00	1.00	1.00
N		0.51	0.38	0.53
L		1.20	1.20	1.80
PLS		0.16	0.24	0.36

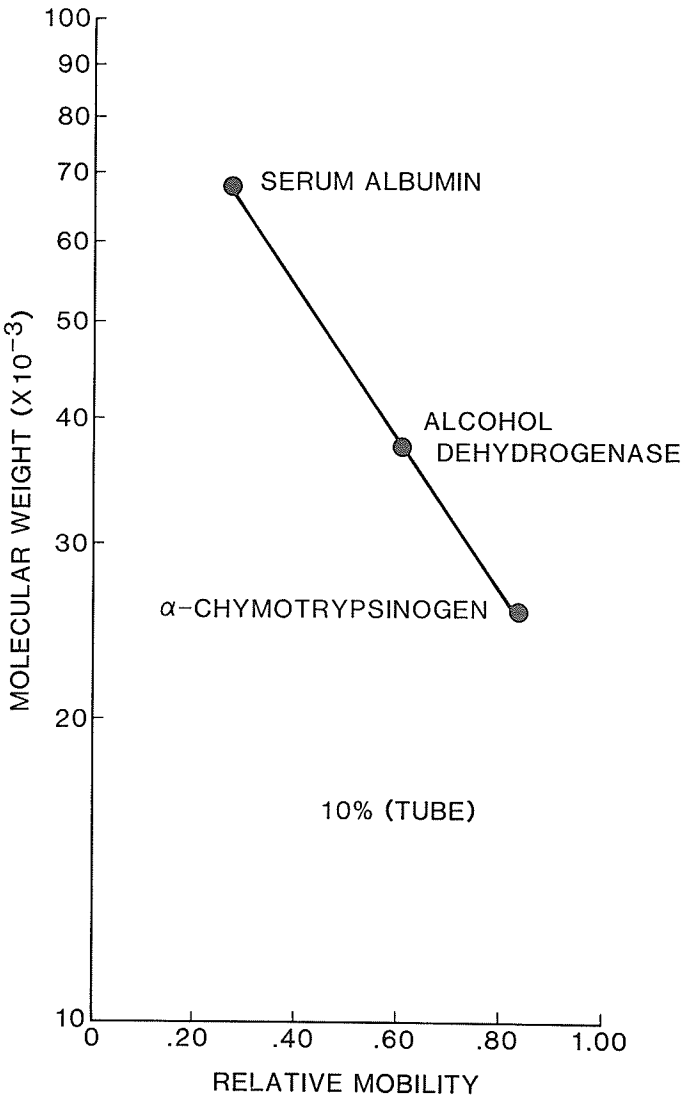


Fig. 1. Molecular weight standard calibration curve for 10% SDS-PAGE tube gels prepared by the method of Pena (1977).

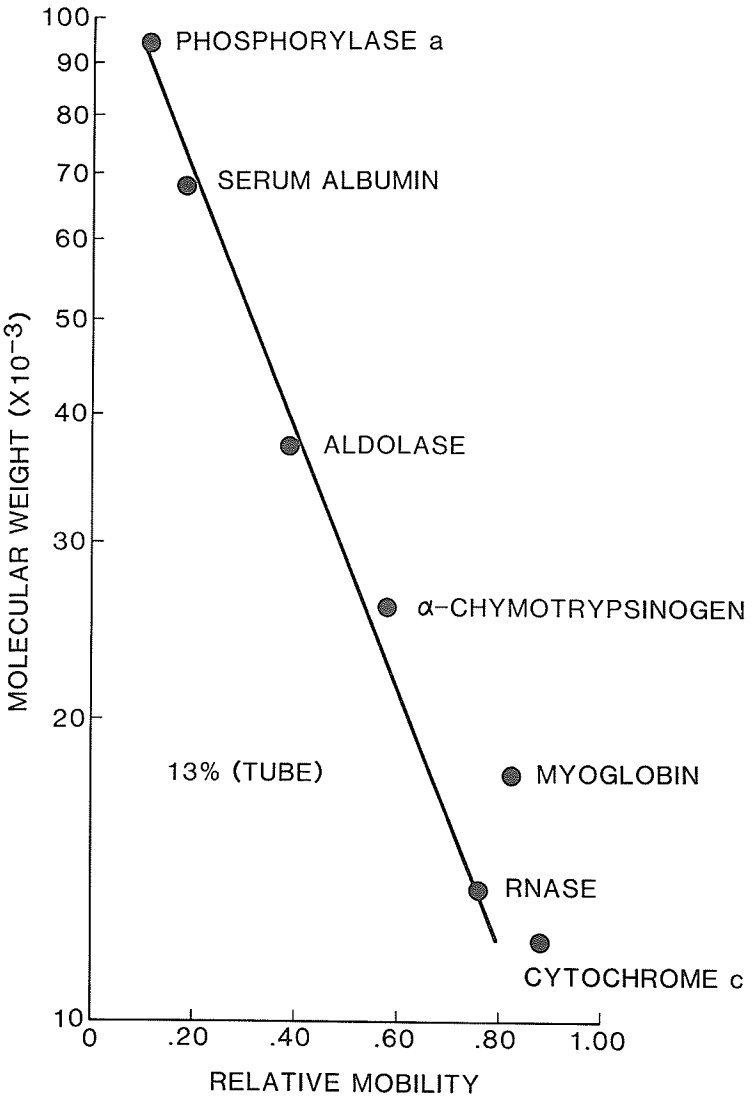


Fig. 2. Molecular weight standard calibration curve for 13% SDS-PAGE tube gels prepared and electrophoresed by the method of Pena (1977).

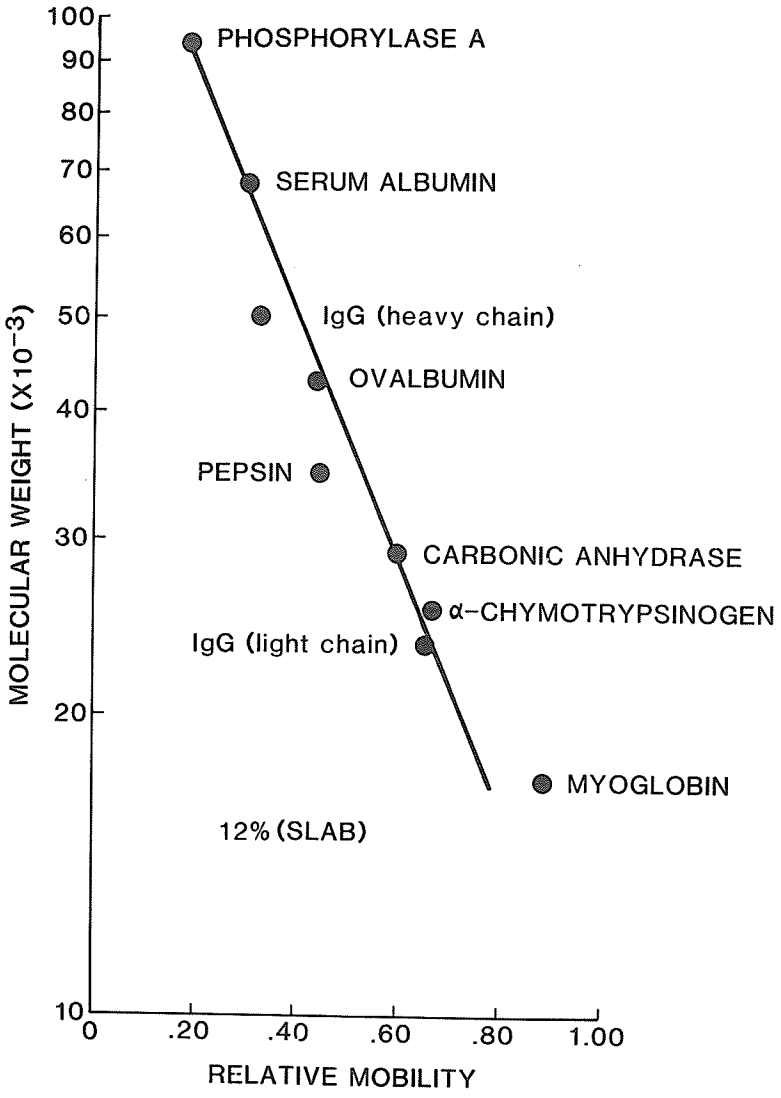


Fig. 3. Molecular weight standard calibration curve for 12% SDS-PAGE slab gels prepared and electrophoresed by the method of O'Farrell (1975).

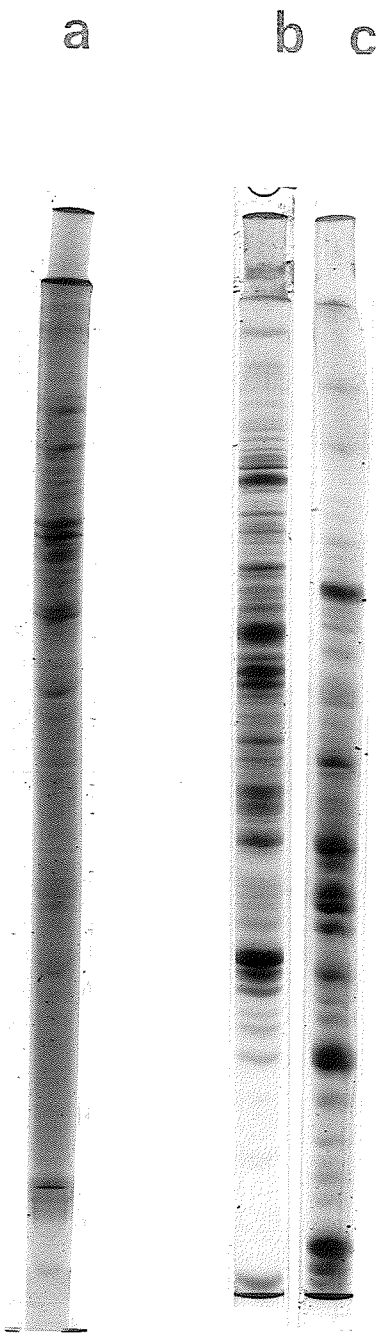


Fig. 4. Separation of unlabeled protein species by SDS-PAGE in tube gels (a) ML fraction from cultured fetal fibroblasts (13% acrylamide) (b) + (c) 100,000xg supernatant from hamster liver homogenate in 13% and 10% acrylamide respectively. Gels were stained with Coomassie blue in TCA.

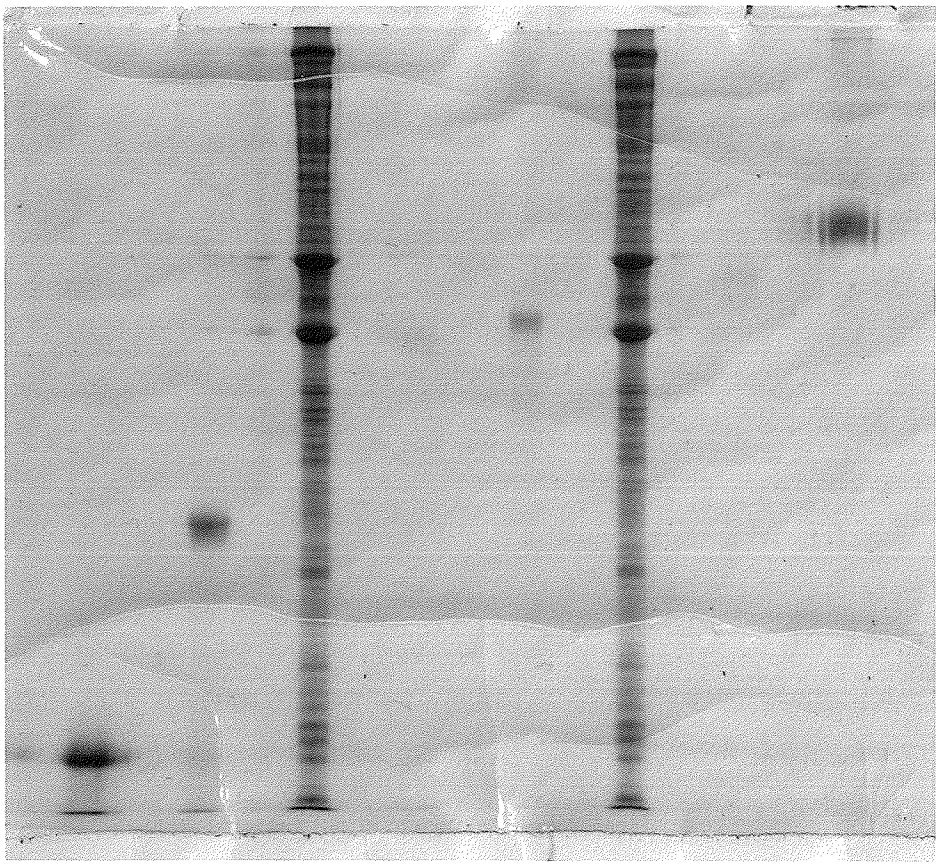


Fig. 5. Separation of unlabeled protein species by SDS-PAGE in slab gels (12% acrylamide). The two prominent lanes represent identical samples of total LMF (fetal) fibroblast protein. The other lanes contain molecular weight marker proteins. The gel was stained with Coomassie blue in TCA.

a.

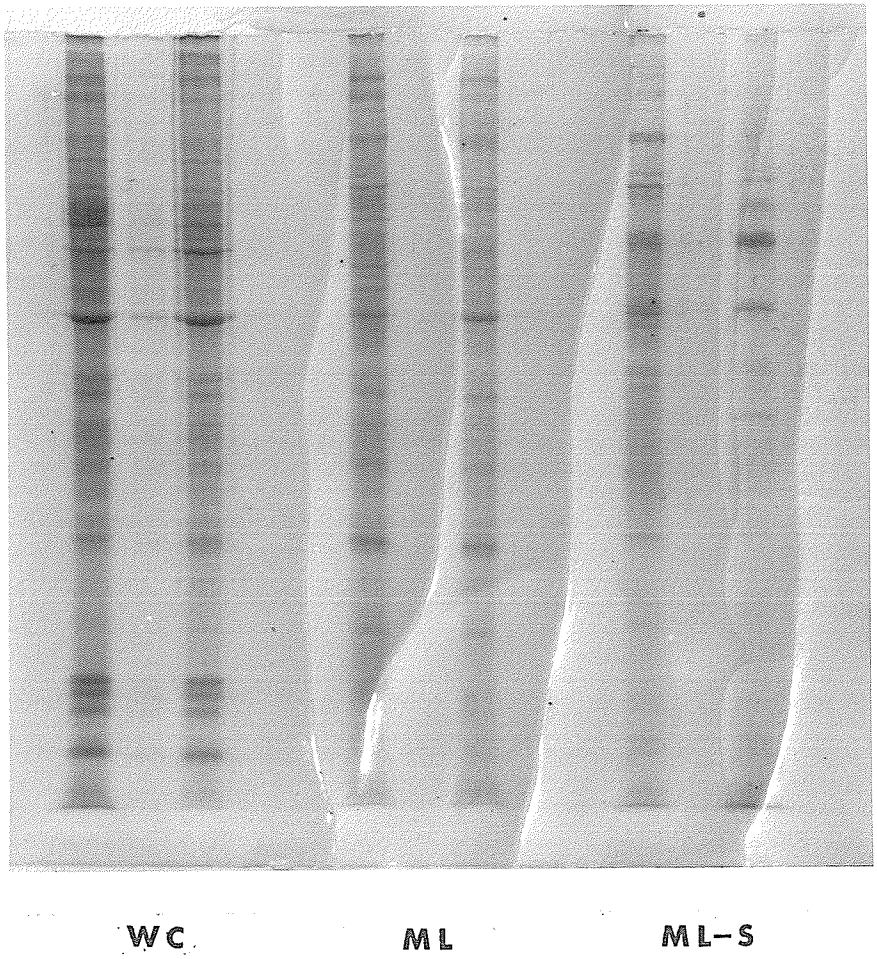


Fig. 6. Separation of unlabeled protein species by SDS-PAGE in slab gels. Normal (left) and Pompe (right) fibroblast preparations are run side by side, with roughly equal amounts of protein in all lanes (a) Strain MCH 7 versus GM 3329; the pairs of lanes represent total cell homogenate, total ML fraction, and ML-S fraction from left to right (b) As in (a), with strain MCH 49 versus WG 482.

b.

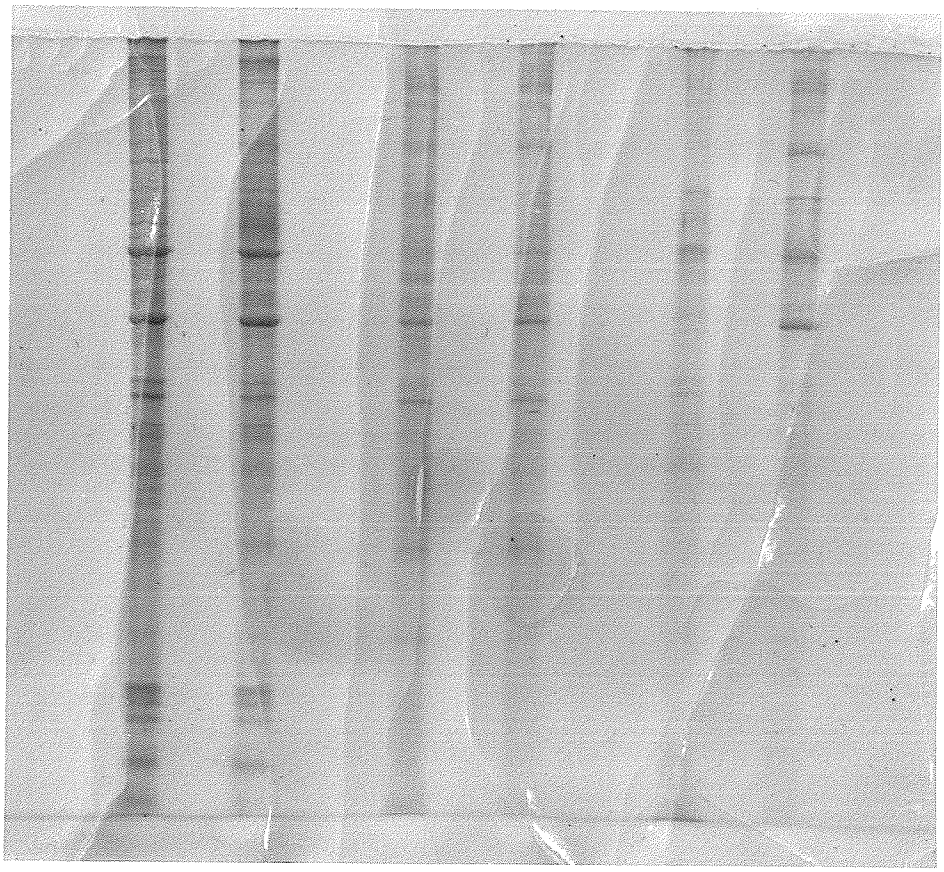


Fig. 6b

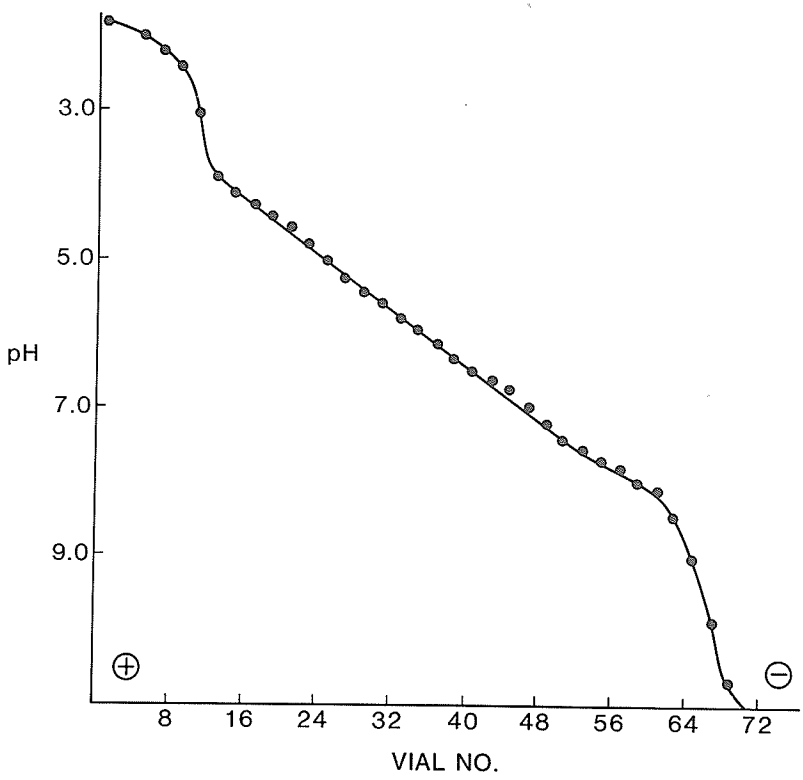


Fig. 7. pH gradient curve for IEF tube gels prepared and electrophoresed by the method of Pena (1977).

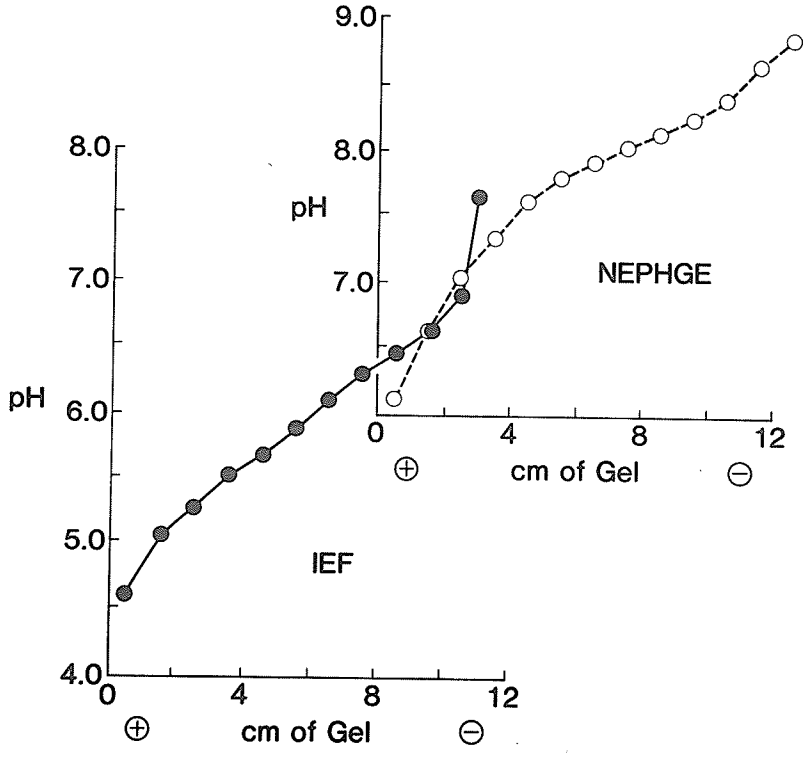


Fig. 8. pH gradient curves for IEF tube gels prepared and electrophoresed by the method of O'Farrell (1975) and for NEPHGE tube gels prepared and electrophoresed by the method of O'Farrell et al. (1977).

B. ONE DIMENSIONAL GEL ELECTROPHORESIS OF DUAL LABEL SAMPLES

1. ISOELECTRIC FOCUSING (IEF) OF POMPE DISEASE SAMPLES

(a) Soluble Fractions

Fig.9 represents a double-label scintillation plot of a cytosolic subcellular fraction that was dialyzed extensively against 1mM KCl. The deviations seen are random, with the resolution of only a few major peaks. This pattern, which roughly resembles the IEF profiles of other soluble fractions has most of the protein incorporation around pH 5.0. The cpm in each vial was very high, and such a profile could be produced if each slice contained several protein bands. This is especially likely since the cytosol represents nearly 50% of the total cellular protein. The above ambiguity is one drawback of arbitrary gel slicing.

Fig. 10 represents a double-label scintillation plot of the 100,000xg supernatant of a microsomal (40S) pellet that had been incubated for 1hr with 10% Lubrol WX, 5mM EDTA and 1mM phenylmethylsulfonyl fluoride(PMSF). Again, the deviations were randomly distributed, except for spurious counts in vials 47 and 52, which were normal upon recounting.

Figs. 11-13 represent double-label scintillation plots of the 100,000xg supernatant from mitochondrial/lysosomal fractions after freeze thawing through five cycles. The deviations are randomly

distributed, and those at either terminus are predominantly due to near background levels of radioactivity. In the latter two gels, a slight deviation is noted in the most basic peak, but this area of the gel would measure approximately pH 9.0. Although the relative proportions of some peaks differ between strains, this could result from minor differences in the labeling and fractionation procedure between different experiments.

(b) Effect of Proteolysis

Figs. 14 and 15 compare IEF gels of two double-label ML-S fractions, one of which was allowed to incubate at room temperature for two hours before polymerizing in the IEF gel. The normally treated sample was comprable to other similar fractions, displaying no major deviations in degree of incorporation between the radioisotopes. The sample which was warm-incubated, however showed degradation of several major peaks, and this degradation was non-uniform in the two cell strains. The gel slices probably contained small peptide fragments with distinctive isoelectric points, which could have contributed to the deviations between the strains. The deviation at the basic terminus also contributed to the spread of the two-standard deviation lines in the ratio plot.

(c) Effect of Growth Stage

Two different pairs of strains were double-labeled and harvested at different stages of in vitro growth, the ML-S fractions were prepared, and submitted to isoelectric focusing. One of these pairs is displayed in Figs. 16 and 17. The only major difference between cells labeled four days after seeding and those labeled on day 11 was a slight increase in a major peak (approx. vial 20) which corresponds to a pH near 5.0. This elevation was from about 3% on day 4 to 4% of the total ML-S protein labeled on day 11. This same observation was less striking in strains MCH 7 vs GM 3329, while the rest of the gel profile was similar between growth stages.

Fig. 9: All 1-D plots are of the same format. The six digit code after the word SCINT represents the date of the computer analysis. This is followed by an internal code, the type of gel, the subcellular fraction analyzed, and the strain designations as they were radiolabeled in the experiment (plus or minus a serum boost). The upper plot represents the percent of total picomoles leucine incorporated for both ^{3}H (O--O) and ^{14}C (X--X) in each vial, and the lower represents the corresponding normalized ratio of ^{14}C-labeled leucine/^{3}H-labeled leucine for each vial. The two lines equidistant from the mean ratio of 1 measure two standard deviations, and thus enclose the 95% confidence interval of the data. Data points in the percent picomoles plot with too few cpm were rejected, and therefore do not appear, in the ratio plot. Major departures from the expected levels of radioactive incorporation may be seen as divergences of O--O and X--X lines in the percent picomoles plot, and the corresponding exit of data points from between the two standard deviation lines of the normalized ratio plot. These are commonly referred to as "deviations" or "abnormalities" in the context of this thesis. This figure: IEF gel plot of ^{3}H-strain GM 498(normal) versus ^{14}C-strain GM 3329(Pompe-infantile). As a convention, all IEF gel plots display acidic protein species on the left and basic protein species on the right, with pI 4.8 occurring at approximately 1/3 and pI 7.9 occurring at approximately 5/6 the total length of the gel. Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the S fraction of fibroblast protein.

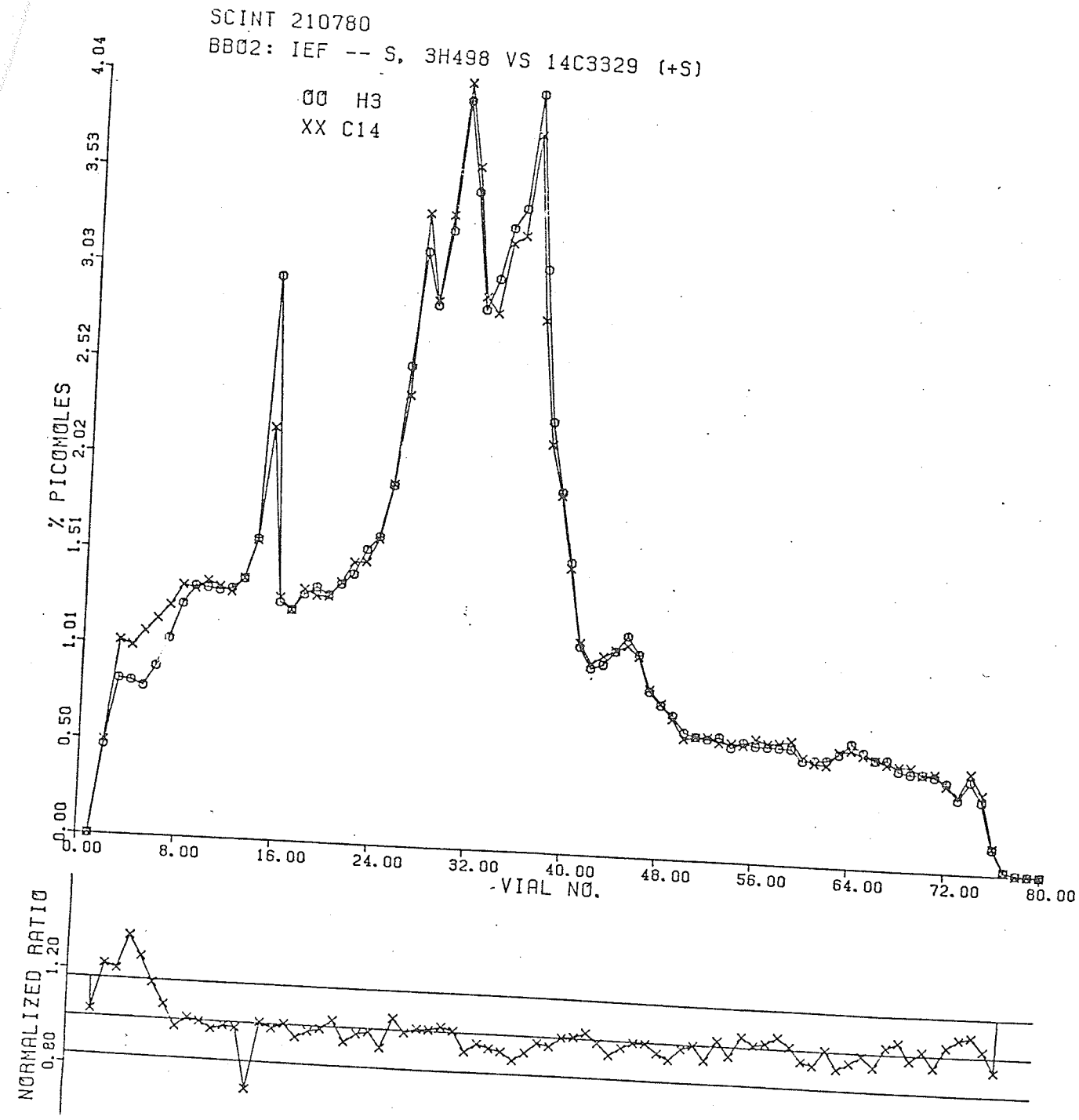

SCINT 210780
BB02: IEF -- S, 3H498 VS 14C3329 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.50
1.01
1.51
2.02
2.52
3.03
3.53
4.04
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
1.20
0.80

Fig. 9

Fig. 10. IEF gel plot of ^{3}H-strain GM 498(normal) versus ^{14}C-strain GM 3329(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the P fraction of fibroblast protein.

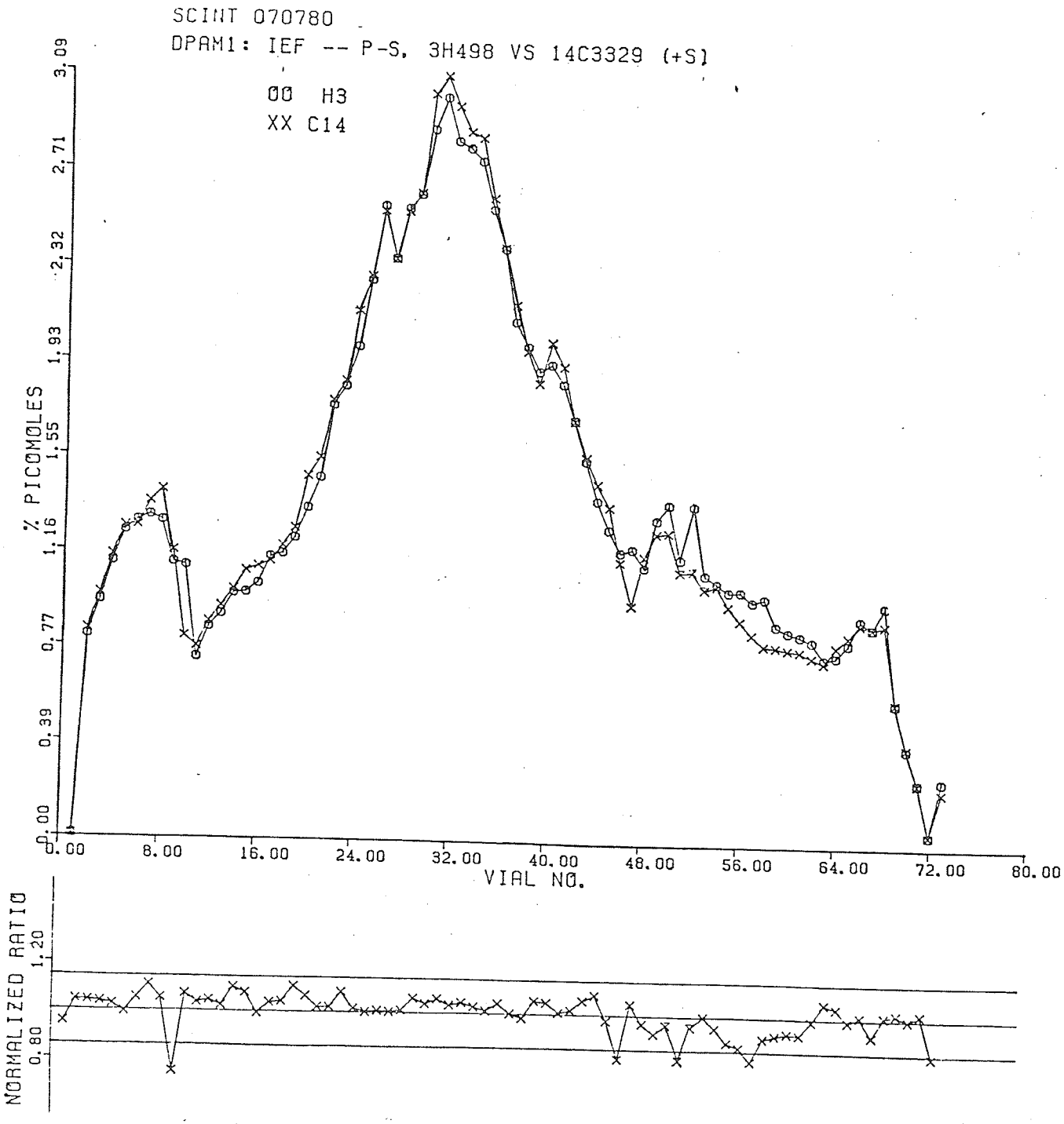

SCINT 070780
DPAM1: IEF -- P-S. 3H498 VS 14C3329 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.39
0.77
1.16
1.55
1.93
2.32
2.71
3.09
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
1.20
0.80

Fig. 10

Fig. 11. IEF gel plot of ^{3}H-strain GM 498(normal) vs ^{14}C-strain GM 3329(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein.

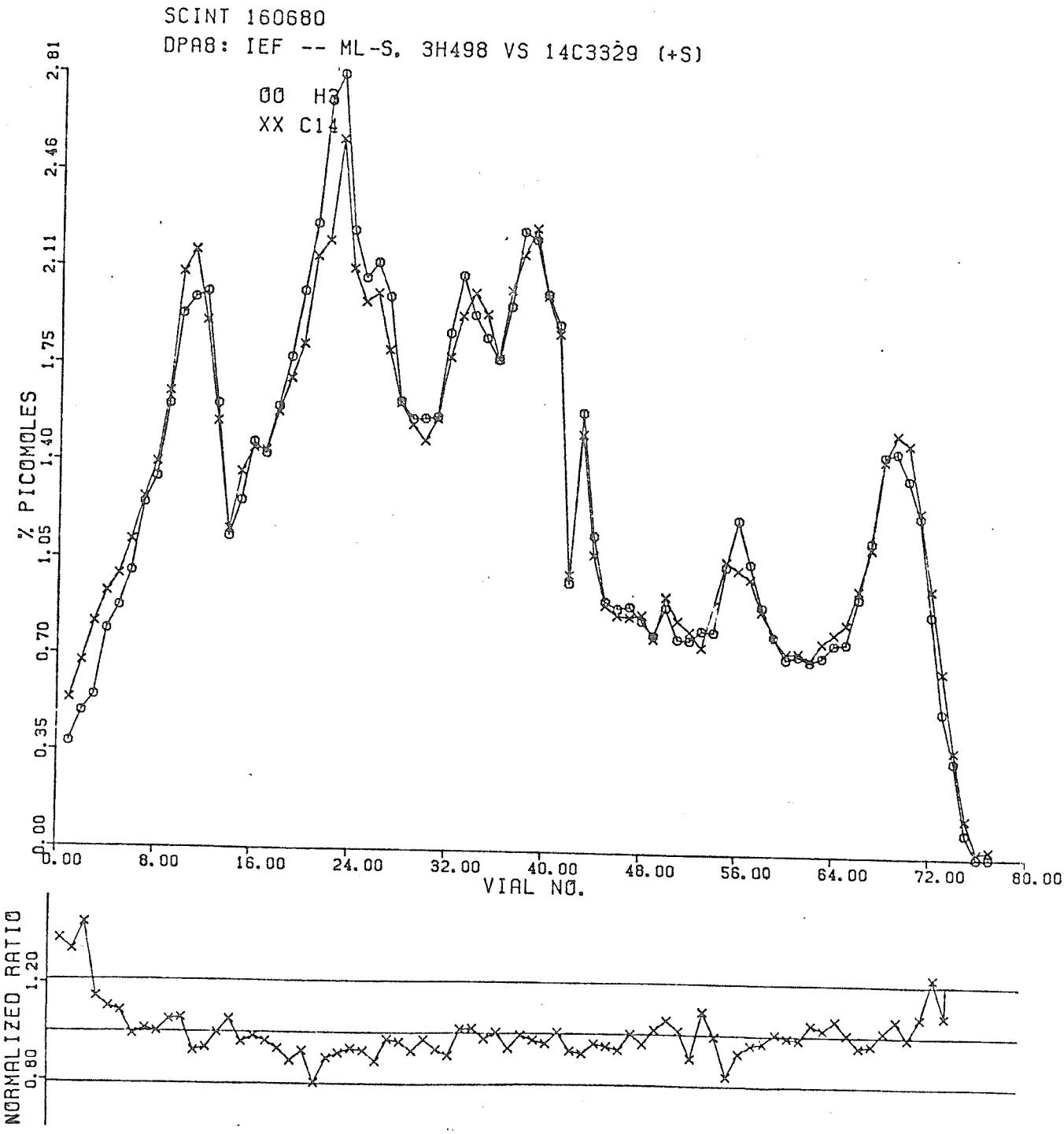

SCINT 160680
DPA8: IEF -- ML-S, 3H498 VS 14C3329 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.35
0.70
1.05
1.40
1.75
2.11
2.46
2.81
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 11

Fig. 12. IEF gel plot of ^{3}H-strain GM 244(Pompe-infantile) versus ^{14}C-strain GM 41(normal). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein.

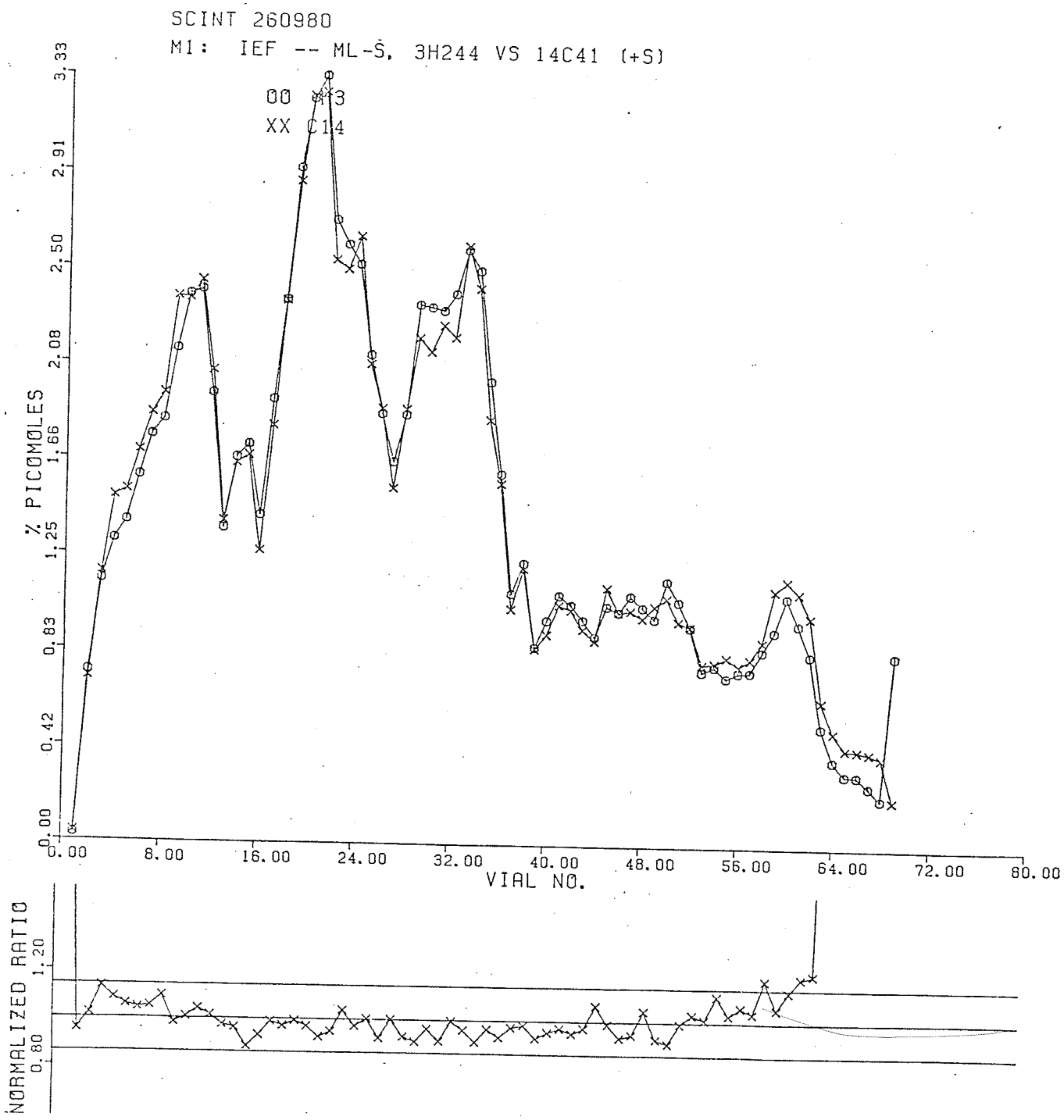

SCINT 260980
M1: IEF -- ML-S, 3H244 VS 14C41 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.42
0.83
1.25
1.66
2.08
2.50
2.91
3.33
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 12

Fig. 13. IEF gel plot of ^{3}H-strain GM1935(Pompe-adult form) versus ^{14}C-strain GM 23(normal). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein.

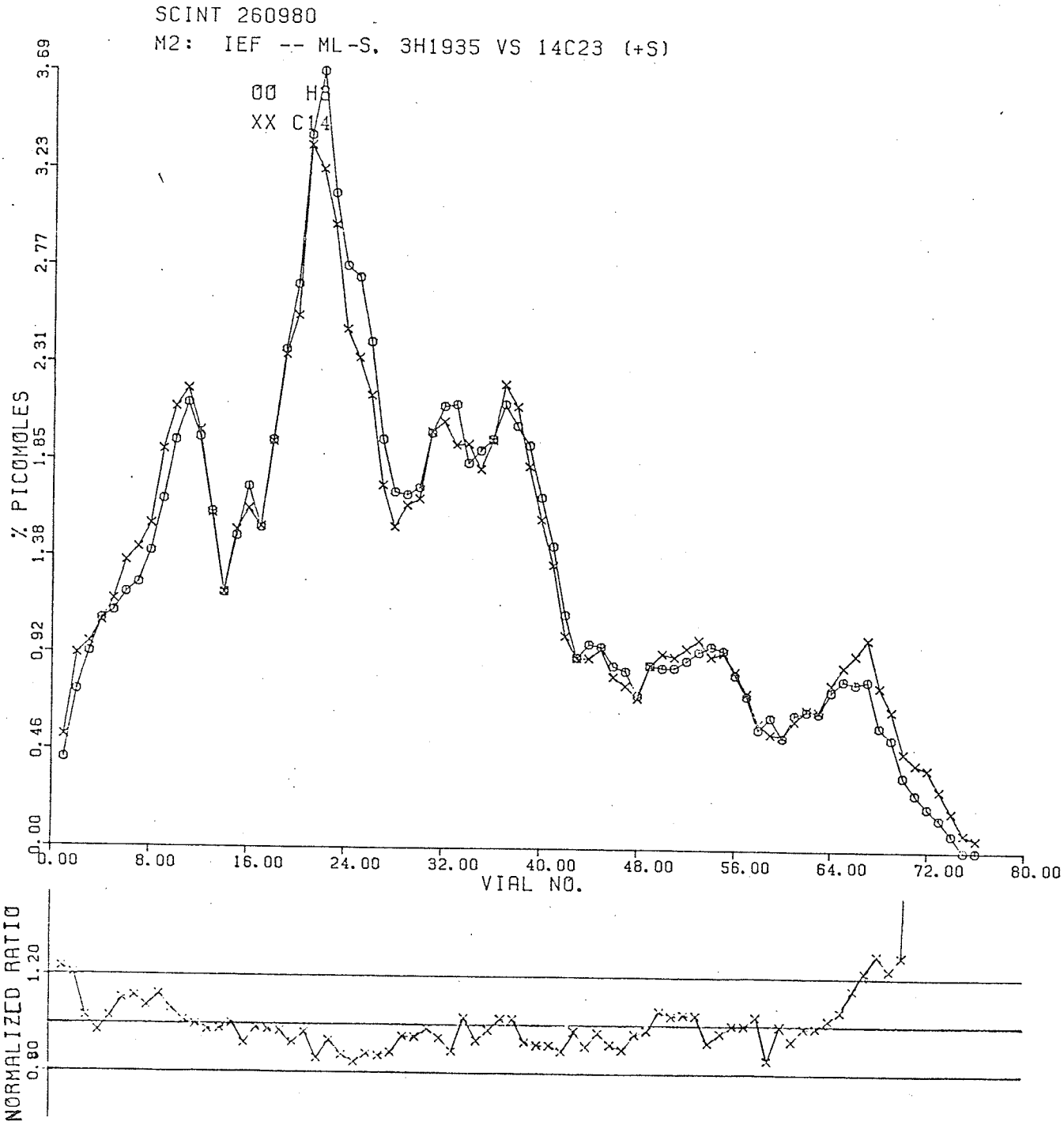

SCINT 260980
M2: IEF -- ML-S. 3H1935 VS 14C23 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.46
0.92
1.38
1.85
2.31
2.77
3.23
3.69
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 13

Fig. 14. IEF gel plot of ^{3}H-strain GM248(Pompe-infantile form) versus ^{14}C-strain GM 302(normal). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. This sample served as a control for the proteolysis experiment (See Fig. 15).

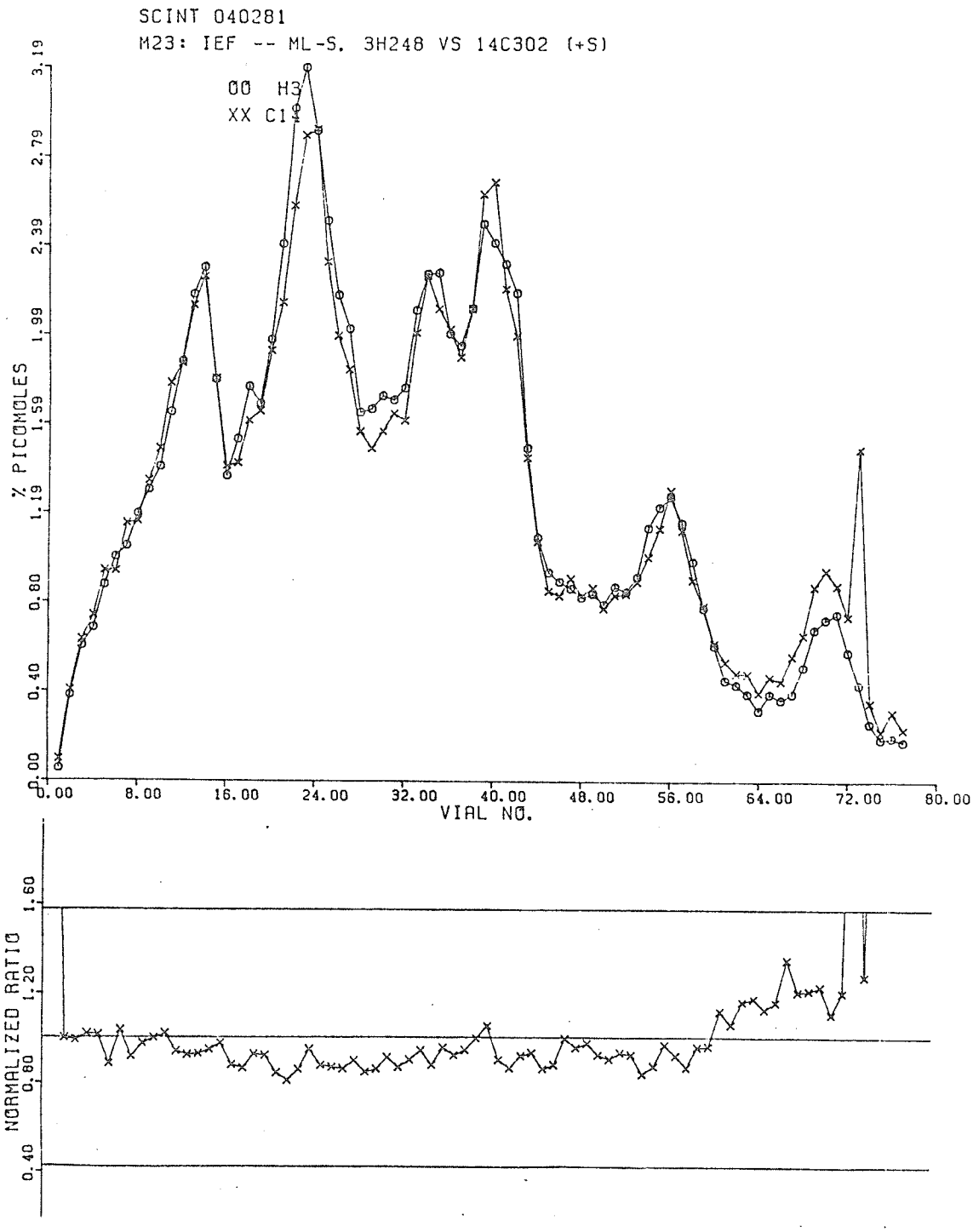

SCINT 040281
M23: IEF -- ML-S. 3H248 VS 14C302 (+S)
00 H3
XX C1
% PICOMOLES
0.00
0.40
0.80
1.19
1.59
1.99
2.39
2.79
3.19
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.40
0.80
1.20
1.60

Fig. 14

Fig. 15. IEF gel plot of ^{3}H-strain GM248(Pompe-infantile form) versus ^{14}C-strain GM 302(normal). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. In this experiment, the total cell homogenate was allowed to incubate at room temperature for 2hrs before subcellular fractionation. Notice the overall loss of resolution compared to Fig. 14.

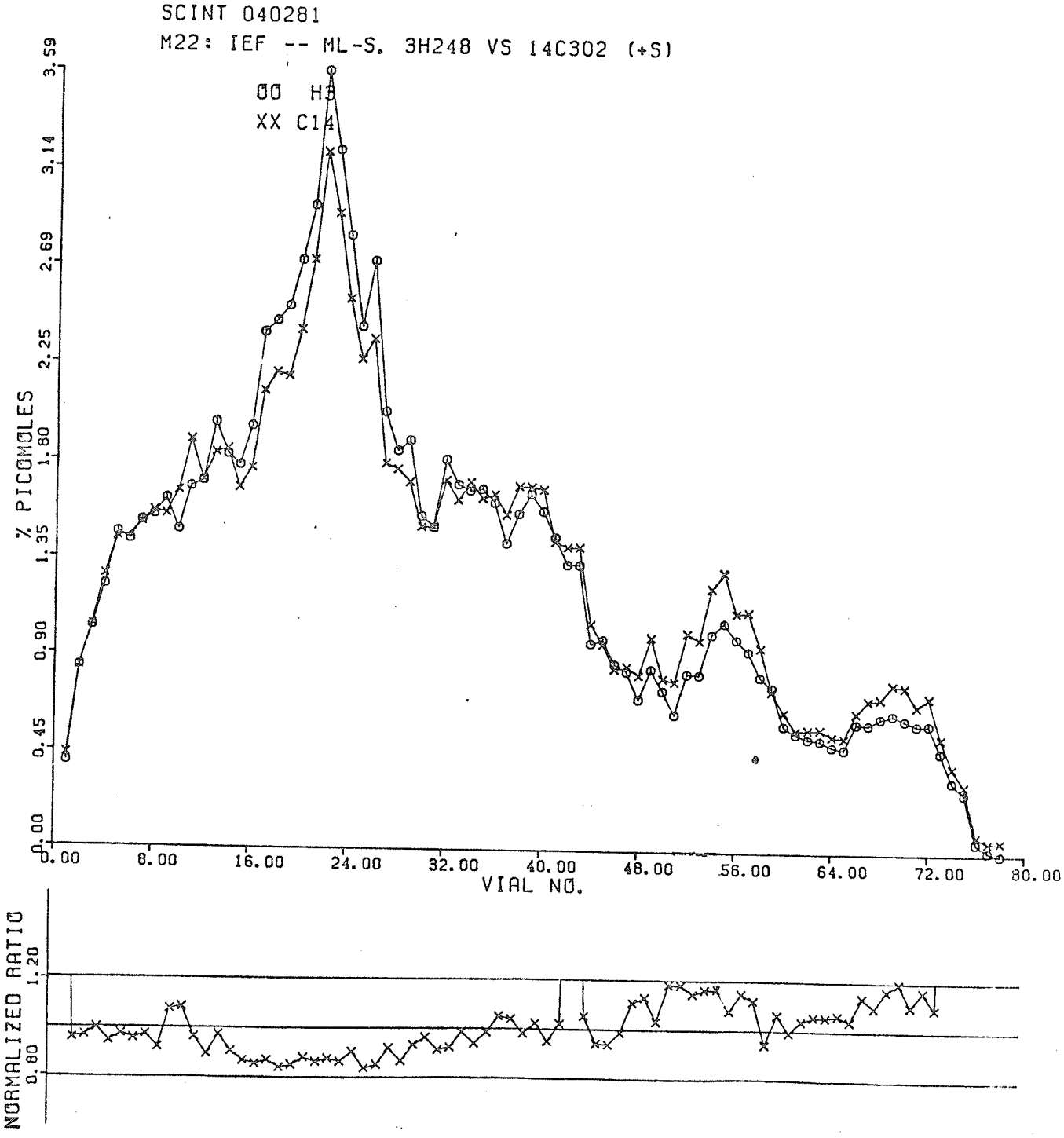

SCINT 040281
M22: IEF -- ML-S. 3H248 VS 14C302 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.45
0.90
1.35
1.80
2.25
2.69
3.14
3.59
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 15

Fig. 16. IEF gel plot of ^{3}H-strain MCH 49(normal) versus ^{14}C-strain WG 482(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 4 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. In this experiment, the cells were not confluent at the time of labelling (day 4), and were presumed to be undergoing logarithmic growth.

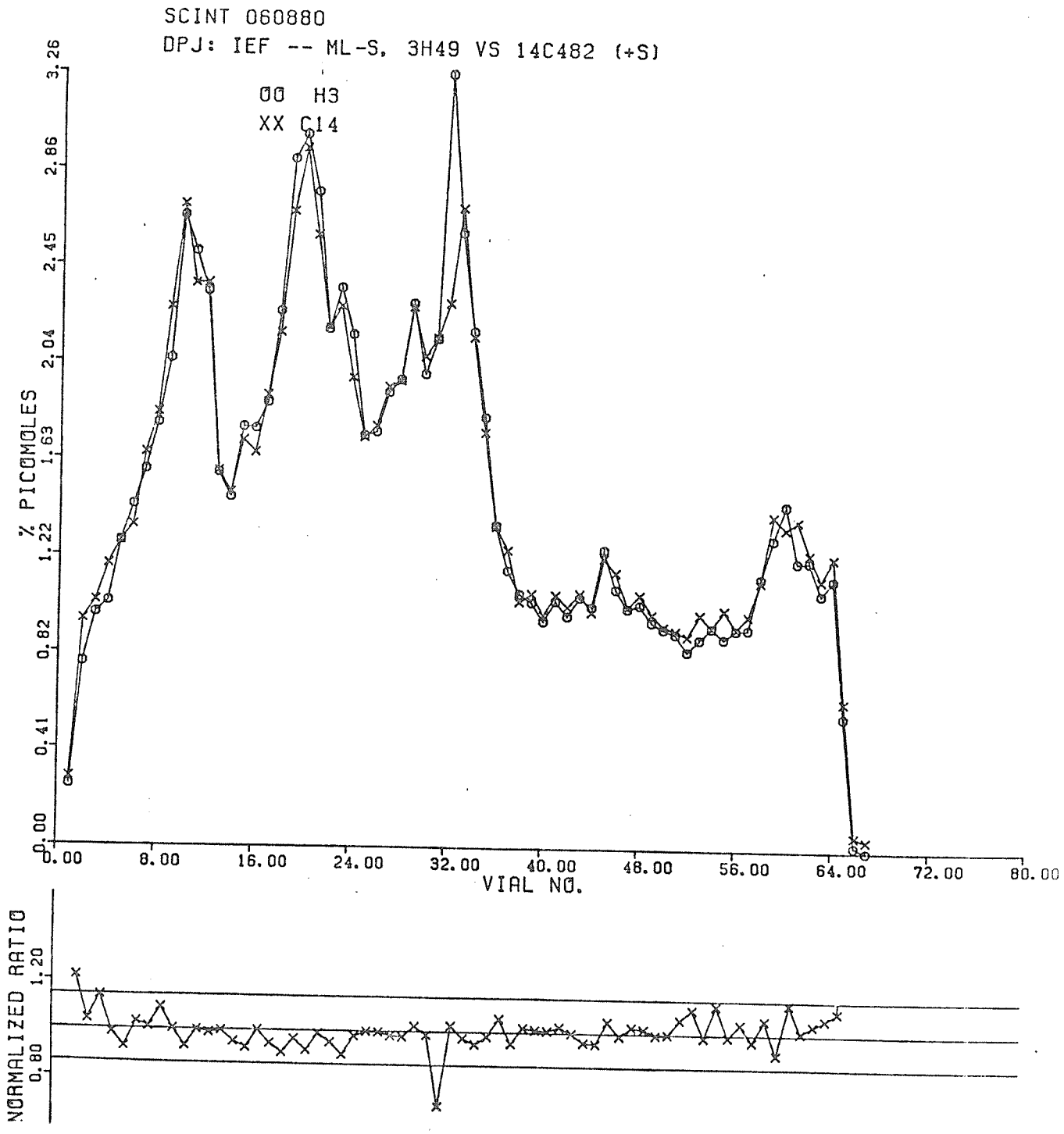

SCINT 060880
DPJ: IEF -- ML-S, 3H49 VS 14C482 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.41
0.82
1.22
1.63
2.04
2.45
2.86
3.26
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 16

Fig. 17. IEF gel plot of ^{3}H-strain MCH 49(normal) versus ^{14}C-strain WG 482(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 11 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. Cells were confluent at the time of labeling (day 11), and display a profile very similar to rapidly dividing cells (Fig. 16).

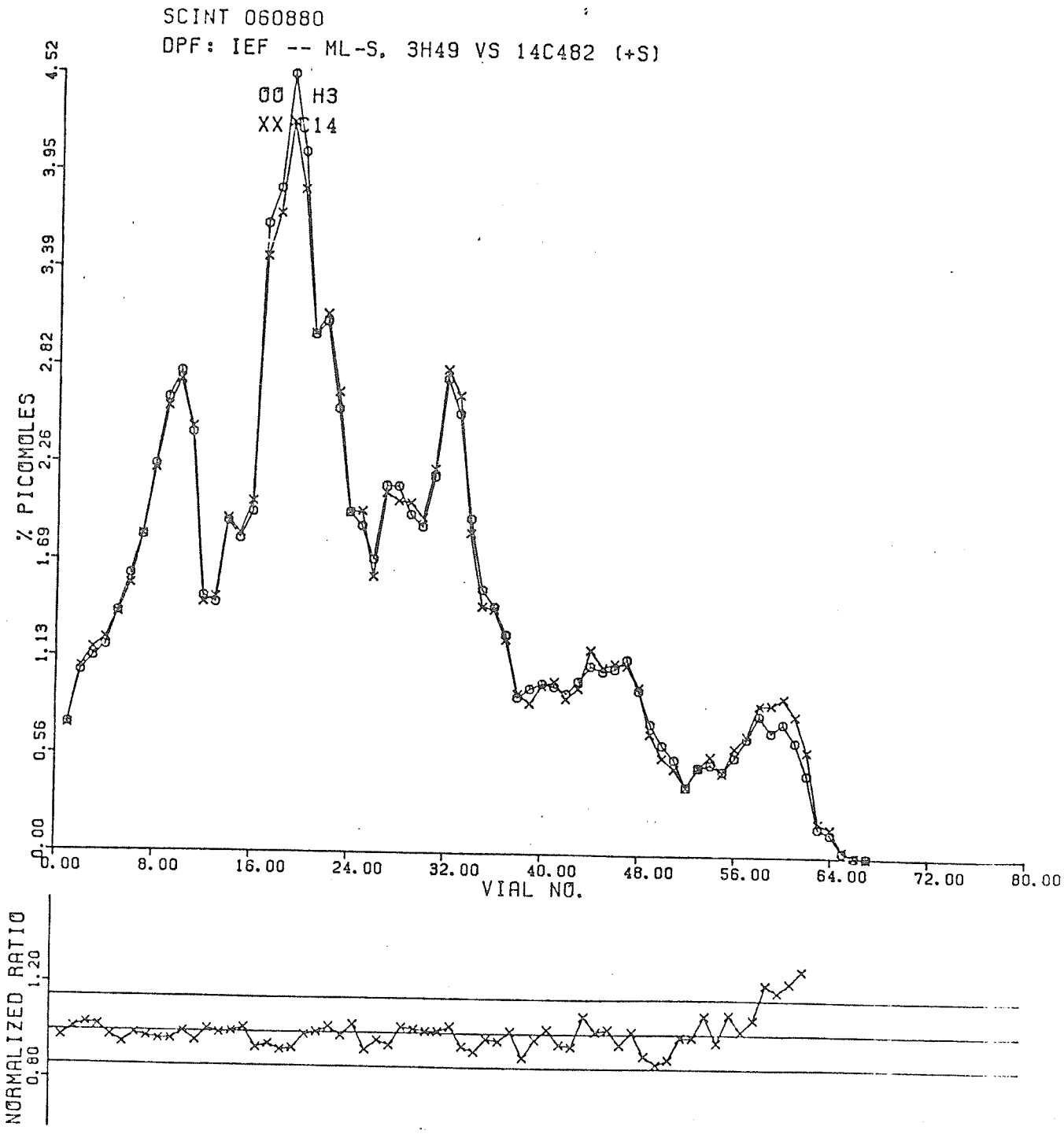

SCINT 060880
DPF: IEF -- ML-S, 3H49 VS 14C482 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.56
1.13
1.69
2.26
2.82
3.39
3.95
4.52
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
64.00
72.00
80.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 17

2. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF POMPE DISEASE SAMPLES

All gels in this section were electrophoresed with the ML-S fraction.

(a) Comparison of Different Cell Strains

Figs. 18-21 display the ML-S fractions from three different pairs of cell strains that were double labeled under different conditions, harvested, fractionated, and electrophoresed on SDS-PAGE gels of different gel concentrations. All display approximately the same proportion of the major component peak, and the same overall profile regardless of serum boost or gel concentration (discussed later). There is no consistent deviation in the 29,000d region (approximately vial 55 in 13% gels and vial 80 in 10% gels) in any of these gels.

(b) Effect of Proteolysis

The same ML-S samples that were used to study the effect of proteolysis on the isoelectric focusing profiles were later prepared in SDS as samples for SDS-PAGE. In addition to the effects of non-uniform proteolysis such as inflation of the variance of the normalized ratio (VNR) and the broadening of peaks, two additional findings were possible:

(i) The gels might have displayed many low molecular weight fragments that were generated at the expense of the high molecular weight peaks, and most of the radioactivity would be seen in these low molecular weight species.

(ii) The 29,000 d deviation of Pena (1977), implicating a lost or missing protein in the Pompe strain, might have been generated.

Neither gel had a recognizable profile, but no reasonable explanation could be found. Nevertheless, the above expectations were not fulfilled, and the treatment did not generate Pena's (1977) findings.

(c) Effect of Fetal Bovine Serum Boost

Greater radioactive incorporation into a particular protein or group of proteins correlated with labeling in the presence of FBS in three different pairs of strains (Table IX,Figs. 22-25). This effect was similar in degree in all strains and thus seems to be a nonspecific effect of a hormone-like substance or growth factor in the serum used. It is possible that such a substance may have affected normal strains only in the experiments of Pena (1977). Fetal bovine serum, a complex undefined growth supplement, has been noted to vary drastically in different batches (Shiigi and Mishell, 1975).

(d) Effect of Human Cord Serum

(i) No preincubation- Since the early experiments of Pena were performed with media containing 5% human cord serum as a growth supplement, it was employed in some final experiments in an effort to reproduce Pena's (1977) findings. In the first experiment, cells were radiolabeled in the presence of 37% FCS, 5% human cord serum. The 13% SDS-PAGE gel of the resulting ML-S fraction is presented in Fig. 26. It displayed an unusual lack of resolution, and coordinate incorporation of the two cell strains was not seen. Inspection of the picomoles plot revealed that the level of incorporation throughout the gel was very low. The high error incurred when incorporation was poor contributed to many small deviations, but the overall profile should not have changed. It is possible that the combined effects of bovine and human serum in the labeling medium were mildly toxic to the cells, which then underwent reduced protein synthesis (Hamm and McKeehan, 1979).

(ii) Preincubation - Conversely, when cells were cultured in medium containing 10% FBS and 5% HCS for two passages prior to seeding, the same radiolabeling procedure led to an ML-S fraction with a normal profile on SDS-PAGE (Fig. 27). It thus appears that cells which grew well in the presence of HCS were conditioned to do so or were selected for by serial passage. However, the presence of HCS did not produce any observation resembling that of Pena (1977).

(d) Effect of Gel Concentration

Using the two different gel concentrations, it was expected that the profile might undergo a telescoping effect, with the occasional resolution of a few peaks at one concentration that were not separated on the other. In particular, 13% gels appeared better at resolving species of low molecular weight, while 10% gels allowed better separation of high molecular weight species although in the latter instance, more protein remained in the ion front (Fig. 4). The fine distinction of sharp adjacent bands is likely lost by arbitrarily cutting the gel in 2mm sections, but many major species are well separated in the relatively long gels. As expected, the serum-effected high molecular weight protein(s) displayed a proportional shift between 10% and 13% gels, showing that it behaved as a true protein and not just an artifact produced at the gel origin. Any true difference between strains would be expected to show such a shift, and adherence to this rule support for the identity of proteins run under different conditions. The missing protein of Pena (1977) also adhered to this rule.

(f) Harvesting with Trypsin vs Scraping

Scraping was originally employed to avoid possible introduction of cellular protein modification (polypeptide cleavage) in sample preparation because of trypsin or warm-incubation. In an effort to reproduce Pena's procedure identically, trypsin was used in most of the experiments to test the hypothesis that Pena's (1978) results were

trypsin induced. This hypothesis was not confirmed. However, more radioactivity was recovered from samples harvested in trypsin, and this could have arisen in two ways: (i) the trypsin may have liberated more cells from the plate than scraping, or (ii) more cells were damaged from the scraping and were washed away as debris early in the fractionation procedure. Protein determinations favoured the latter hypothesis.

Fig. 18. 10% SDS-PAGE gel plot of ^{3}H-strain GM 23(normal) versus ^{14}C-strain GM 1935(Pompe-adult form). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding without a serum boost. The plot represents the ML-S fraction of fibroblast protein.

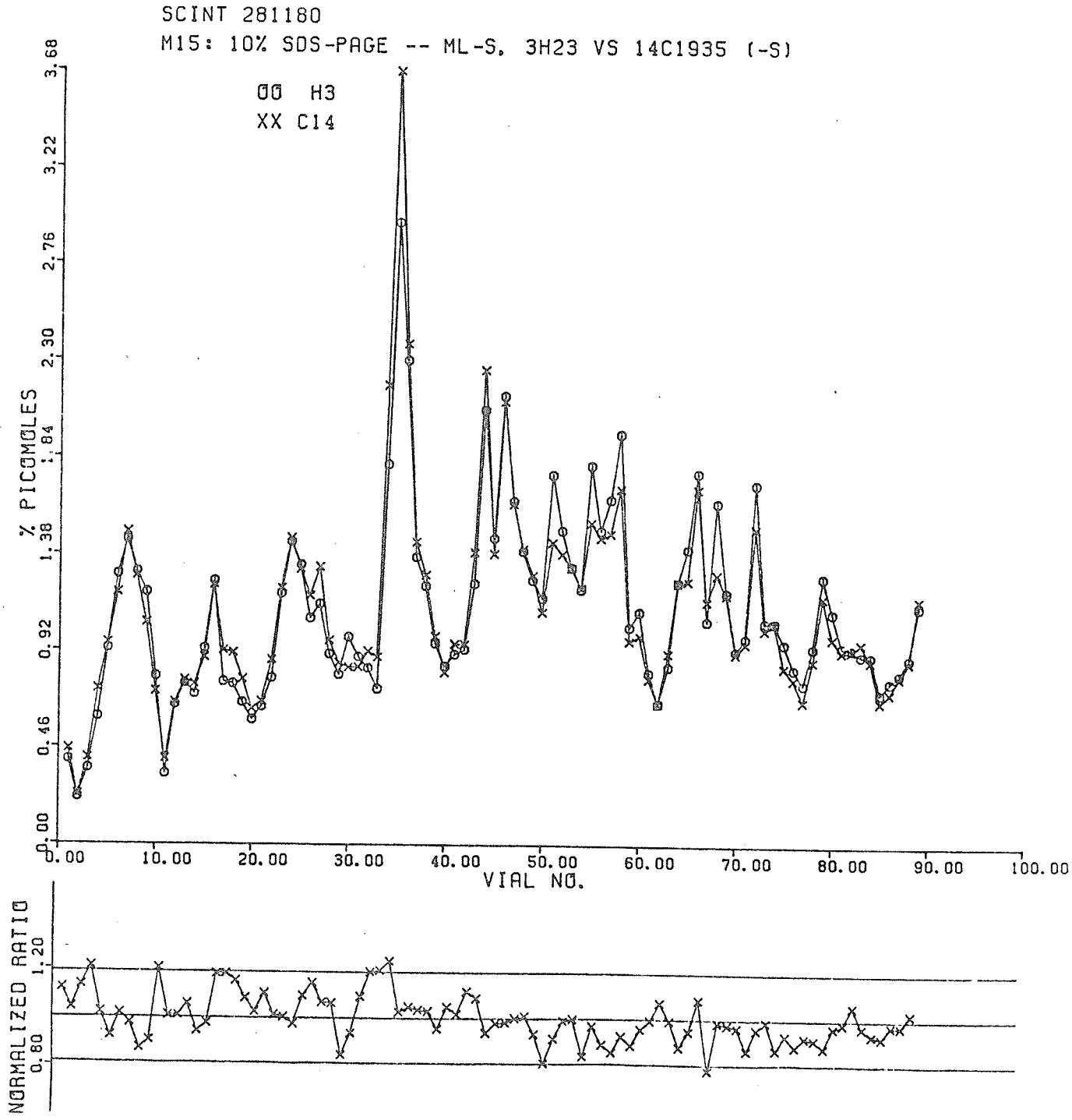

SCINT 281180
M15: 10% SDS-PAGE -- ML-S, 3H23 VS 14C1935 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.46
0.92
1.38
1.84
2.30
2.76
3.22
3.68
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 18

Fig. 19. 10% SDS-PAGE gel plot of ^{3}H-strain WG 482(Pompe-infantile) versus ^{14}C-strain MCH 49(normal). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein.

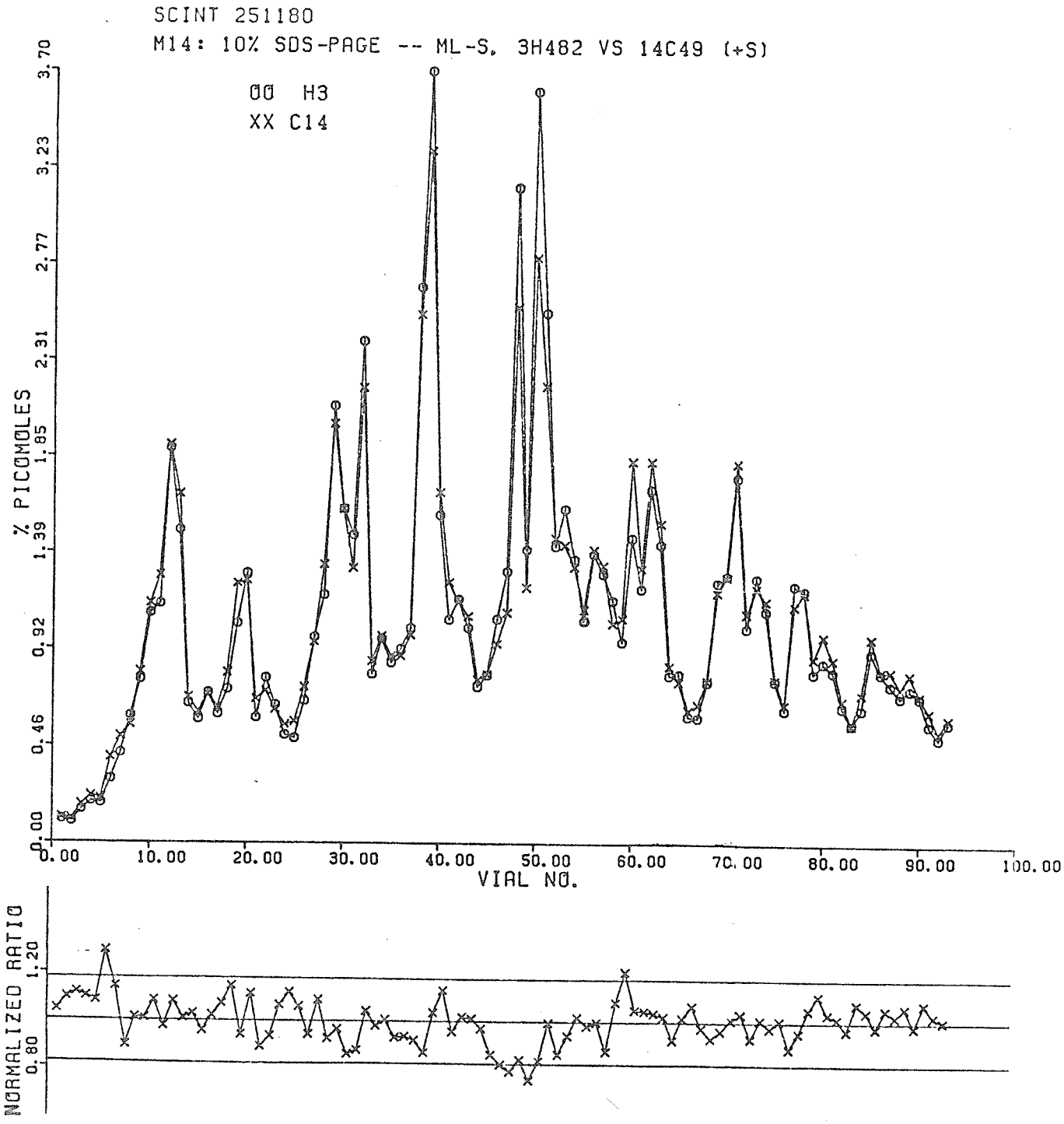

SCINT 251180
M14: 10% SDS-PAGE -- ML-S, 3H482 VS 14C49 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.46
0.92
1.39
1.85
2.31
2.77
3.23
3.70
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 19

Fig. 20. 13% SDS-PAGE gel plot of ^{3}H-strain GM 23(normal) versus ^{14}C-strain GM 1935(Pompe-adult form). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding without a serum boost. The plot represents the ML-S fraction of fibroblast protein.

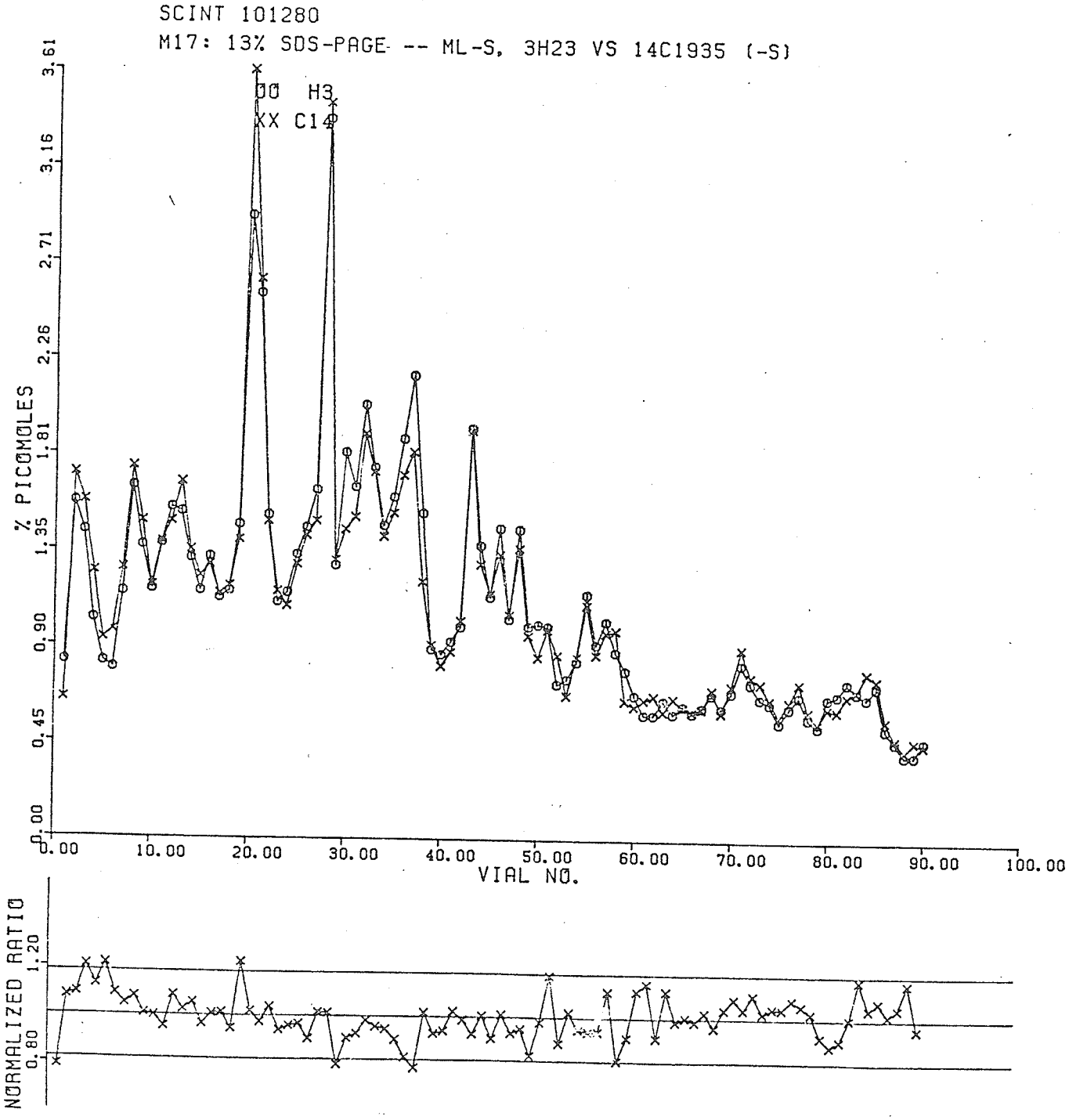

SCINT 101280
M17: 13% SDS-PAGE -- ML-S, 3H23 VS 14C1935 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.45
0.90
1.35
1.81
2.26
2.71
3.16
3.61
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 20

Fig. 21 . 13% SDS-PAGE gel plot of ^{3}H-strain GM 3329(Pompe-infantile) versus ^{14}C-strain MCH 7(normal). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein.

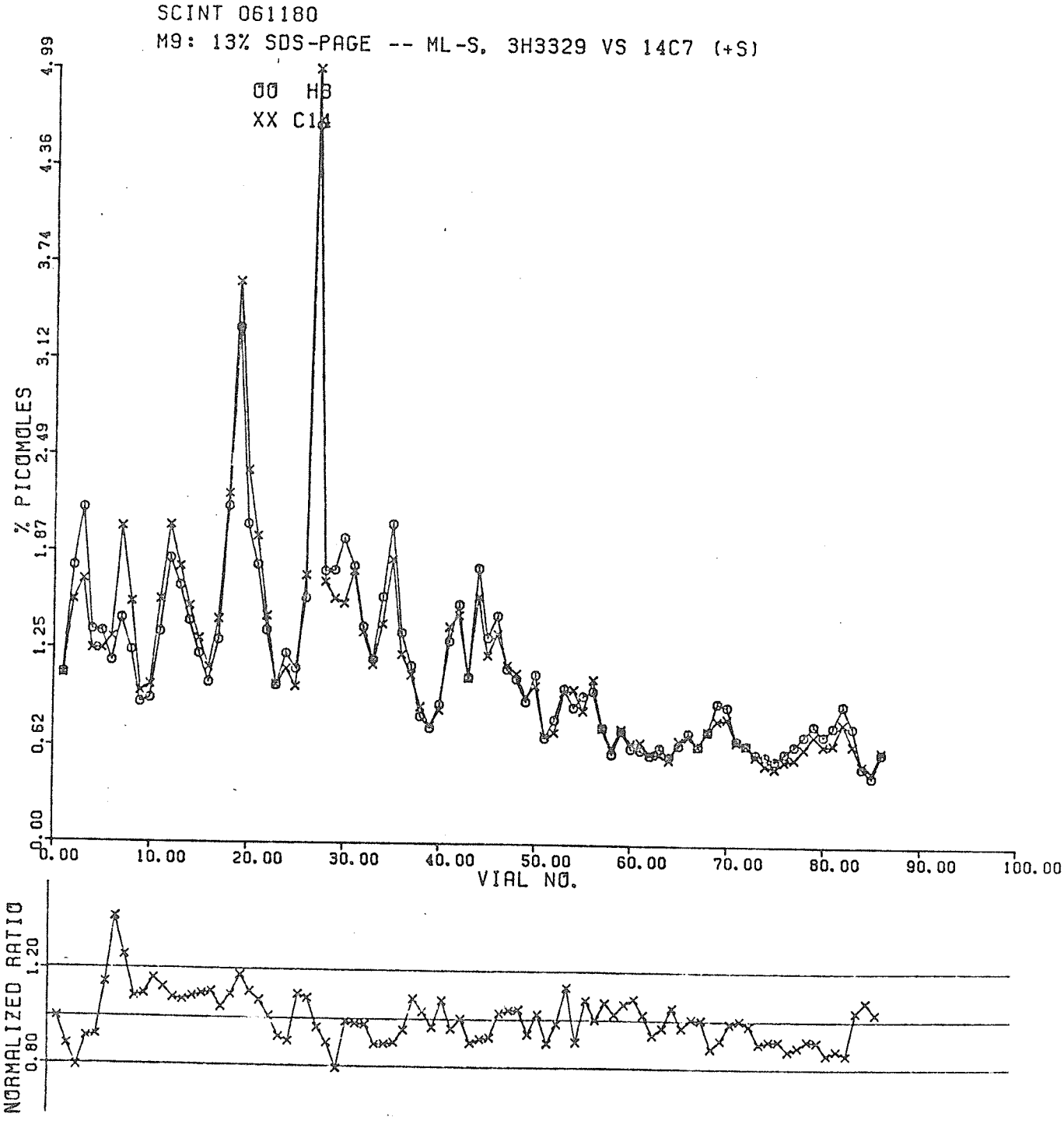

SCINT 061180
M9: 13% SDS-PAGE -- ML-S, 3H3329 VS 14C7 (+S)
00 HB
XX C14
% PICOMOLES
0.00
0.62
1.25
1.87
2.49
3.12
3.74
4.36
4.99
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 21

Fig. 22. 10% SDS-PAGE gel plot of ^{3}H-strain GM 41(normal) versus ^{14}C-strain GM 244(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding without a serum boost. The plot represents the ML-S fraction of fibroblast protein.

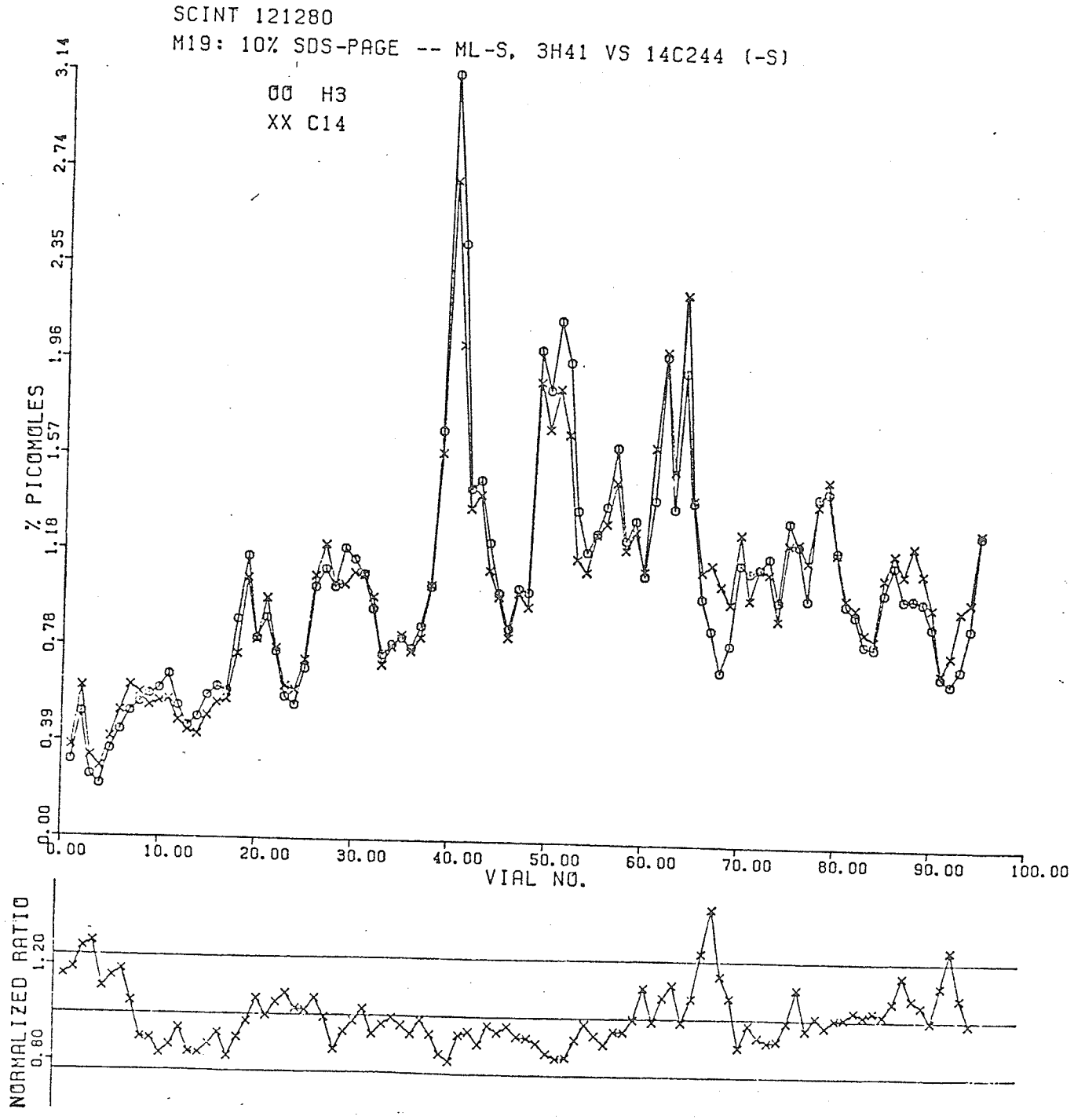

SCINT 121280
M19: 10% SDS-PAGE -- ML-S, 3H41 VS 14C244 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.39
0.78
1.18
1.57
1.96
2.35
2.74
3.14
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 22

Fig. 23. 10% SDS-PAGE gel plot of ^{3}H-strain GM 41(normal) versus ^{14}C-strain GM 244(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. Note the elevated incorporation of the peak around vial 15, which without a serum boost (Fig. 22) was lower.

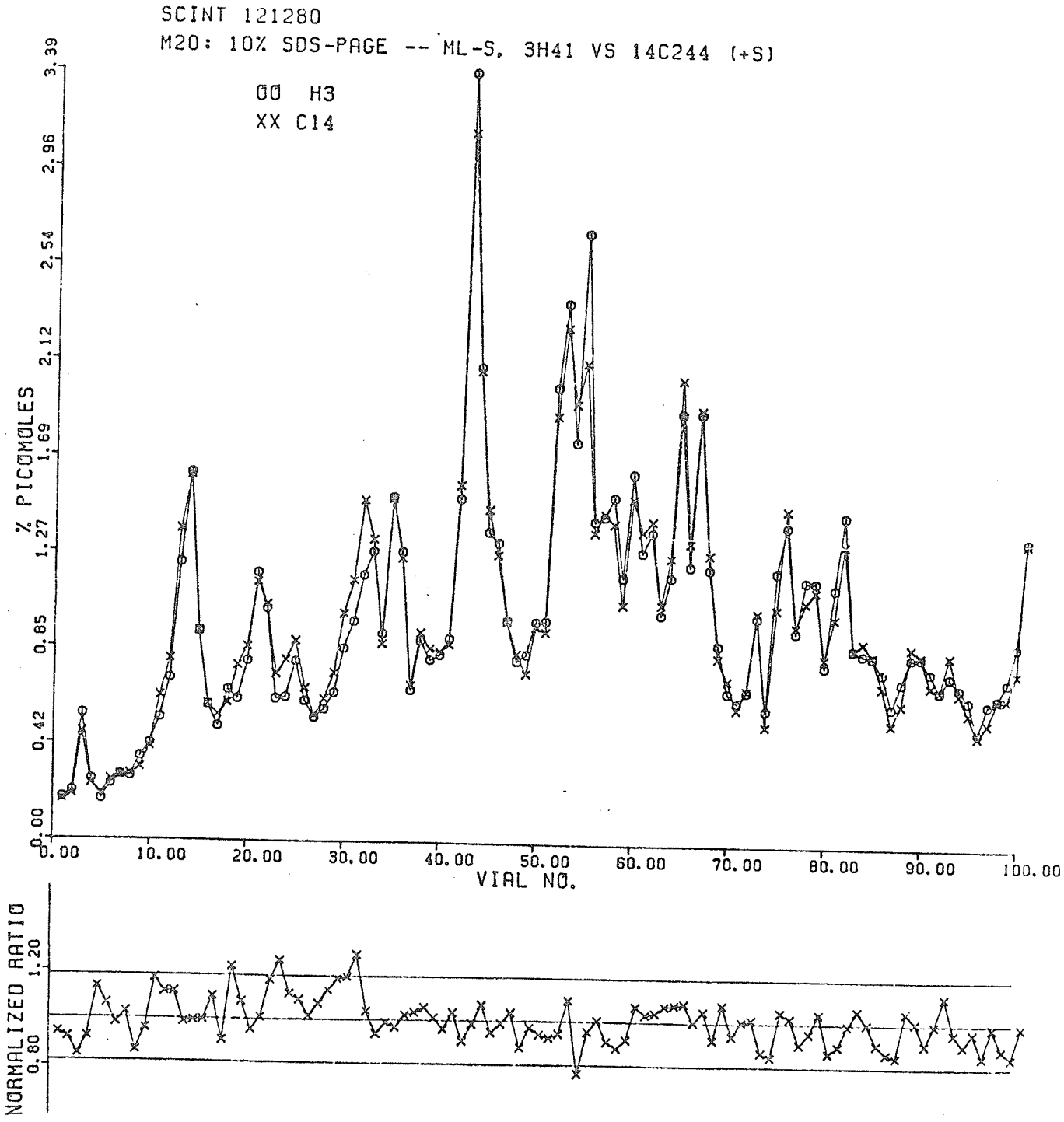

SCINT 121280
M20: 10% SDS-PAGE -- ML-S, 3H41 VS 14C244 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.42
0.85
1.27
1.69
2.12
2.54
2.96
3.39
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 23

Fig. 24. 13% SDS-PAGE gel plot of ^{3}H-strain GM 41(normal) versus ^{14}C-strain GM 244(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding without a serum boost. The plot represents the ML-S fraction of fibroblast protein.

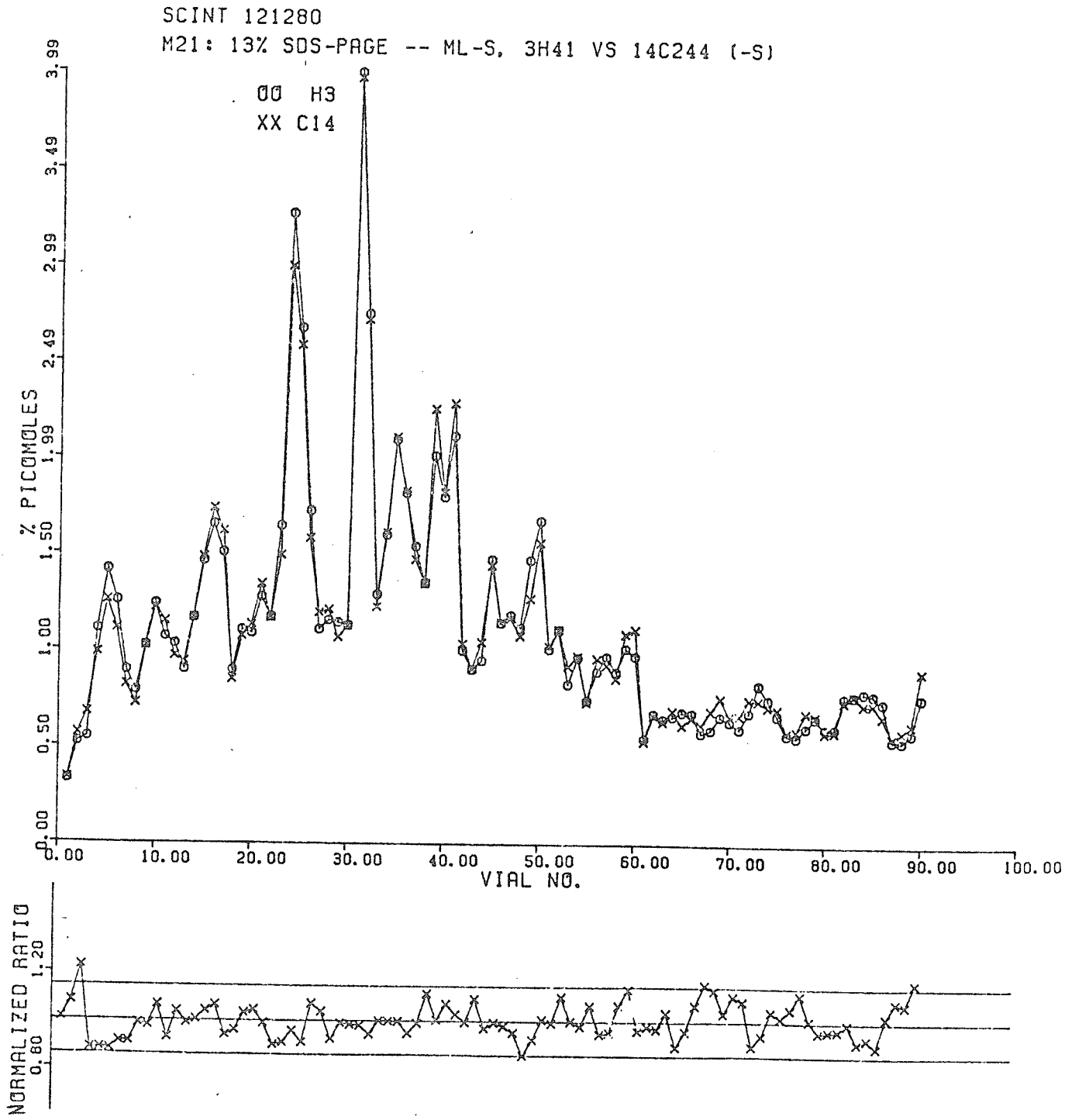

SCINT 121280
M21: 13% SDS-PAGE -- ML-S, 3H41 VS 14C244 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.50
1.00
1.50
1.99
2.49
2.99
3.49
3.99
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
1.20
0.80

Fig. 24

Fig. 25. 13% SDS-PAGE gel plot of ^{3}H-strain GM 41(normal) versus ^{14}C-strain GM 244(Pompe-infantile). Cells were seeded at a density of 3 x 10^{5}/100mm dish and labeled 14 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. Note the elevated incorporation of the peak around vial 6, which without a serum boost (Fig. 24) was lower.

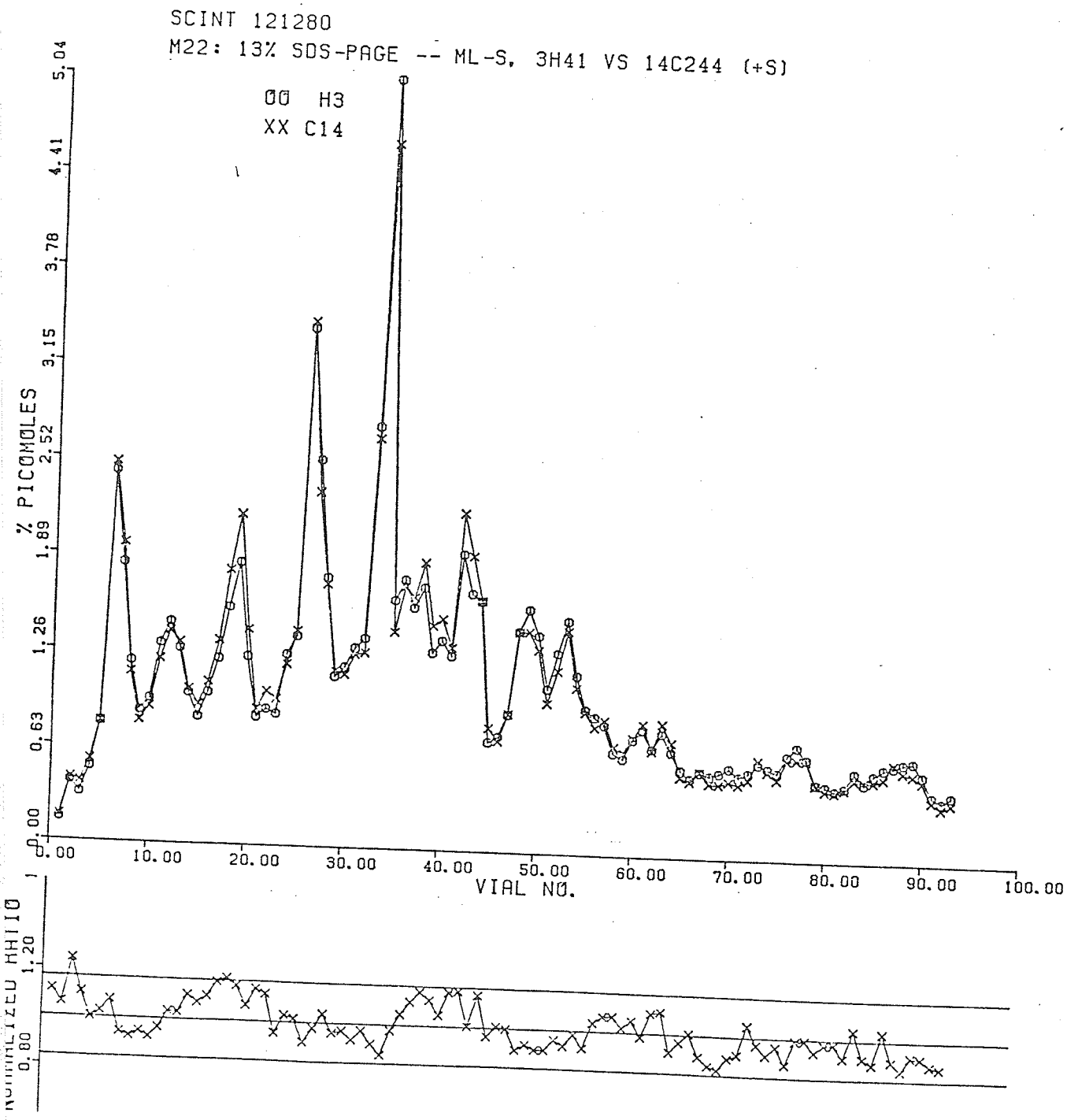

SCINT 121280
M22: 13% SDS-PAGE -- ML-S, 3H41 VS 14C244 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.63
1.26
1.89
2.52
3.15
3.78
4.41
5.04
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
0.80
1.20

Fig. 25

Table IX: Serum-effected leucine incorporation into high molecular weight protein(s) in six cell strains. Summary including Figs. 22-25.

Strain (^{3}H vs ^{14}C)	Gel Concentration	Approximate increase* in high molecular weight species.
GM41 vs GM244	10%	1%
	13%	1%
GM23 vs GM1935	10%	0.5%
	13%	0.5% in GM1935 only
WG482 vs GM49	10%	1%
	13%	2%

* Relative difference in percent picomoles of leucine incorporated during labeling, serum vs no serum boost.

Fig. 26. 13% SDS-PAGE gel plot of ^{3}H-strain GM 248(Pompe-infantile) versus ^{14}C-strain GM 302(normal). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. In addition to the usual serum boost given upon labeling (final concentration of 37% FBS), a 5% human cord serum (HCS) was included.

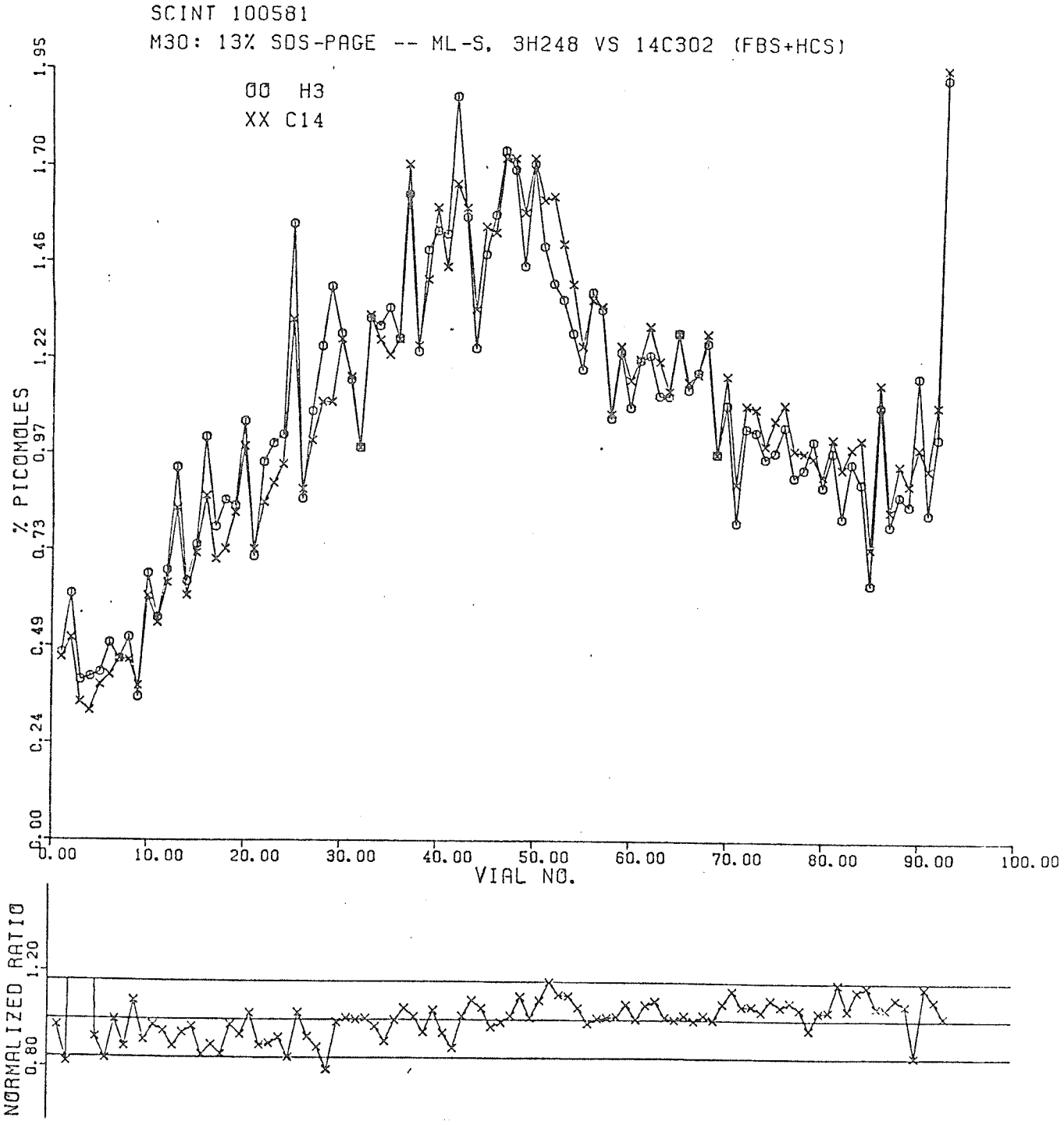

SCINT 100581
M30: 13% SDS-PAGE -- ML-S, 3H248 VS 14C302 (FBS+HCS)
00 H3
XX C14
% PICOMOLES
0.00
0.24
0.49
0.73
0.97
1.22
1.46
1.70
1.95
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 26

Fig. 27. 13% SDS-PAGE gel plot of ^{3}H-strain GM 248(Pompe-infantile) versus ^{14}C-strain GM 302(normal). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the ML-S fraction of fibroblast protein. In this experiment, cells were cultured for several generations in HuMc5A medium containing 10% FBS and 5% HCS prior to labeling, and then labeled as above in medium containing 37% FBS, 5% HCS.

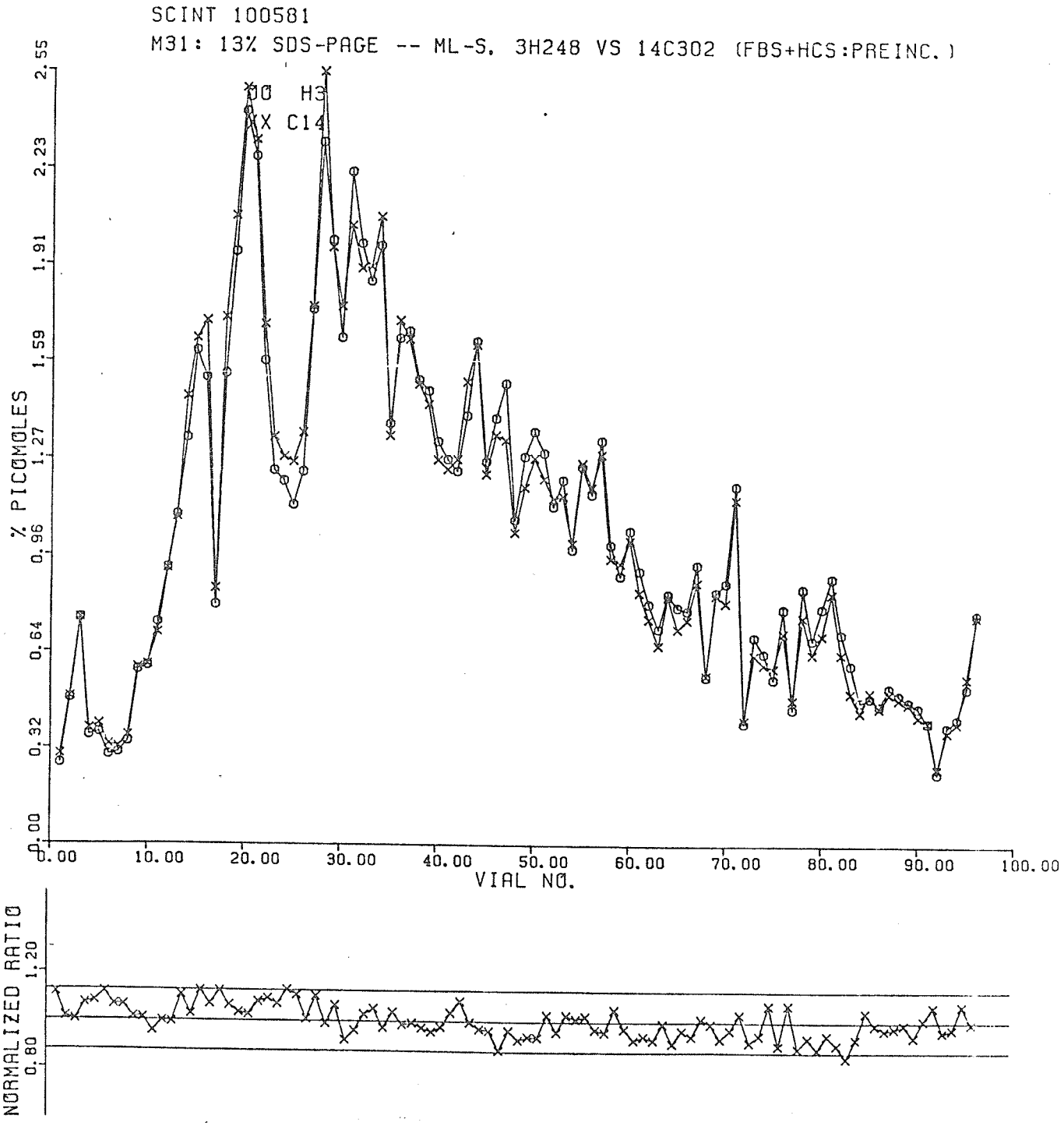

SCINT 100581
M31: 13% SDS-PAGE -- ML-S, 3H248 VS 14C302 (FBS+HCS:PREINC.)
00 H3
XX C14
% PICOMOLES
0.00
0.32
0.64
0.96
1.27
1.59
1.91
2.23
2.55
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 27

SDS-PAGE OF GAUCHER DISEASE SAMPLES

Two pairs of Gaucher and normal fibroblasts strains were examined by double label 1-D SDS-PAGE in view of the biochemical uncertainties of the disease and its analogy to Pompe disease. The evidence of both membrane-bound and soluble forms of beta-glucosidase activity favoured the preparation of the complete ML pellet in SDS, which restricted the separation in these experiments to SDS-PAGE. Fig. 28 displays a plot of a pair of strains that was labeled 5 days after seeding. The most notable differences were slight deficiencies in vial 22 (60,000d) of the Gaucher strain and vial 31 (50,000d) of the normal strain. This observation was especially interesting since a cytosolic form of the enzyme has been found to have a molecular weight of 50,000d while the membrane bound form had a molecular weight of 60,000d (Barret and Heath, 1977). The differences, although minor, persisted upon recounting, and the experiment was therefore repeated. The results were ambiguous in the two subsequent experiments, since the gel profiles were different from the first, and many minor deviations were seen. The final Gaucher experiment (Figs. 29,30), however clarified many of the ambiguities. These profiles were different from the original (Fig. 28), but plots from the second and third experiments (Figs. 31,32). This differnce apparently resulted from labeling at a later stage of growth. There was a single major deficiency in vials 48-52 (40-38,000d) of the Gaucher sample electrophoresed in a 10% gel (Fig. 29), which resolved as two distinct peaks in the corresponding vials (29-32) of the 13% gel. When the profile of the 13% gel was

matched with profiles from the three preceding experiments (all 13% SDS-PAGE) the same deviations were seen, but were no more striking than the numerous other deviations of the high molecular weight proteins. The persistence of minor deviations was especially confusing between the second and third experiments (Fig. 31,32) which displayed an unusual reversal of the profile at approximately vial 20 (note that in all Gaucher experiments, the normal strain was labeled with ^{3}H-leucine). One modification that may have been critical in the final experiment was that the fibroblasts were not labeled in leucine-deficient medium, but in the standard culture medium with an FBS boost. The total incorporation of radioisotope into the normal strain was lower, but the Gaucher strain incorporated radioisotope just as well as in experiments employing leucine deficient medium. It is not certain that the leucine deficient medium obscured the defect in the early experiments, but it may have contributed to asynchrony of protein synthesis and the resulting inconsistent differences between cell strains. Nevertheless, the defect is more striking in 10% polyacrylamide gels. Since the deficiency was unequivocal in only GM 877, the difference might have been purely due to genetic variation.

Fig. 28. 13% SDS-PAGE gel plot of ^{3}H-strain GM 495(normal) versus ^{14}C-strain GM 372(Gaucher-type 3). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 5 days after seeding with a serum boost. The plot represents the total ML fraction of fibroblast protein. Leucine deficient medium was employed to enhance the incorporation of radioisotope.

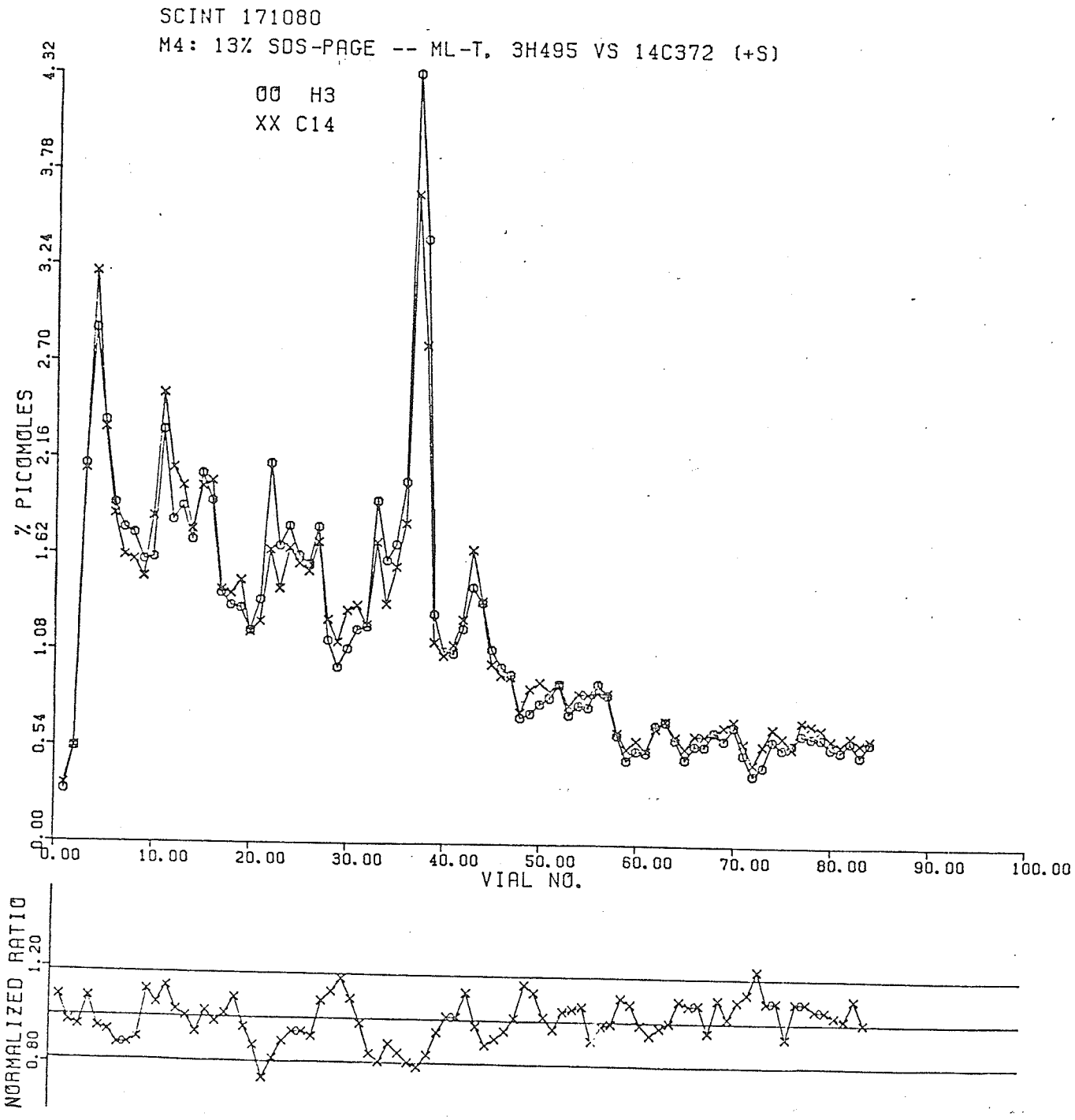

SCINT 171080
M4: 13% SDS-PAGE -- ML-T. 3H495 VS 14C372 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.54
1.08
1.62
2.16
2.70
3.24
3.78
4.32
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 28

Fig. 29. 10% SDS-PAGE gel plot of ^{3}H-strain GM 302(normal) versus ^{14}C-strain GM 877(Gaucher-type 2). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding without a serum boost. The plot represents the total ML fraction of fibroblast protein. The protein species deficient in the Gaucher strain is visible as one peak between vials 49-52 (40-38,000d).

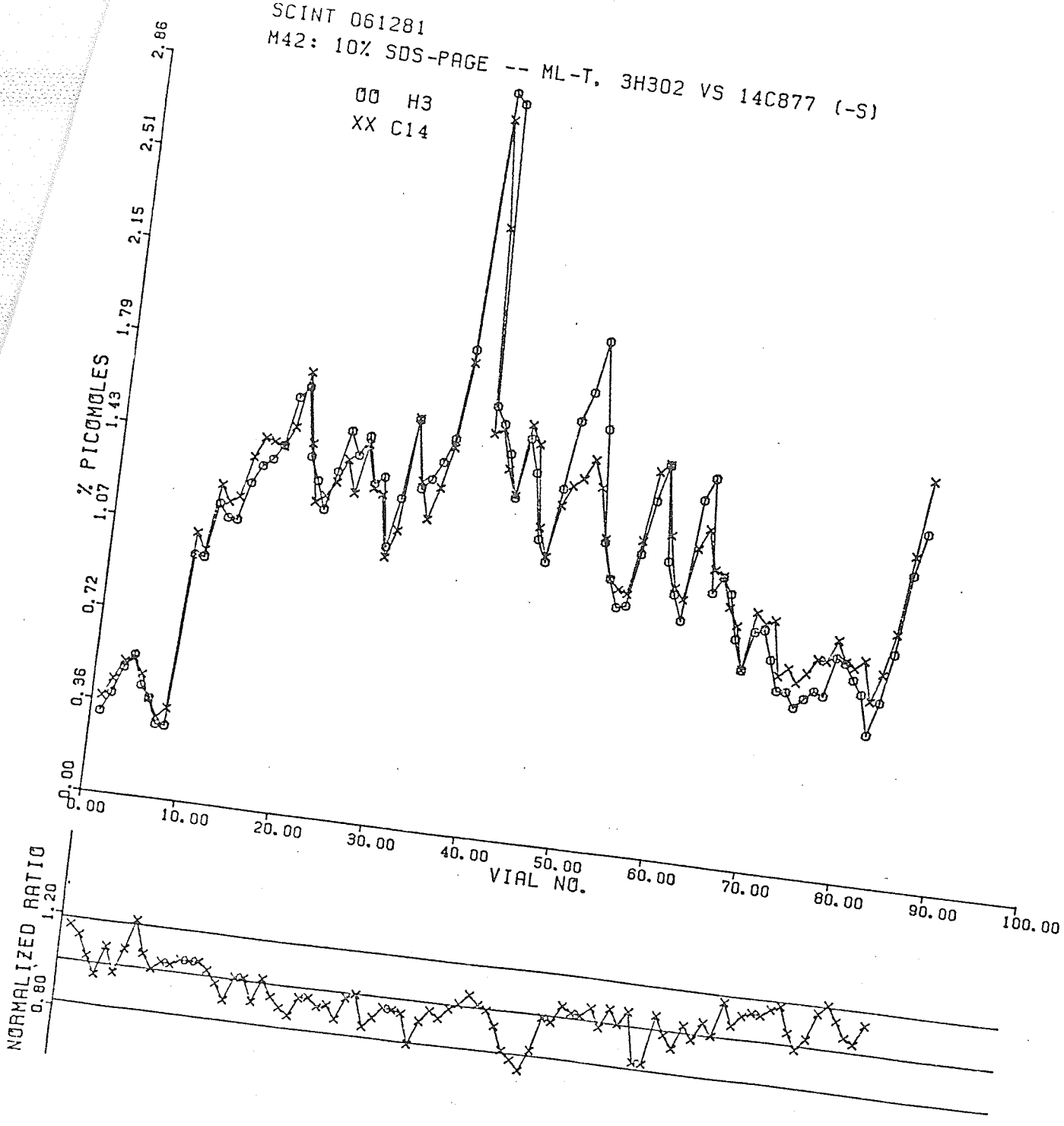

SCINT 061281
M42: 10% SDS-PAGE -- ML-T. 3H302 VS 14C877 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.36
0.72
1.07
1.43
1.79
2.15
2.51
2.86
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 29

Fig. 30. 13% SDS-PAGE gel plot of ^{3}H-strain GM 302(normal) versus ^{14}C-strain GM 877(Gaucher-type 2). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding without a serum boost. The plot represents the total ML fraction of fibroblast protein. The protein species deficient in the Gaucher strain is visible as two peaks between vials 29-32 (43-39,000d).

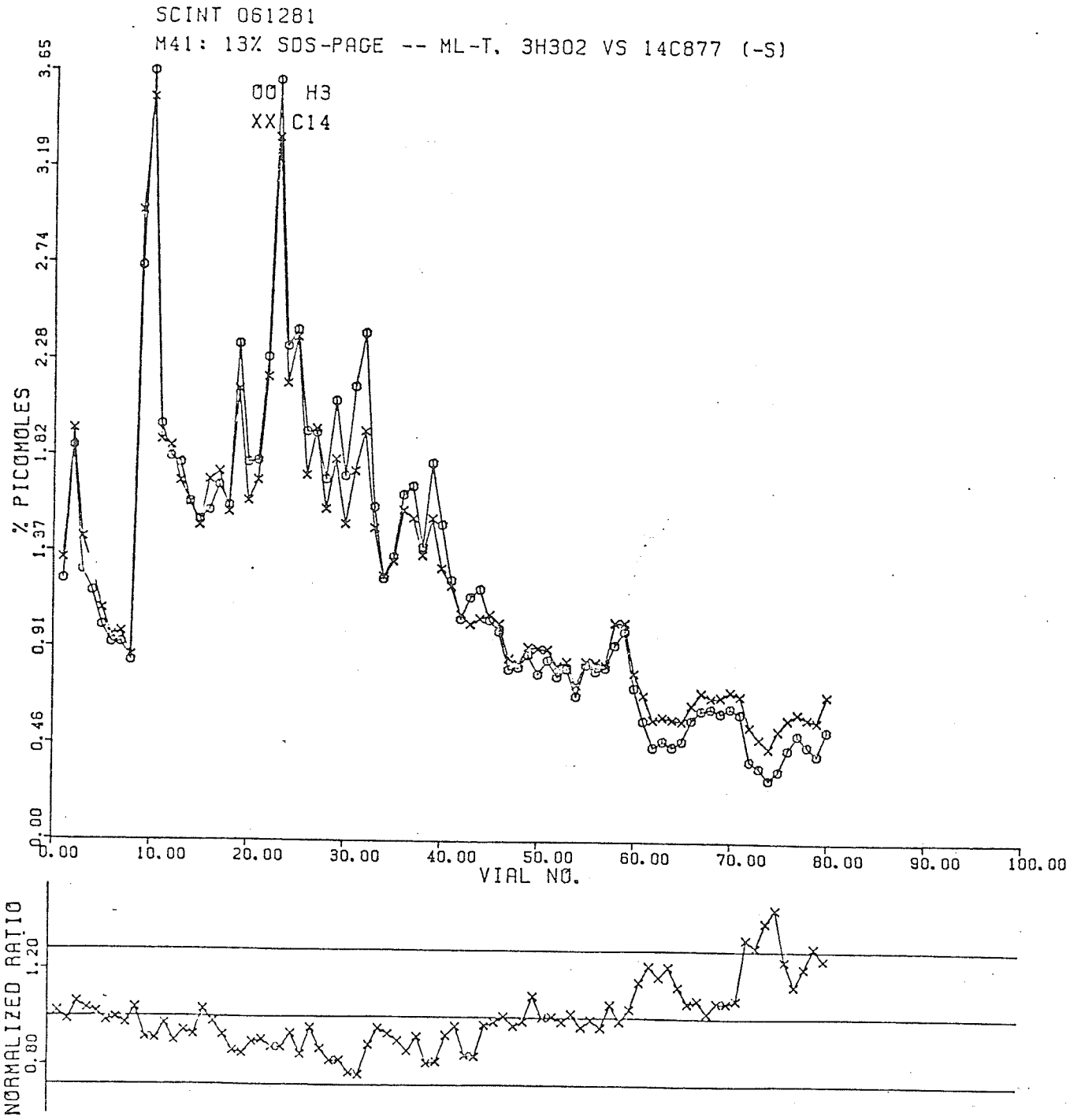

SCINT 061281
M41: 13% SDS-PAGE -- ML-T, 3H302 VS 14C877 (-S)
00 H3
XX C14
% PICOMOLES
0.00
0.46
0.91
1.37
1.82
2.28
2.74
3.19
3.65
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 30

Fig. 31 . 13% SDS-PAGE gel plot of ^{3}H-strain GM 302(normal) versus ^{14}C-strain GM 877(Gaucher-type 2). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the total ML fraction of fibroblast protein. Leucine deficient medium was employed to enhance the incorporation of radioisotope.

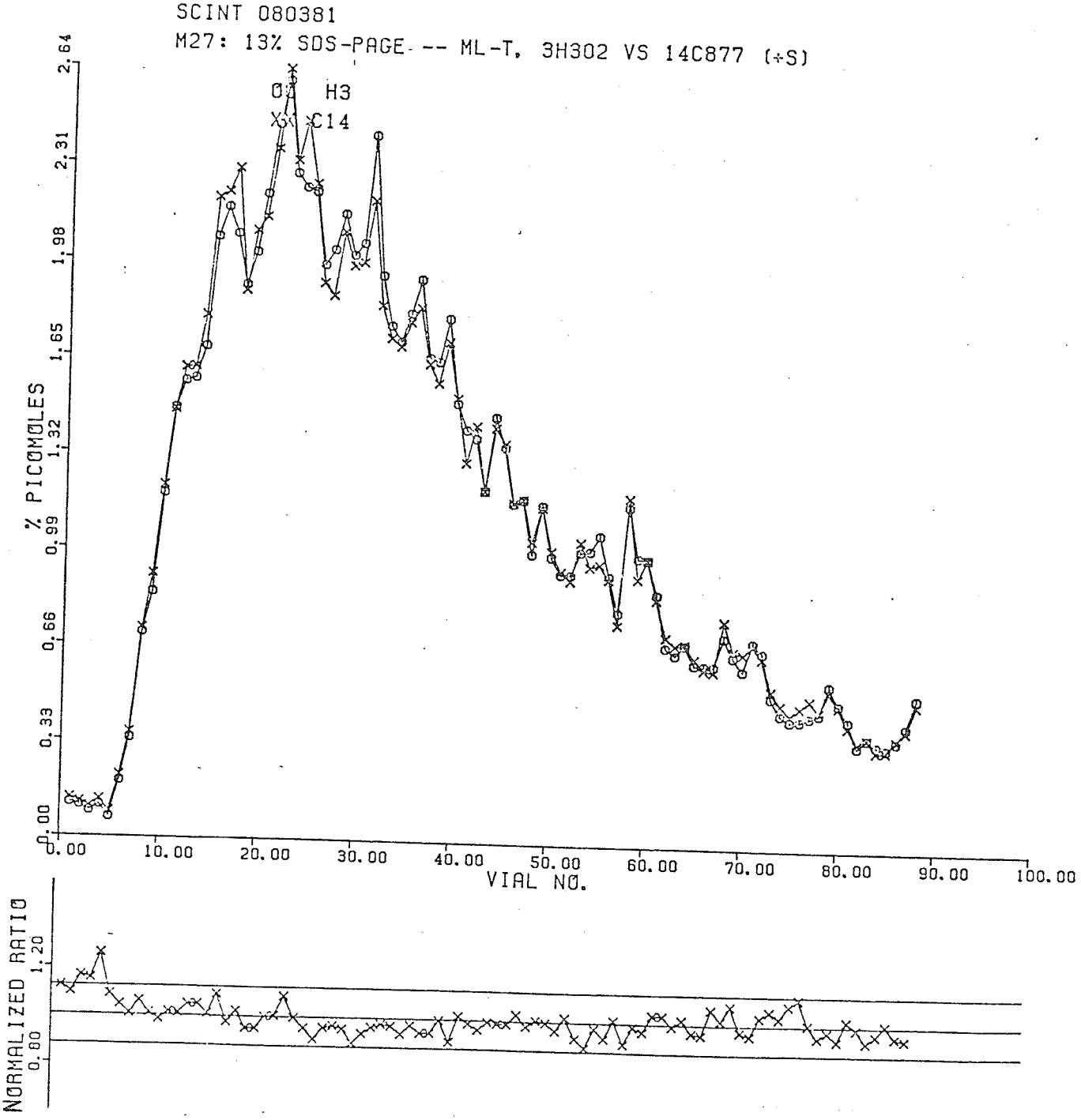

SCINT 080381
M27: 13% SDS-PAGE -- ML-T, 3H302 VS 14C877 (÷S)
H3
C14
% PICOMOLES
0.00
0.33
0.66
0.99
1.32
1.65
1.98
2.31
2.64
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 31

Fig. 32. 13% SDS-PAGE gel plot of ^{3}H-strain GM 495(normal) versus ^{14}C-strain GM 372(Gaucher-type 3). Cells were seeded at a density of 5 x 10^{5}/100mm dish and labeled 7 days after seeding with a serum boost. The plot represents the total ML fraction of fibroblast protein. The protein species deficient in the Gaucher strain are visible in two peaks between vials 32-35 (42-39,000d). Leucine deficient medium was employed to enhance the incorporation of radioisotope.

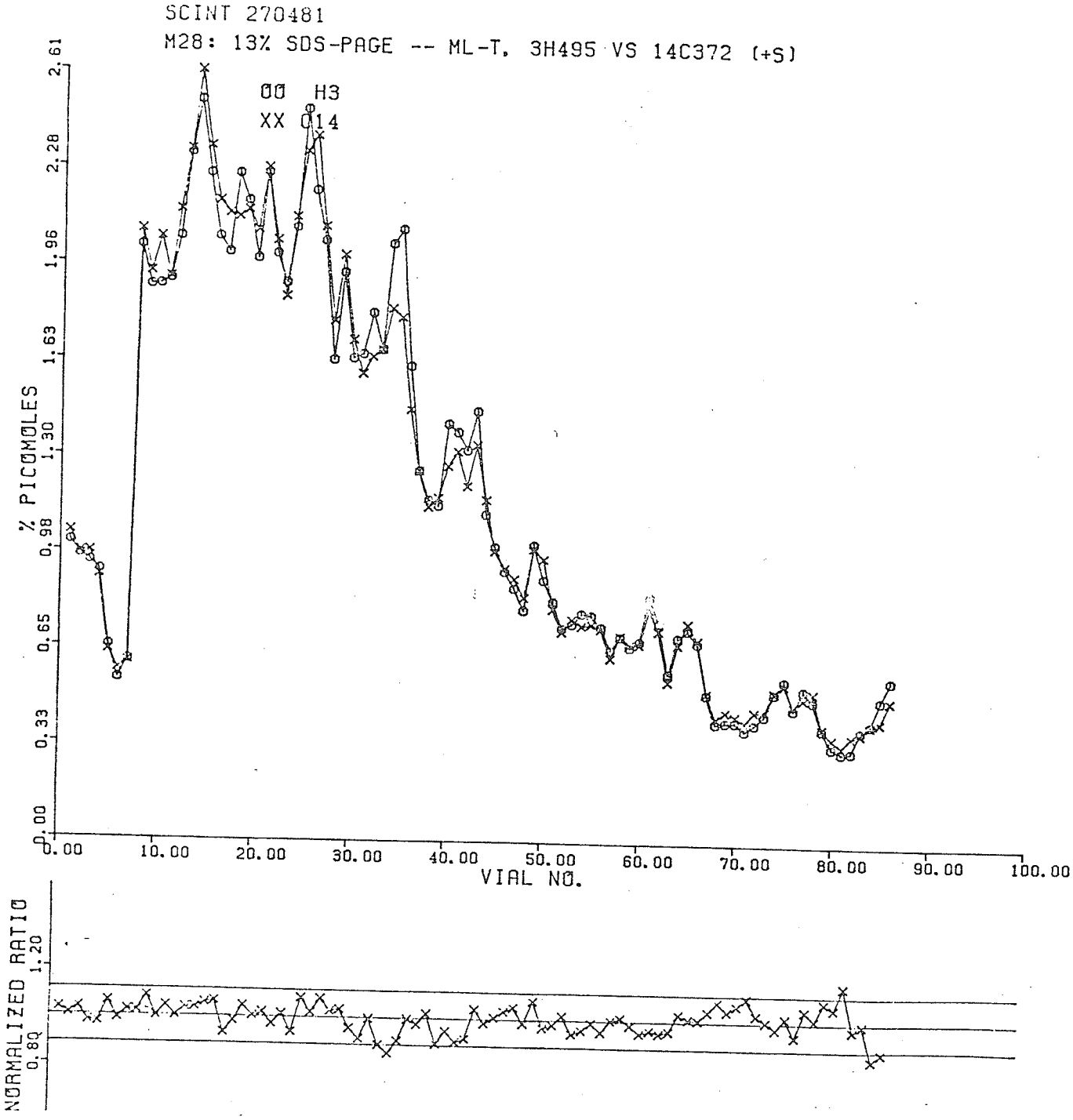

SCINT 270481
M28: 13% SDS-PAGE -- ML-T, 3H495 VS 14C372 (+S)
00 H3
XX C14
% PICOMOLES
0.00
0.33
0.65
0.98
1.30
1.63
1.96
2.28
2.61
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
VIAL NO.
NORMALIZED RATIO
0.80
1.20

Fig. 32

C. TWO DIMENSIONAL GEL ELECTROPHORESIS - UNLABELED AND SINGLE LABELED SAMPLES

Two dimensional gel electrophoresis offered a means of studying the problem of protein synthesis in Pompe disease with much greater resolution. Pena's basic defect might have been subdued, and therefore less obvious, in 1-D gels but be visible clearly as a spot missing or quantitatively reduced in a 2-D gel. The prospect of demonstrating other differences between Pompe fibroblasts and their control strains, or perhaps multiple differences among the Pompe strains would provide good basis for the speculations of genetic heterogeneity. With this in mind, the technique was employed to complement the results of 1-D gels and to generate data that could assist in interpreting the disease's etiology.

1. SELECTING THE OPTIMAL SYSTEM

The primitive two dimensional gel system used by Pena (1977) involved removal of a basic segment from a IEF tube gel and applying it to an SDS-PAGE tube gel. With the many 1-D IEF gels that did not reveal any abnormality, I was not predisposed to one particular area of the IEF gel. However, a more complete examination was attempted by applying entire IEF gels (Pena type) to thick O'Farrell type second dimension slab gels. The resulting stained gels revealed fewer than 6 spots in addition to streaks and smears. Work in our laboratory suggested that this might have been due to incomptability of tube gel diameter (6.0mm) and slab gel thickness (1.5mm). To avoid this problem, IEF gels of the Pena (1978) type were cast at a diameter of

2.1mm, but the modification did not improve resolution at all. It was concluded that the overall differences in sample preparation, gel composition, and electrophoretic conditions rendered the Pena method for IEF incompatible with the O'Farrell second dimension.

A method for 2-D gel electrophoresis developed by Garrels (1979) seemed a major improvement over the method of O'Farrell in terms of spot number and quality. The requirement for radioactively labeled protein samples was absolute, since the IEF gels were of only 1.5mm diameter, and could not accommodate enough protein for staining. The complete ML fractions from a normal (Fig. 33) and a Pompe (Fig. 34) strain were separately labeled and electrophoresed in both dimensions as recommended by Garrels. The overall similarity of autoradiograms from these gels did not compromise the narrow pH range established in the first dimension and the poor resolution of low molecular weight spots in the second dimension. Alteration of the ampholyte composition enabled extension to the pH range to the acidic, but not the basic side.

The simple, rapid, and ultrasensitive silver staining technique that had just been developed (Oakley et al., 1981) was ideally suited to study unlabeled protein samples in thin slab gels of the O'Farrell (1975) type. The application was first made using protein samples treated with nucleases and SDS (Garrels sample preparation). Figures 35 and 36 display total fibroblast protein maps of the fetal cell strain LMF. The first was stained with Coomassie blue by the method of Anderson and Anderson (1977), and the second,

containing the same amount of protein, by the method of Oakley et al. (1981). The sensitivity of the silver staining was striking, detecting incompletely focused proteins in contaminating levels. This interference was later eliminated by regulating the degree of staining, or destaining the overstained gels. Two more total fibroblast samples of hamster origin, CHW (Fig. 37) and 1103 (Fig. 38) illustrate the quality of the separation. Unfortunately in three different sample preparations of the many Pompe strains and their control strains, the resolution achieved in the above gels could not be attained. Samples prepared by the standard O'Farrell method (in the absence of SDS) later proved to be the most reliable in electrophoresis, and this method was therefore uniformly adopted.

The use of silver staining and the unmodified O'Farrell 2-D gel system provided gel patterns of better but inconsistent quality. Most gels displayed one or more of the following sporadic problems:
(a) Horizontal streaks occurring in the 50,000-60,000d region of the second dimension gel, (b) Blank or "washed-out" regions at the top of the second dimension separating gel, or (c) Poor resolution of low molecular weight spots.
These may be seen in the stained gels in the following section. Various concentrations of acrylamide slabs and different exponential gradients of acrylamide were employed to sharpen low molecular weight spots without excluding the well resolved higher molecular weight spots, but none were effective in improving the overall spot distribution.

The use of protein samples radiolabeled with ^{35}S-methionine finally proved to be the method of choice for independent 2-D gel electrophoresis. Apparently, several artifacts viewed by silver staining did not incorporate radioactivity, while many low molecular weight proteins that were invisible on silver stained gels incorporated enough radioactivity to be seen in autoradiograms.

2. APPLYING THE OPTIMAL SYSTEM

The second of the two dimensions adequately resolved spots of both 100,000d and 30,000d, a range that would include undissociated or dissociated alpha-glucosidase molecules, and the small deficient molecule described by Pena and Wrogemann (1978). The range of proteins included in the first dimension of the standard O'Farrell 2-D system was from pI 4.5 to 7.0, which would include acidic but not basic proteins. To examine basic cellular proteins, a modification of the O'Farrell procedure called non-equilibrium pH gradient electrophoresis (NEPHGE; O'Farrell *et al.* 1978) was employed. In this procedure, protein samples were applied to the acidic rather than the basic terminus, and current was applied for a shorter period of time. The spots resolved in NEPHGE gels included proteins that were focused, and spots that were migrating from pH 7 to pH 9. Since the IEF and NEPHGE first dimension gels have few spots in common (little pH overlap, see Fig. 8), it was possible to generate a continuous montage of spots when both types first dimension gel were electrophoresed similarly in the second dimension. It is important to emphasize, however, that only the

IEF spots could be considered truly focused, while NEPHGE spots may be of higher pI's than their locations indicated.

Such montages are illustrated in Figs. 39-43. Fig. 39 is the protein map of the fibroblast strain GM302, which served as a control for the Pompe disease strain GM248 (Fig. 40) and the Gaucher strain GM877 (Fig.41). Of the 738 spots compared in each of the normal and Pompe patterns (Figs. 39,40), only 12 spots were found to be clearly present in one strain and absent in the other. None of these differences were consistent in either a second infantile Pompe strain (Fig. 42) or an adult form Pompe strain (Fig. 43).

The Gaucher fibroblast pattern (Fig. 41) was also very similar to the control pattern, with only 4 spots clearly present in one strain and absent in the other. One difference which was more striking than most is noted by an arrow. This particular spot is discussed further in the following section. Interestingly, the deficiency of the 40,000d protein species found in 1-D gels was not apparent in the 2-D gel autoradiograms.

Since the most discrete spot resolution was achieved with strains WG 482 and GM 1935, these were compared for a more objective determination of differences. The comparisons are summarized in Table X. This observation is consistent with the very low levels of polymorphism detectable by 2-D gel electrophoresis (McConkey et al., 1979; Walton et al., 1979).

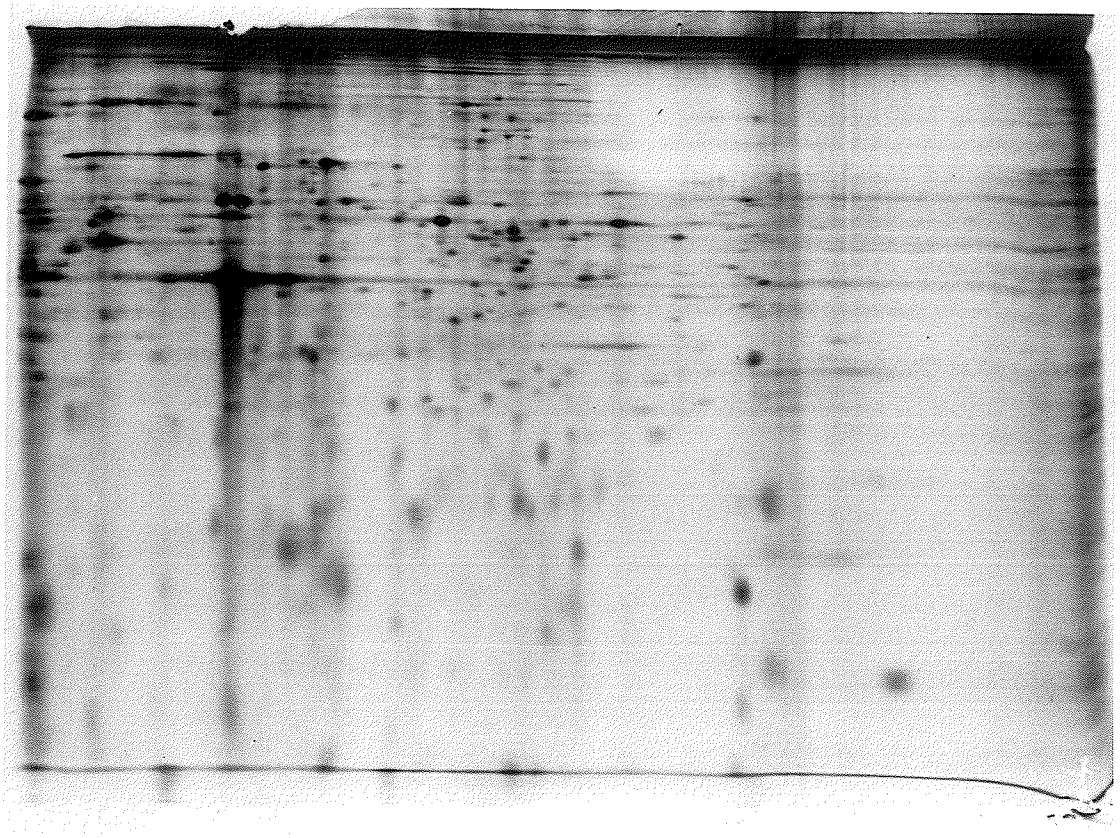


Fig. 33. An autoradiogram of a gel containing ^{35}S-methionine labeled MCH 49 (normal) proteins separated by 2-D gel electrophoresis (Garrels, 1979). The first dimension gel contained 2% pH 6-8 ampholytes and was electrophoresed for 19000 Vhr. This gel was applied to a 12% polyacrylamide slab gel and electrophoresed in the second dimension. Protein spots are more basic toward the right and of smaller molecular weight toward the bottom of the autoradiogram.

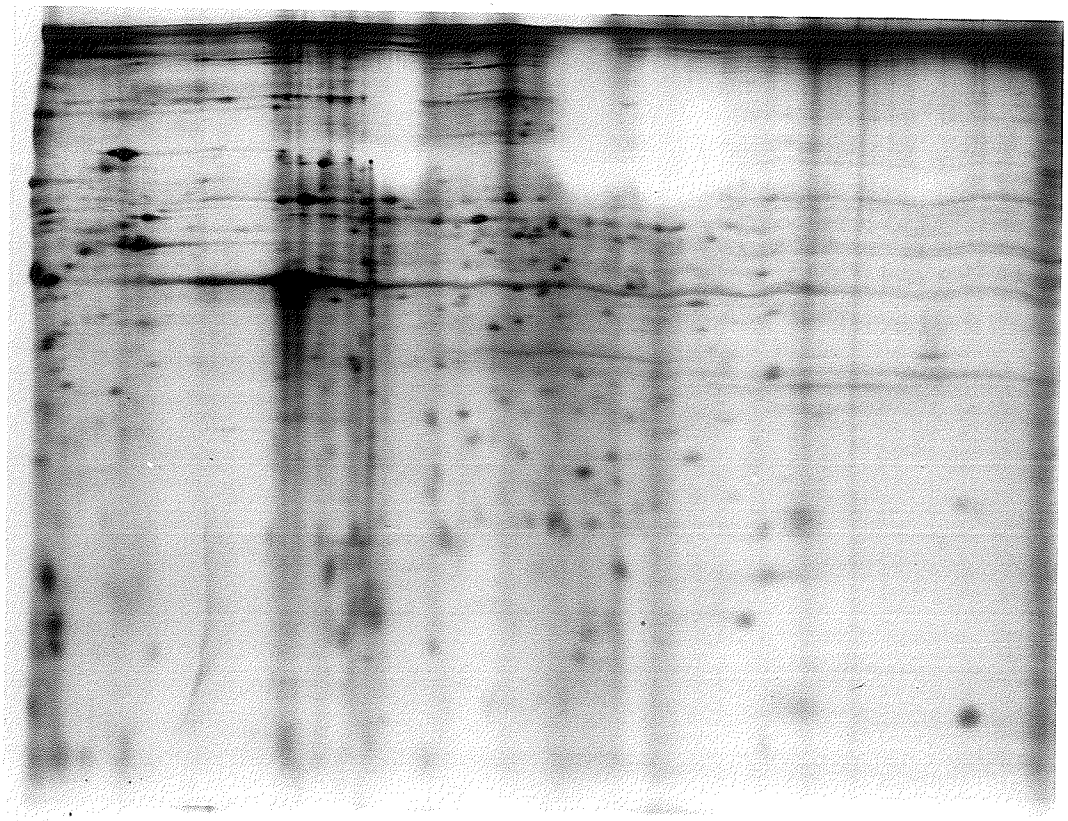


Fig. 34. An autoradiogram of a gel containing ^{35}S-methionine labeled WG 482 (Pompe-infantile) proteins separated by 2-D gel electrophoresis (Garrels, 1979). Electrophoresis was performed as described in the legend to Fig. 33.

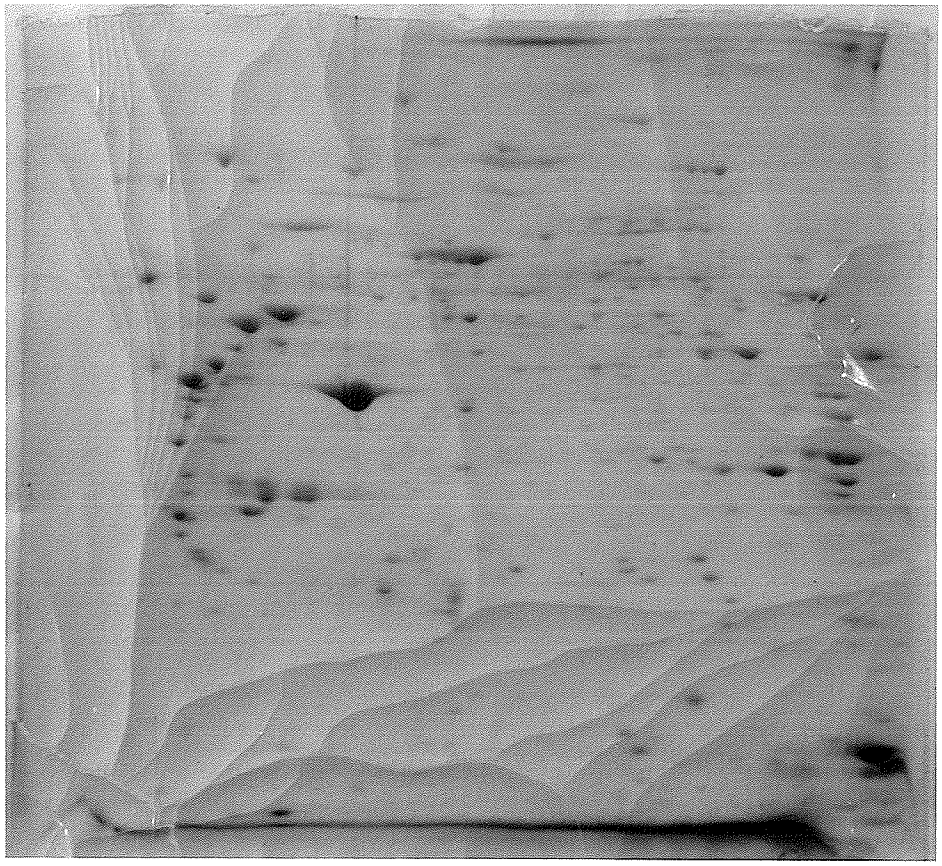


Fig. 35. A Coomassie blue stained gel of unlabeled total LMF fibroblast protein prepared by the method of Garrels (1979). The first dimension gels were 3.0mm in diameter and contained 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes. Electrofocusing was performed for 5600 Vhr. This gel was applied to a 10% polyacrylamide slab gel and electrophoresed in the second dimension.

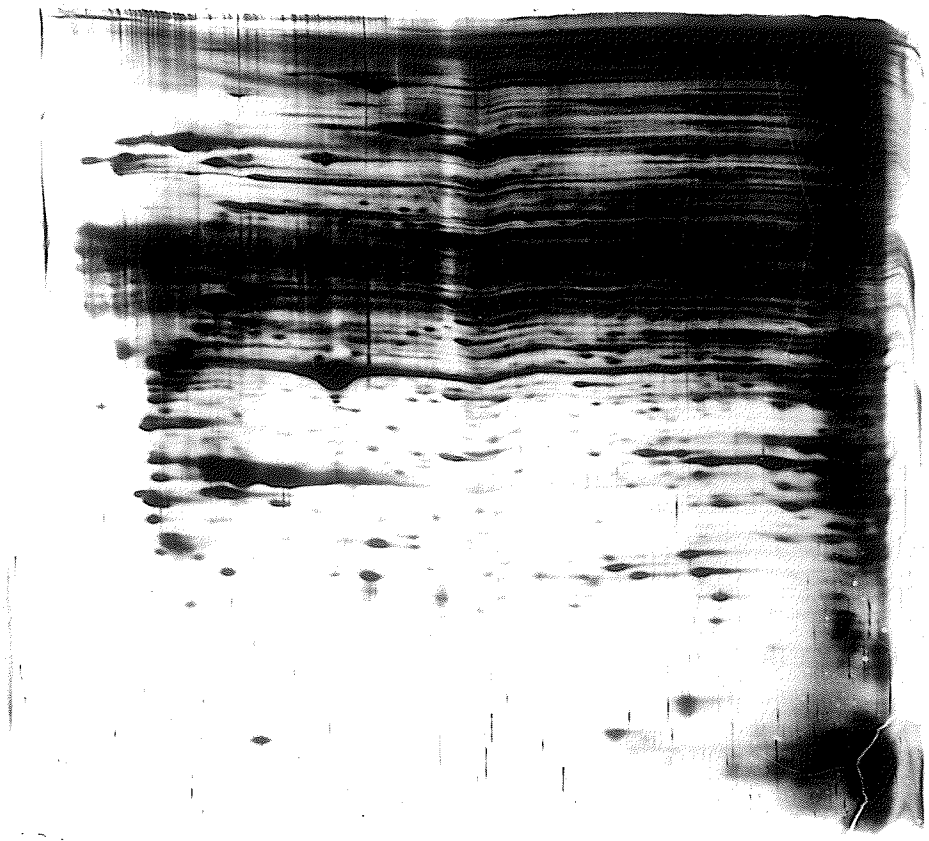


Fig. 36. A silver stained gel of the same sample electrophoresed in parallel described with the gel shown in Fig. 35. The sensitivity of the staining is much greater.

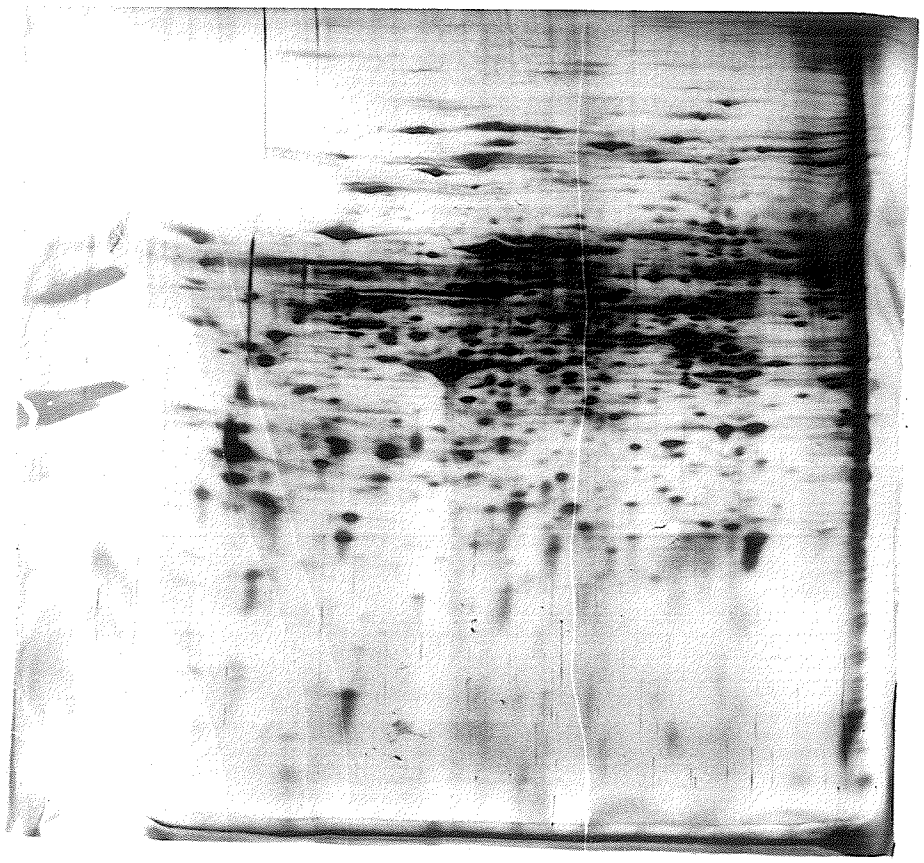


Fig. 37. A silver stained gel of unlabeled total CHW fibroblast protein (hamster) prepared by the method of Garrels (1979). Electrophoresis was performed as described in the legend to Fig. 35

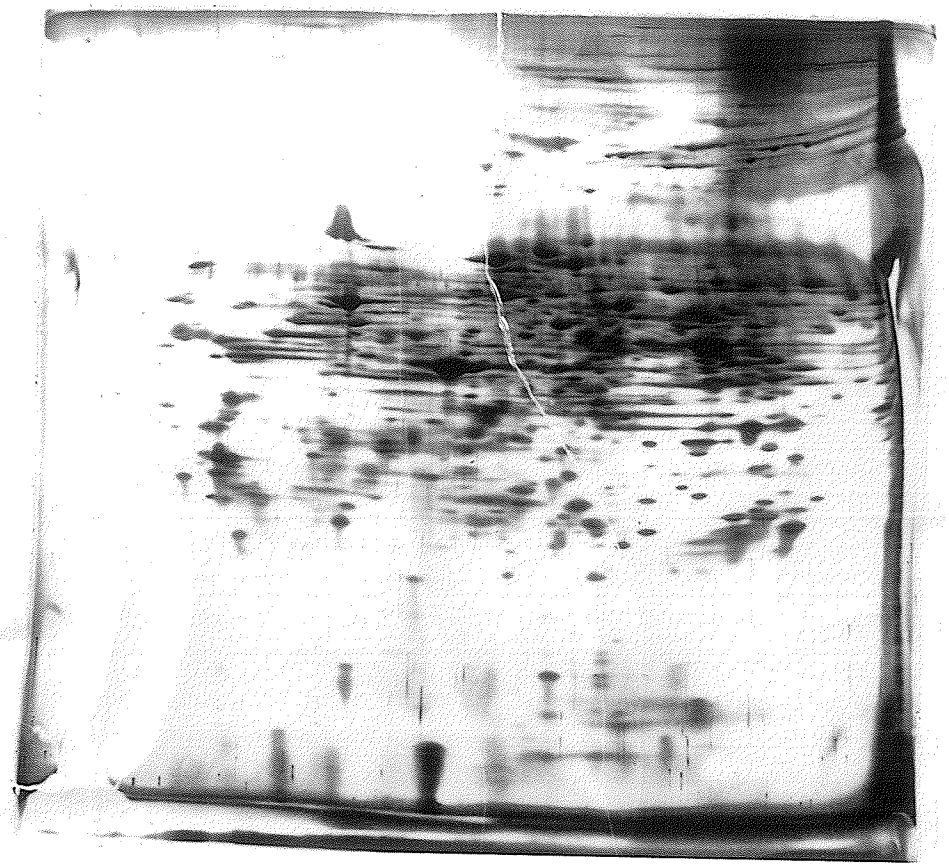


Fig. 38. A silver stained gel of unlabeled total 1103 fibroblast protein (hamster) prepared by the method of Garrels (1979). Electrophoresis was performed as described in the legend to Fig. 35.

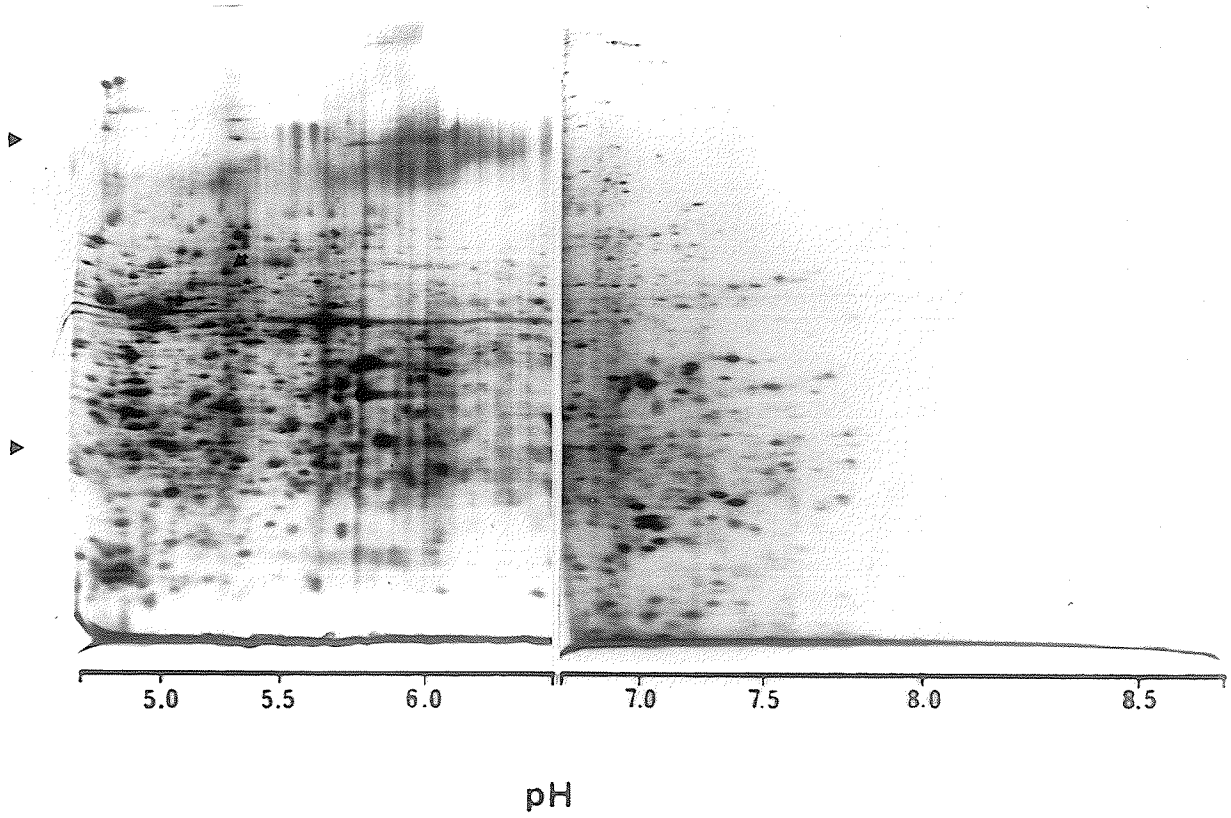


Fig. 39. Montage of ^{35}S-labeled GM 302 (normal) proteins separated by two complementary types of 2-D gel electrophoresis. The first dimension IEF gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 5600 Vhr. The first dimension NEPHGE gel contained 2% pH 7-9 ampholytes and was electrophoresed for 1600 Vhr. Both IEF and NEPHGE gels were applied to 10% polyacrylamide slab gels and electrophoresed in the second dimension. The autoradiograms of these gels displayed acidic (left side) and basic (right side) protein species respectively. The mobilities of two molecular markers, phosphorylase a (94,000; upper arrowhead) and carbonic anhydrase (29,000; lower arrowhead) are indicated along the left gel margin. Approximately 10^6 TCA-precipitable cpm were applied to each of the first-dimension gels, and the autoradiograms exposed for seven days. The solid arrow within the gel indicates a spot that is missing in DMD strains (Fig. 41).

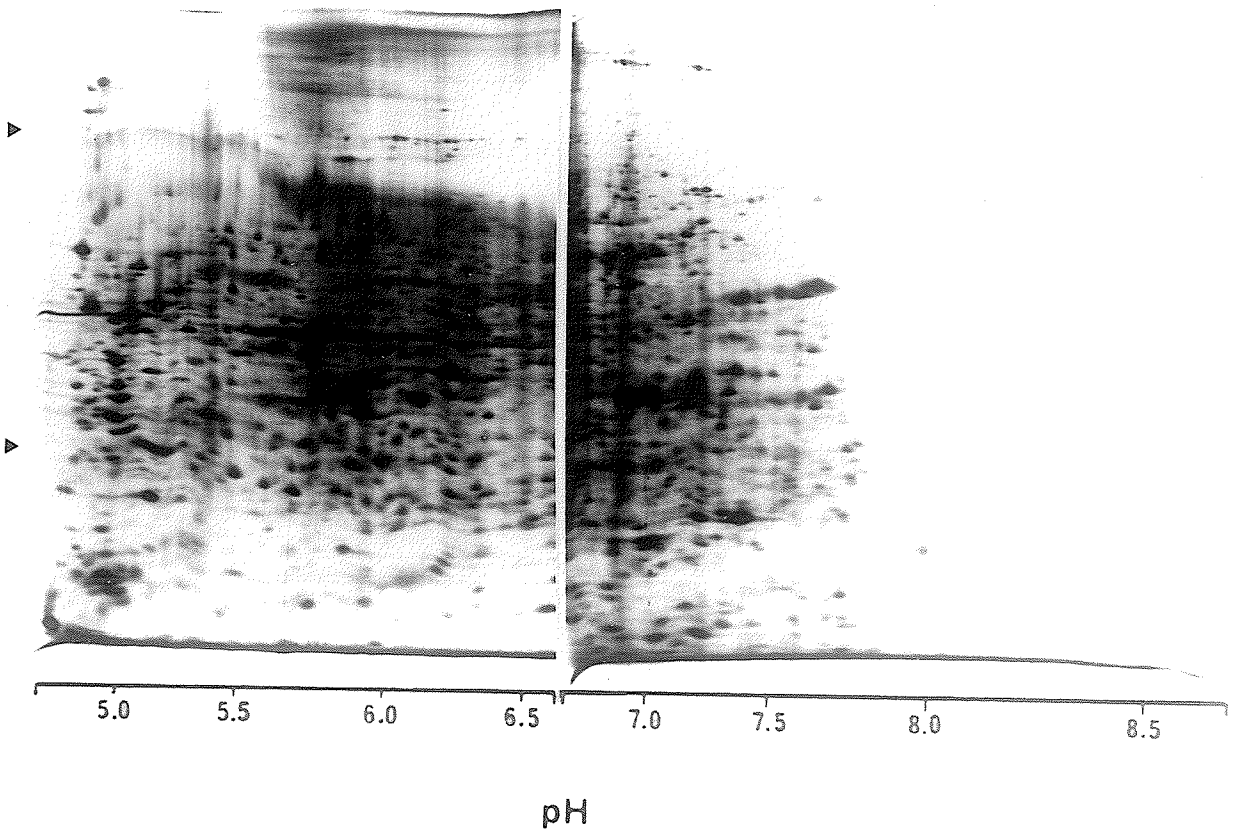


Fig. 40. Montage of ^{35}S-labeled GM 248 (Pompe-infantile) proteins separated by two complementary types of 2-D gel electrophoresis. The first dimension IEF gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 5600 Vhr. The first dimension NEPHGE gel contained 2% pH 7-9 ampholytes and was electrophoresed for 1600 Vhr. Both IEF and NEPHGE gels were applied to 10% polyacrylamide slab gels and electrophoresed in the second dimension. The autoradiograms of these gels displayed acidic (left side) and basic (right side) protein species respectively. The mobilities of two molecular markers, phosphorylase a (94,000; upper arrowhead) and carbonic anhydrase (29,000; lower arrowhead) are indicated along the left gel margin. Approximately 10^6 TCA-precipi table cpm were applied to each of the first-dimension gels, and the autoradiograms exposed for seven days.

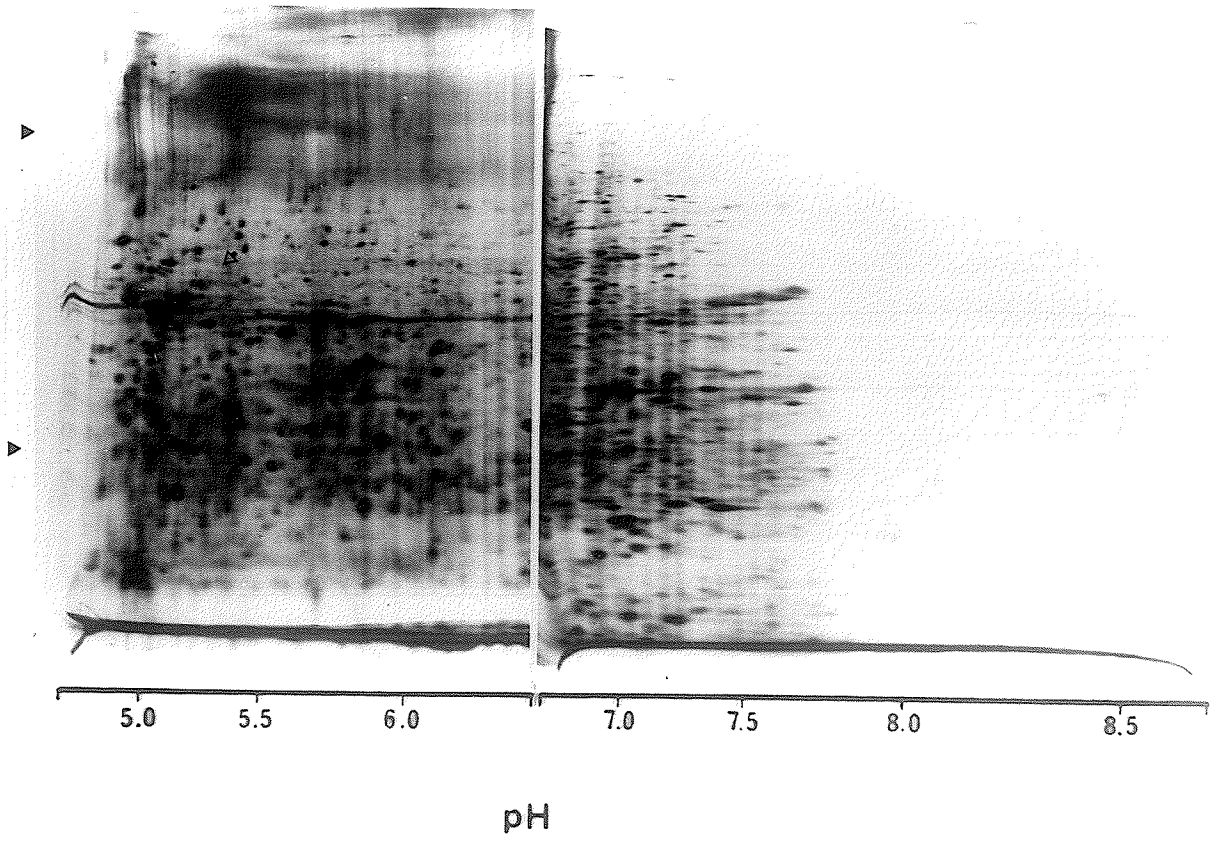


Fig. 41. Montage of ^{35}S-labeled GM 877 (Gaucher-type 2) proteins separated by two complementary types of 2-D gel electrophoresis. The first dimension IEF gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 5600 Vhr. The first dimension NEPHGE gel contained 2% pH 7-9 ampholytes and was electrophoresed for 1600 Vhr. Both IEF and NEPHGE gels were applied to 10% polyacrylamide slab gels and electrophoresed in the second dimension. The autoradiograms of these gels displayed acidic (left side) and basic (right side) protein species respectively. The mobilities of two molecular markers, phosphorylase a (94,000; upper arrowhead) and carbonic anhydrase (29,000; lower arrowhead) are indicated along the left gel margin. Approximately 10^6 TCA-precipitable cpm were applied to each of the first-dimension gels, and the autoradiograms exposed for seven days. The hollow arrow within the gel indicates the position of a spot which is present in the control strain, GM 302 (Fig. 39).

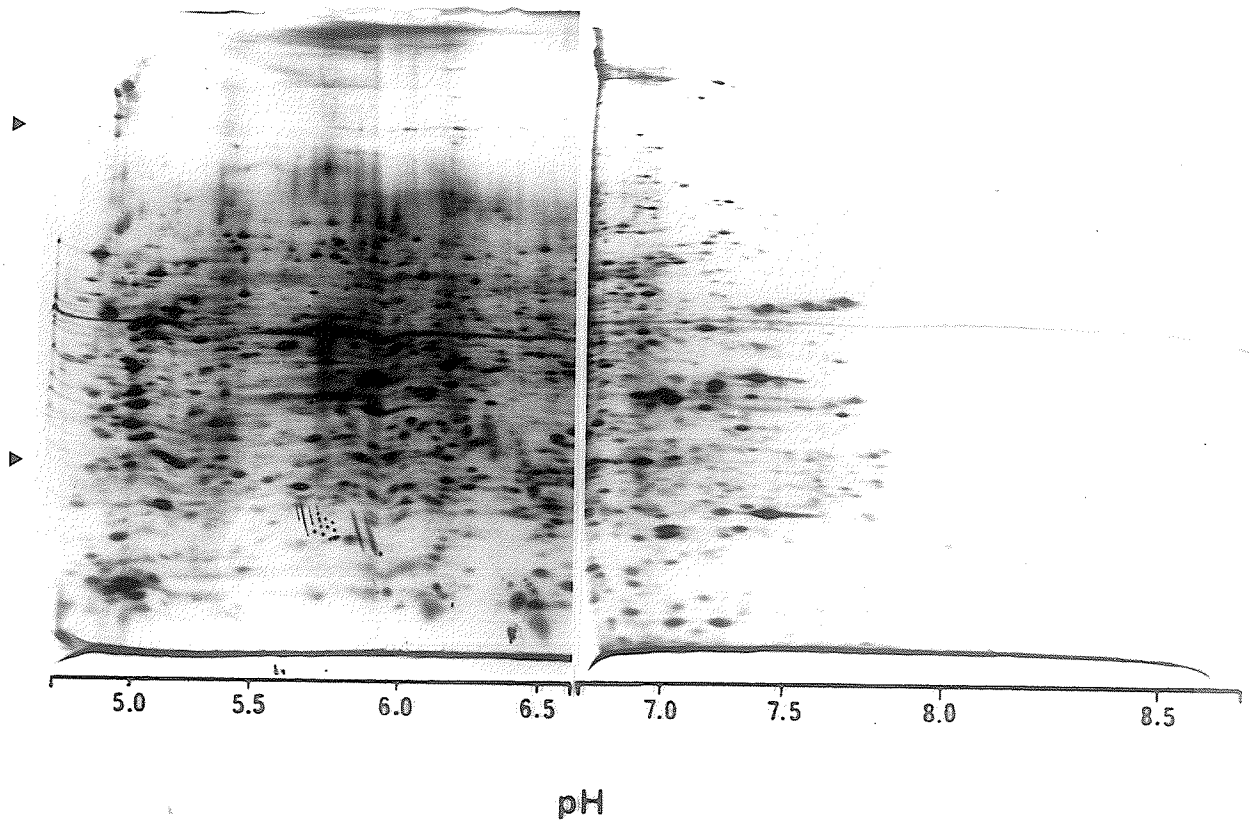


Fig. 42. Montage of ^{35}S-labeled WG 482 (Pompe-infantile) proteins separated by two complementary types of 2-D gel electrophoresis. The first dimension IEF gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 5600 Vhr. The first dimension NEPHGE gel contained 2% pH 7-9 ampholytes and was electrophoresed for 1600 Vhr. Both IEF and NEPHGE gels were applied to 10% polyacrylamide slab gels and electrophoresed in the second dimension. The autoradiograms of these gels displayed acidic (left side) and basic (right side) protein species respectively. The mobilities of two molecular markers, phosphorylase a (94,000; upper arrowhead) and carbonic anhydrase (29,000; lower arrowhead) are indicated along the left gel margin. Approximately 10^6 TCA-precipitable cpm were applied to each of the first-dimension gels, and the autoradiograms exposed for seven days.

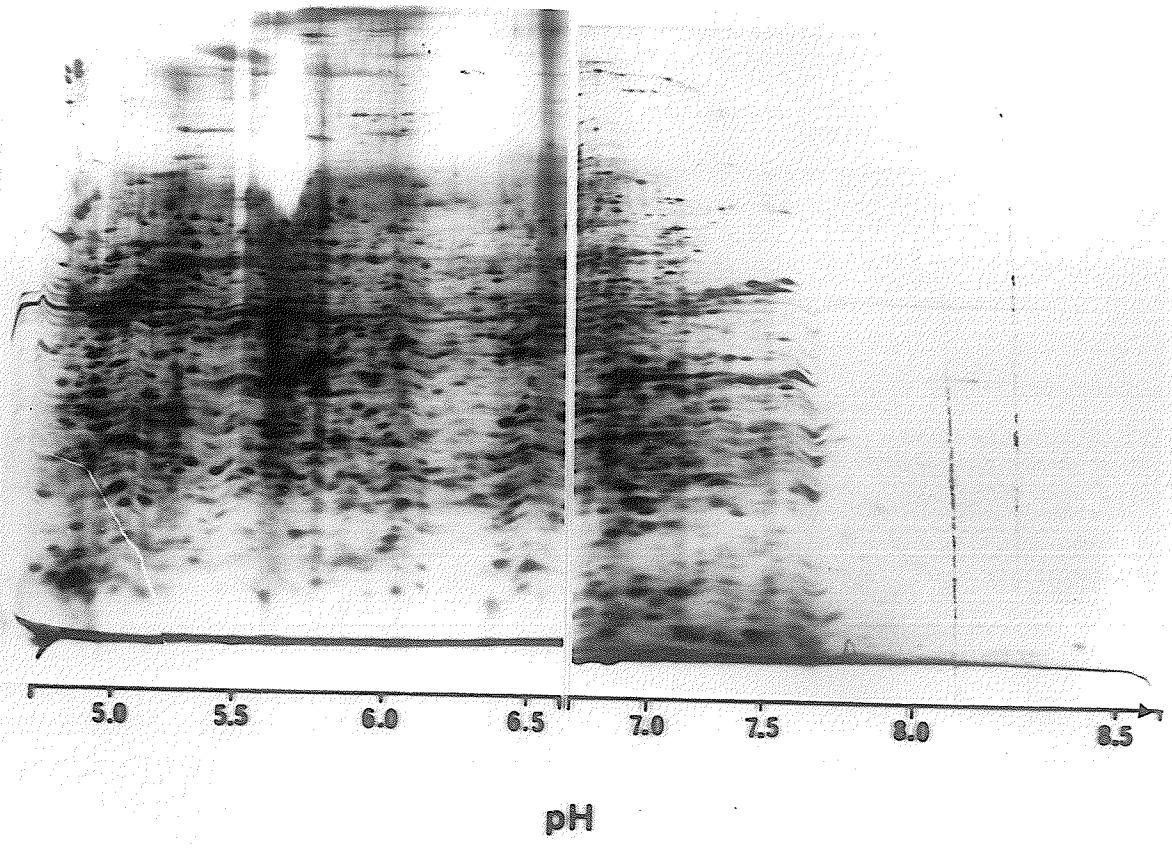


Fig. 43. Montage of ^{35}S-labeled GM 1935 (Pompe-adult form) proteins separated by two complementary types of 2-D gel electrophoresis. The first dimension IEF gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 5600 Vhr. The first dimension NEPHGE gel contained 2% pH 7-9 ampholytes and was electrophoresed for 1600 Vhr. Both IEF and NEPHGE gels were applied to 10% polyacrylamide slab gels and electrophoresed in the second dimension. The autoradiograms of these gels displayed acidic (left side) and basic (right side) protein species respectively. The mobilities of two molecular markers, phosphorylase a (94,000; upper arrowhead) and carbonic anhydrase (29,000; lower arrowhead) are indicated along the left gel margin. Approximately 10^6 TCA-precipitable cpm were applied to each of the first-dimension gels, and the autoradiograms exposed for seven days.

Table X: Summary of qualitative differences between 2-D gel autoradiograms of ^{35}S-labeled fibroblast proteins.

Strains compared	Spots matched	Spots unmatched	Unmatched/ Total Spots (%)
GM 248 vs GM 302	738	12	1.6%
GM 1877 vs GM 302	720	4	0.5%
WG 482 vs GM 1935	681	10	1.4%

D. TWO DIMENSIONAL GEL ELECTROPHORESIS - DOUBLE LABELED SAMPLES

The ability to quantitate differences between protein species is an advantage of the 1-D gel electrophoresis/scintillation counting approach that is not shared in the analysis of 2-D gel patterns. The most comparable method to achieve this has been the development of optical scanning and computerized integration systems to standardize the distribution and intensity of spots on different gels (Anderson et al., 1979a; Bossinger et al., 1979; Garrels, 1979; Lester et al., 1980; Lipkin and Lemkin, 1980). Unfortunately, these systems are not widely available, and are most reliable when using the double label method of McConkey (1979). This procedure involves the production of different radiograms for ^{14}C and ^{3}H so that the differently labeled proteins can be visualized in the same gel. The production of comparable radiograms , especially the ^{3}H fluorogram is regrettably a lengthy procedure, and often yeilds a diffuse image. In analogy to the 1-D method of Pena (1977), however, individual double labeled protein spots can be stained, excised,and prepared for liquid scintillation counting. The analyses performed by the SCINT program (Wrogemann et al., 1977) are well suited to detect minor differences within the well resolved proteins. The technique was performed successfully on a spot found consistently missing in strains from patients with DMD. (Rosenmann et al., manuscript in preparation). Figs. 44 and 45 display 2-D gels of ^{3}H normal and ^{14}C dystrophic fibroblast protein with the suspected abnormality and Fig. 46 shows the punched gel that was electrophoresed

with both samples present. Fig. 47 is a diagrammatic representation of a gel that was used to mesure the reduction of leucine incorporated into spot #35 of the dystrophic strain DC. Approximately 50 spots were excised, including several from different blank areas of the gel, and those with less than twice background cpm were rejected. The normalized ratio of spot number 35 indicated that the leucine incorporation of this spot was approximately five times greater in the normal control strain, MCH 40, than in the dystrophic strain DC (Fig. 48), while spots 11 and 12 (the next greatest deviants from the normalized ratio) were visible as minor quantitative differences.

Naturally, the technique is too laborious to randomly search for qualitative differences, but it is well suited to verify suspected abnormalities detected by simpler and more rapid methods. It was therefore intended for use with Pompe disease fibroblasts, since a small subset of protein spots could be selected on the basis of size and molecular weight. Unfortunately, failure to demonstrate any such abnormality among Pompe disease strains in ^{35}S-labeled proteins in 2-D gels (previous section), combined with the negative results of the 1-D gels, made this excercise unwarranted.

Gaucher disease fibroblasts clearly did show a defect at approximately 40,000d in 1-D SDS-PAGE gels, and it was of interest whether this defect would be easily detected in 2-D gels. The gel autoradiograms from single labeled samples failed to show any spots near 40,000d to be missing in strain GM877, but an acidic protein of approximately 50,000d was absent in this Gaucher strain compared to its

control and the Pompe strains. With a double labeled sample, a similar gel was electrophoresed, silver stained, and many spots excised, including the 50,000d acidic protein, and many 40,000d proteins. Only two of the spots excised had what was considered much reduced leucine incorporation, and both were in the Gaucher strain. The punched gel and the spots mentioned are displayed in Fig. 49. The first was the 50,000d acid protein, and the second was a protein of approximately 64,000d, with a slightly higher pI. For both spots, the leucine incorporated was 2.5 times greater in the normal strain GM302 than in the Gaucher strain.

The significance of this finding was twofold. First, the technique was used to confirm and quantitate a deficiency that was observed in other gel systems, as was the case in the DMD experiment. Second, the two spots may have represented cytosolic and lysosomal forms of beta-glucosidase, which have been shown to have molecular weights of 50,000d (rat kidney) and 60,000d (human placenta) respectively (Barret and Heath, 1978). This would corroborate clinical evidence that both activities are reduced in some patients. Alternatively, the observation could represent quantitative variation in protein levels, which was observed to be common in the analysis of gel autoradiograms. Since no other Gaucher strains were subsequently tested, neither explanation could be favoured.

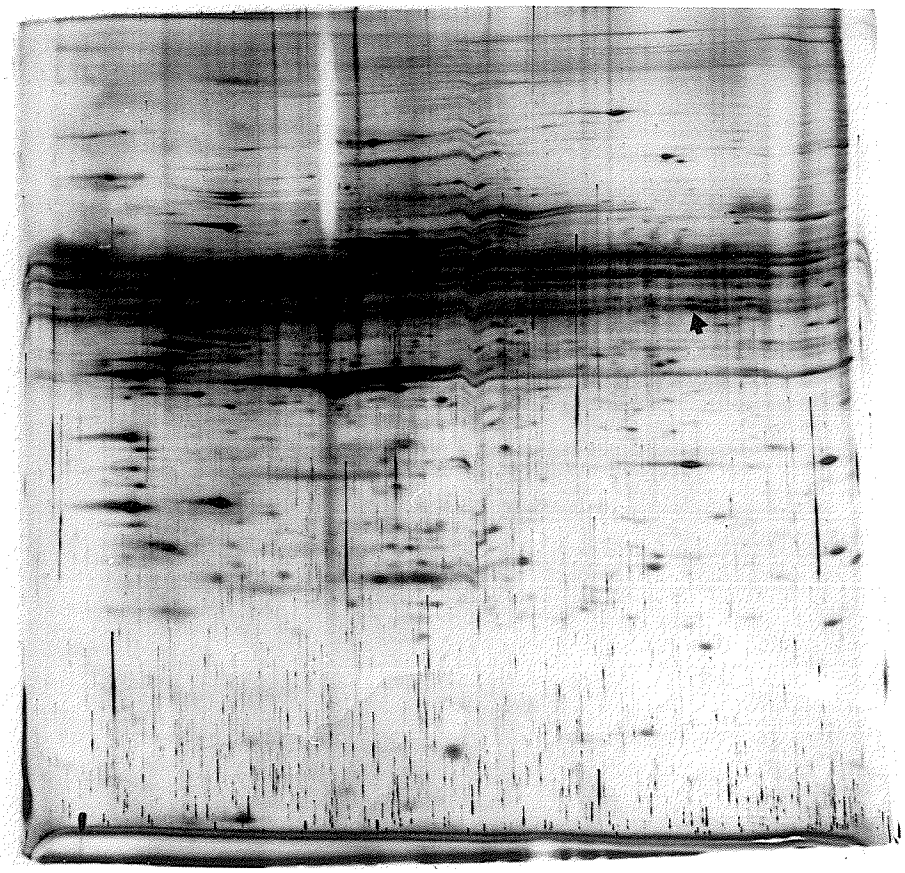


Fig. 44. A silver stained gel of ^{3}H-labeled MCH 40 proteins separated by 2-D gel electrophoresis (O'Farrell 1975). The first dimension gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 7200 Vhr. This gel was applied to a 10% polyacrylamide slab gel and electrophoresed in the second dimension. The solid arrow indicates the spot that is missing in DMD strains. 3.5

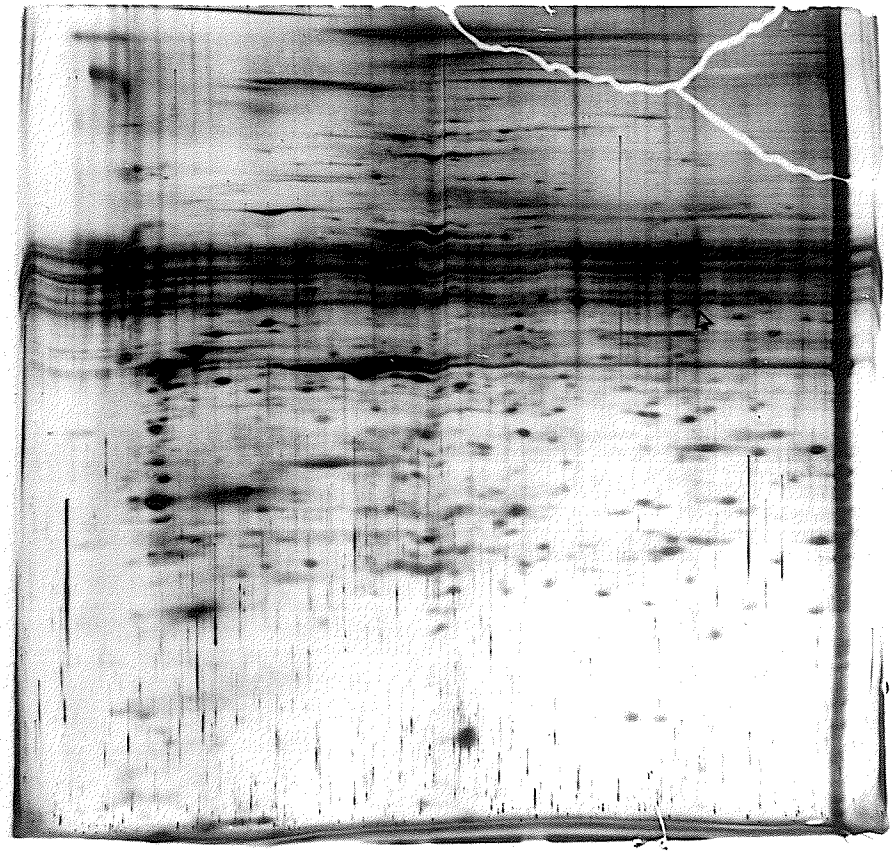


Fig. 45. A silver stained gel of ^{14}C labeled DC proteins separated by 2-D gel electrophoresis (O'Farrell 1975). The first dimension gel containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes was electrophoresed for 7200 Vhr. This gel was applied to a 10% polyacrylamide slab gel and electrophoresed in the second dimension. The hollow arrow indicates the position of the spot that is present in normal strains.

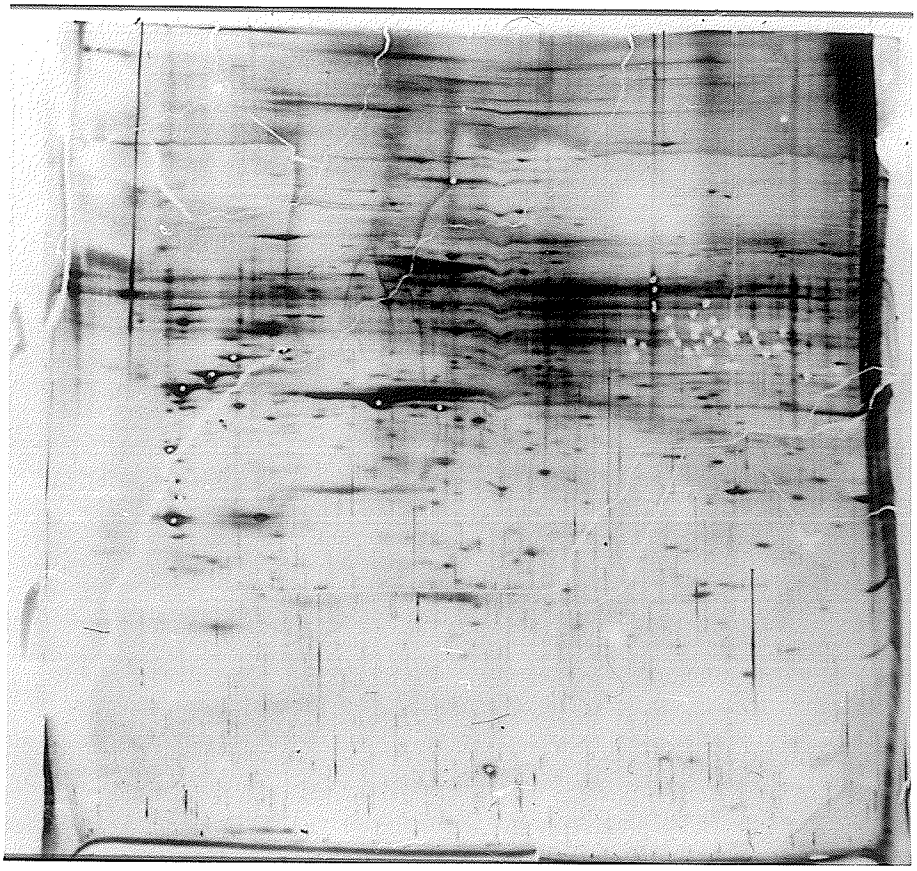
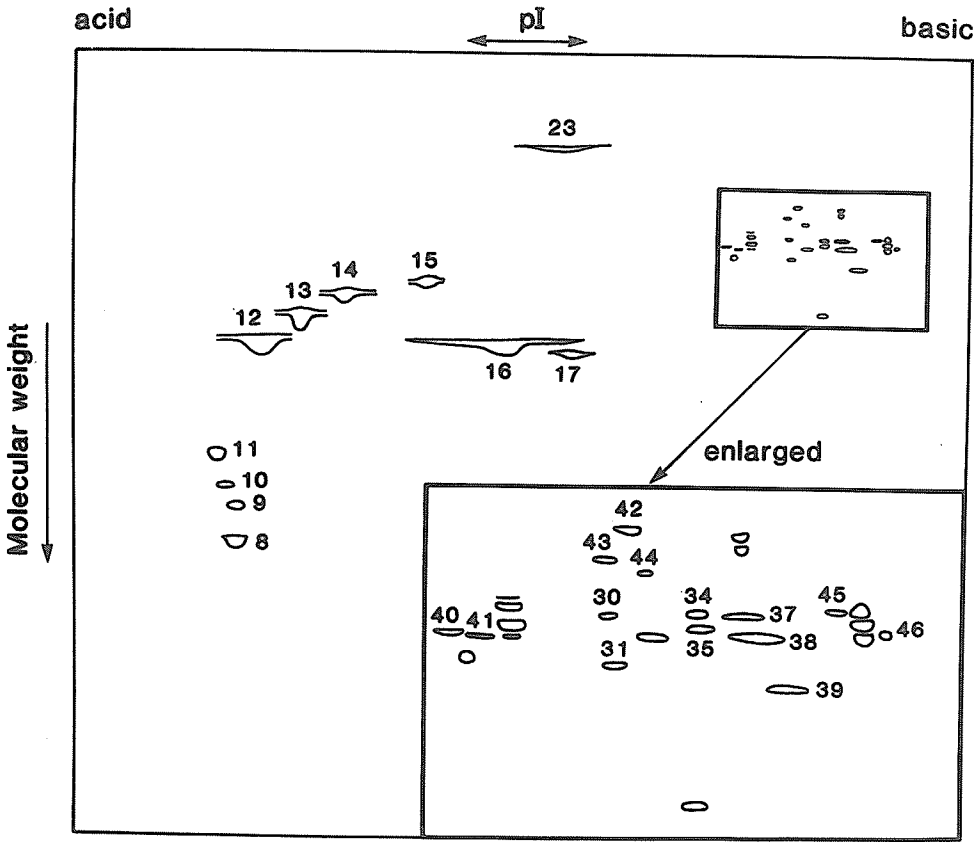
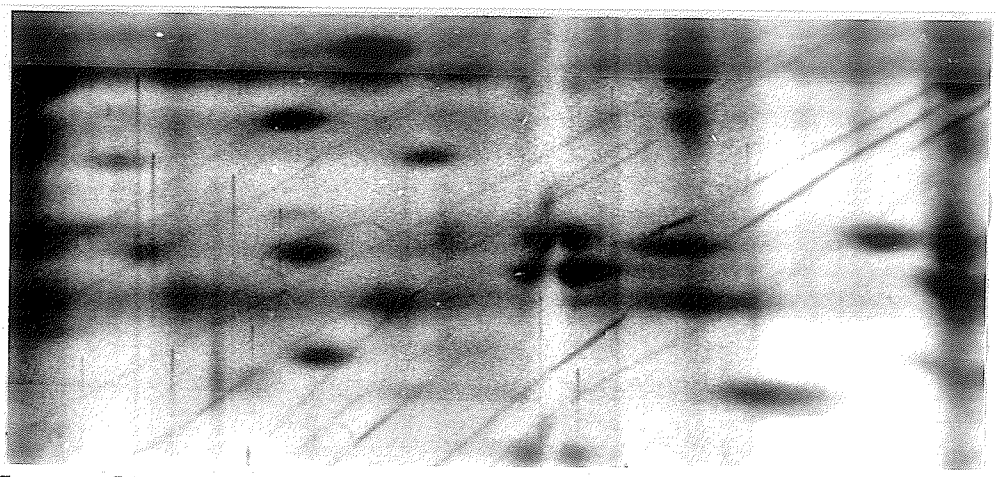


Fig. 46. A silver stained 2-D gel after excision of spots. This gel contained both ^{3}H-labeled (MCH 40) normal proteins and ^{14}C-labeled dystrophic (DC) fibroblast proteins. The gel electrophoresis was performed as described in the legends to Figs. 44 and 45. Many dark spots were punched out, including the spot observed to be missing in dystrophic fibroblast strains.



a.



b.

Fig. 47. a. Diagrammatic representation of the gel in Fig. 46. Spots were numbered arbitrarily during the course of the experiment. The area of the expected protein deficiency is enlarged for clarity, and a photograph of this area displayed in (b). The diagonal marks are shadows cast by the transilluminator.

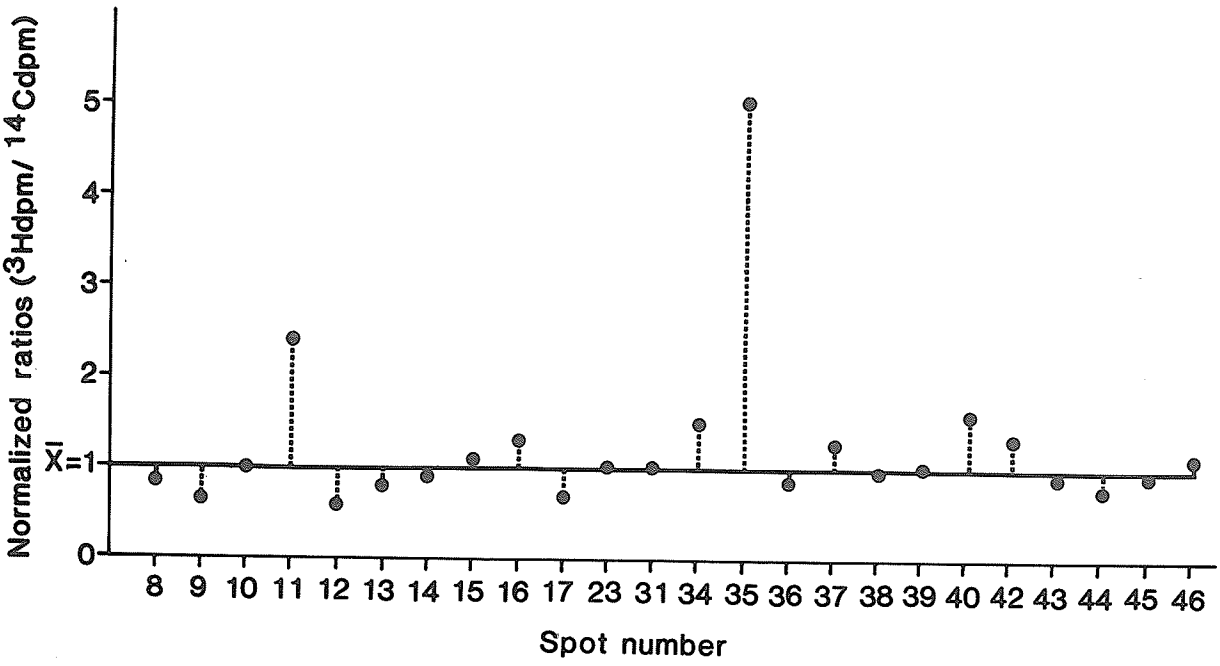


Fig. 48. Plot of the normalized ratio of ^{3}H dpm/^{14}C dpm for spots excised from the gel in Fig. 47. The mean ratio was adjusted to 1, and the dotted lines represent deviations from the mean. The plot displays that spot number 35 has thus incorporated 5 times more radioactive leucine into control fibroblast protein (^{3}H) than into dystrophic fibroblast protein (^{14}C).

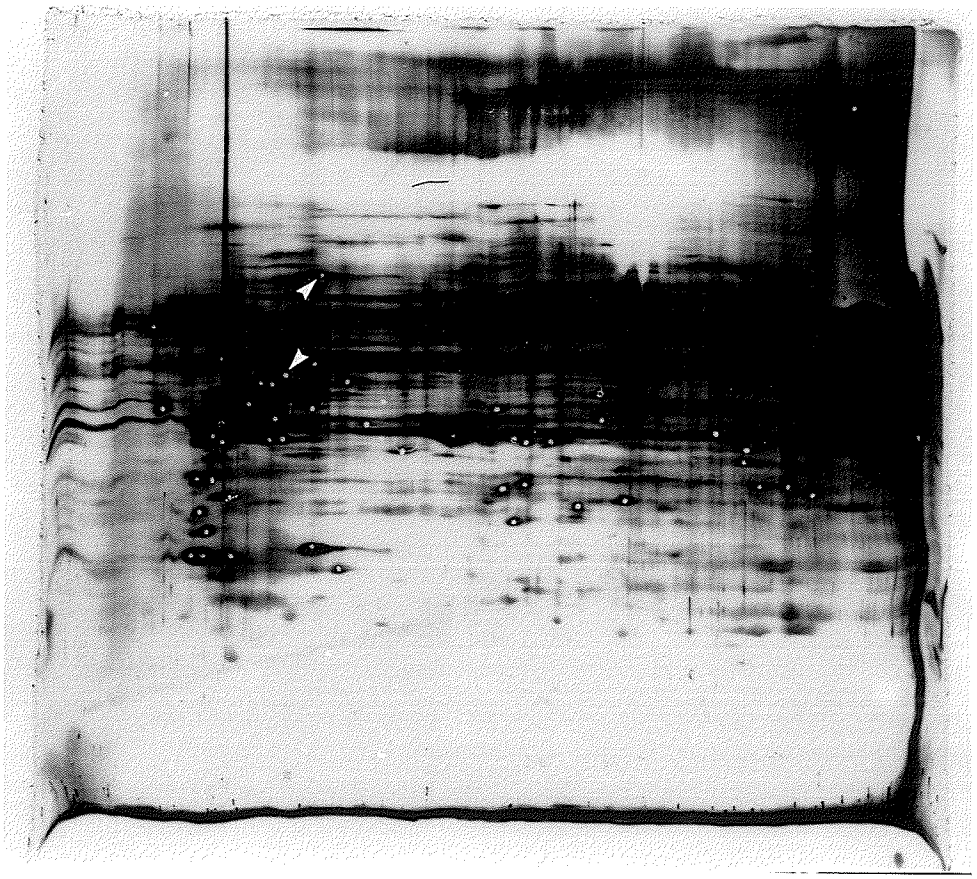


Fig. 49. A silver stained 2-D gel after excision of spots. This gel contained both ^{3}H-labeled Gaucher proteins (GM 877) and ^{14}C-labeled normal (GM 302) fibroblast proteins. Electrophoresis was performed as described in the legend to Fig. 44. The two spots highlighted were shown to contain a 2.5 fold reduction in leucine incorporation in the Gaucher strain.

DISCUSSION

The initial purpose of my research was to verify and further investigate a basic protein defect in Pompe disese, and develop an explanation for the clinical heterogeneity of the disease by clarifying its biochemical etiology. The course of my study, however, led me to search for sources of variability in laboratory procedures, and to learn and evaluate more comprehensive techniques for idenntifying biological peculiarities. In doing so, I found nothing to suggest the existence of a basic protein defect in fibroblasts from individuals with Pompe disease. I cannot easily dismiss the finding of Pena as fallacious, because of the inherent inconsistency of biological material and its responsiveness to what one often considers rigorous and reproducible treatments. Several of my observations have reminded me that cells are not yet ready to be defined. In attacking a specific problem and failing, I was forced to carefully examine what the methods employed were capable of demonstrating, both technically and theoretically. I have also gained an awareness of the limitations in applying techniques as opposed to logical strategies in the solving of problems. Each of these elements is considered in the following discussion.

A. COMPLEXITY OF FIBROBLAST GROWTH AND METABOLISM IN CULTURE

1. CELL GROWTH CHARACTERISTICS

Human cells removed in the form of a skin biopsy and cultured as fibroblasts have been regarded as biological models which might bear only minimal resemblance to the same cells in the intact organism. Certainly, the proliferation of cells in culture is testament to the retention of constituitive function, but many observations support the notion that cell growth characteristics strongly influence the morphology and metabolism of fibroblasts in vitro. The initiation of primary cell cultures from biopsy specimens has proved critical in determining the characteristics of the resulting strain. Comings (1981) emphasized that control donors must be carefully selected to match experimental donors in age, sex, state of health, and general environment. Several enzymes display lower specific activity with increasing donor age (Dobbs et al., manuscript in preparation [Appendix B]; Vanneuville et al., 1978). The natural differences in fibroblasts from different skin layers, in addition to differences in exposure to sunlight and disinfectants or local anaesthetics has confounded studies on Huntington disease fibroblasts that were not obtained in a standardized fashion (Goëtz et al., 1981). Conditions such as length of time in culture, population density, and nutritional status have long been known to modulate the expression of various enzyme activities, and have lead to daily fluctuations of several lysosomal

enzymes (Shapiro, 1981; Vanneuville et al., 1978). The phenomenon of in vitro cellular senescence is also an important consideration in biochemical studies of non-transformed fibroblast strains. Human cells are known to proliferate from the primary explant (phase 1), grow exponentially for approximately 60 generations with regular subcultivation (phase 2), and then gradually cease to divide (phase 3, Melman, 1971). This behaviour has classically been regarded as in vitro aging, but one theory holds that phase 3 cells have reached a state of further differentiation (Bell et al., 1978). Either phenomenon might be expected to give rise to a change in the pattern of protein synthesis. The major observation of (Bell et al., 1978) was that different cells from the same lineage ceased dividing after different numbers of divisions so that in vitro age was not necessarily linear with passage (1:2 subcultivation) number (for example) the most frequently used measure. Such nonuniformity and selection for dividing cells may have been a further complication in Huntington disease growth studies (Comings, 1981). Hornsby and Gill (1980) have argued against the evolution of further differentiated fibroblasts in culture, claiming that cell types induced to undergo terminal differentiation (e.g. bovine adrenocortical cells) show clear and dramatic changes in protein synthesis in contrast to the changes seen in phase 3 fibroblasts. Whether differentiated fibroblast changes are more difficult to detect, or endocrine changes are a unique instance (Bell et al.,1980) remains to be decided.

2. CELLULAR BIOCHEMISTRY

(a) The Variability of Lysosomal Storage Diseases.

There is growing evidence that genes other than those coding for lysosomal enzymes can substantially alter the enzyme's apparent activity. The dogma that inborn errors of metabolism arise from enzyme deficiencies in metabolic pathways has now to accommodate non-enzymatic activator proteins, and further unconventional regulatory systems will undoubtedly be discovered. Tissues from patients suffering with the same disease often possess enzymes with different kinetic properties, or different immunologic specificity from the normal enzyme, and many cannot be explained by simple mutation (Bootsma and Galjaard, 1979). The clinical and pathological nature of particular diseases are similar enough within families, however, to suggest that the heterogeneity is genetically determined (Bootsma and Galjaard, (1979).

The clinical diagnosis of Pompe disease is complicated by the interaction of isoenzymes whose relative activities vary according to the substrate used and the length of time fibroblasts are in culture (Fujimoto and Fluharty, 1978). Although three major forms of alpha-glucosidase deficiency have been recognized, five biochemical classes are now distinguishable (La Badie et al., 1981): (a) alpha glucosidase molecules with severely reduced catalytic activity are present, (b) both enzyme activity and crossreative material are absent, (c) the level of enzyme activity is proportional with the amount of enzyme protein present, (d) the enzyme activity is differentially

reduced in fibroblasts and muscle, (e) the enzyme protein is reduced and has a reduced catalytic activity for glycogen only.

The tissue specific isoenzymes of B-galactosidase are produced nongenetically by sialylation (Lussis et al.) although two structural loci on different chromosomes have been identified for the enzyme. Infantile form B-galactosidase deficient mutants appear to carry a mutation at one of these loci, while adult form mutants carry mutations in neither (Hoeksema et al., 1980).

Heterogeneity in the mucolipidoses could result from defects in several genes of lysosomal enzyme processing, and several complementation groups within each disease have recently been identified (Mueller et al., 1981). The variability in this group of diseases, which displays multiple enzyme abnormalities, could also arise from different degrees of mosaicism (Toomey et al., 1981) for a single gene defect involved in enzyme processing, such as cathepsin B (Murnane and Mellman, 1981).

Mutations in both A- (Grebner and Jackson, 1981) and B-chains (Kaback and O'Brien, 1981) of hexosaminidase have been shown responsible for unstable tetramers, and the A-locus mutation not involving the active site might cause chronic rather than acute G_{M2}-gangliosidosis.

(b) Other Disorders

It is likely that the etiology of poorly understood inherited metabolic disorders arise from defects in types of regulatory systems that have not yet been described. Numerous examples scratch the surface of this complexity. Certainly, there is no solid explanation for the variability in severity and ages at onset within "single" hereditary disorders, even with the discovery of activator proteins. Emery et al.(1979) described two groups of severe DMD patients in which physical incapacity was more severe in boys of normal intelligence than those who were mentally retarded. Human pyruvate kinse is known to undergo a proteolytic maturation process which improves its regulatory properties, but the regulation of this maturation is not understood (Kahn and Marie, 1979). On the contrary, hexokinase changes during senescence in vivo from a phosphate sensitive form to a phosphate insensitive form, and mutant insensitive hexokinase molecules have been found in young individuals (Stahl and Rijksen, 1979). Gelman et al. (1980) believe that DMD arises from a defect in the lysosomal membrane and by interfering with lysosomal chloride transport, leading to inhibition of the chloride dependent enzyme dipeptidyl aminopeptidase I. In many instances, intracellular regulation could occur translationally or pretranslationally. The solving of such unique problems in genetic diseases will likely contribute to the understanding of metabolic regulation and its consequences.

B. ONE DIMENSIONAL GELS

1. STRENGTH OF THE INITIAL OBSERVATION

The work of Pena (1977) provided the following essential observations:

(1) A protein or group of proteins of MW 29,000 was deficient in two different Pompe disease fibroblast strains.

(2) A protein or group of proteins of pI 7.9 was deficient in three different Pompe disease fibroblast strains.

(3) The defect was only observed in soluble proteins from mitochondrial/lysosomal preparations.

(4) The defect was more marked in infantile onset strains then in the adult form strain.

The hypothesis was made that the deficient species was the same in both IEF gels and SDS-PAGE gels, and that it was unlikely to represent alpha-glucosidase, a stable multimeric protein of acidic pI. The most favourable of several explanations was that a small basic activator protein was missing in these strains, although no other evidence had suggested such an occurrence in Pompe disease.

The reliability of the data lay in the consistency of the observation in different cell strains, regardless of whether the Pompe strain was labeled with ^{3}H-leucine or ^{14}C-leucine. The defect was more clearly seen when an IEF gel segment containing pH 7.9 ampholytes was run in a SDS-PAGE tube (primitive 2-D gel system). To my knowledge, the result was observed when the experiments were done by either of two individuals, and experiments performed several months apart were successful. It was especially attractive in the light of new protein activator systems described in other lysosomal storage diseases (cf. INTRODUCTION). The only doubt that could be raised was that no subsequent publications had commented on the validity of the finding.

2. POSSIBLE REASONS FOR THE IRREPRODUCIBILITY OF PENA'S DATA

The following explanations are presented as potential causes of irreproducibility. The possibility of technical error on my part is assumed although all reasonable efforts and precautions were taken to perform experiments in the original fashion.

(a) Systematic Positive Error

A systematic positive error refers to some inadvertant or undocumented treatment which in this case could have enhanced the defect or maintained it if it were labile. Such an error may have been

incurred by the inclusion of an antiproteolytic agent in the fractionation buffers, which was used in some fucose-labeling experiments in the study of DMD strains (Pena, 1977).

(b) Systematic Negative Error

A systematic negative error, conversely, refers to some inadvertant or undocumented treatment which in this case could have led to degradation of a protein in Pompe strains or the accumulation of a particular labeled protein species in normal strains. Either of these situations could give rise to a consistent and true defect whose expression is dependent on the environment in the cell or cell preparation, but that arises as a result of a non-regulated procedure. For example, proteolysis could have occurred at any one of many stages of the analysis, including cell harvesting, different stages of cell fractionation, sample storage, and even during slicing at room temperature. The attempt to artifically reproduce such an error by letting fresh homogenate incubate at room temperature for two hours (proteolysis experiments) tested only one of these stages, and failed to generate the observations of Pena. The conditions of the treatment, however, were arbitrary, and may have been too harsh or too mild. One might expect that the generation of labeled species in one region of the gel at the expense of another radioactive species would be clearly shown in gel profiles. Such an event might not be seen for two reasons: (a) Either the new protein species or the original protein

species could lie outside resolvable regions of the gels, or (b) Either the new protein species or the original protein species could be distributed among several regions of the gel; these differences may not be discernable within the precision of the technique.

(c) Cultured Fibroblasts

The variability of fibroblasts in cell culture has already been described. There is a small chance that the defect observed by Pena (1977) was real, and that in the populations of cells being observed now the defect is not present. Whether cells are of clonal origin is questionable, since primary cultures are most often used to directly establish commercial stocks without the use of a cloning step. One might imagine that every established culture contains a random sample of the cells biopsied, but this is not necessarily so. Further, it is likely that the growth characteristics of the different cell lineages varies, and the recovery of each from storage in liquid nitrogen could be sensitive to time in storage. Since the in vitro lifespan of the cells is relatively short, each strain was initiated from frozen subcultures several times. It is therefore unlikely that my observations were typical of rare cell populations from these strains.

(d) Fetal Calf Serum

The effects of fetal calf serum on protein synthesis have already been considered. Nevertheless, it must be emphasized that individual lots of sera are collected from many animals, and that they can be quite different. The quality of the serum could depend on the state of health of the animal, the method of obtaining the whole blood, and the preparation and storage methods, all of which might differ between commercial suppliers. It is also conceivable that major shifts in the nature of the livestock's feed could have an impact on serum quality, and thus on longitudinal studies requiring serum. The timespan between my experiments and Pena's (1977) was at least four years. Recently Willard and Anderson (1981) have shown that urine contains many lymphocyte effectors, and it is likely that serum also contains a multitude of tissue effectors (Hamm and McKeehan, 1979). Such an effector response in a high molecular weight species was demonstrated here in one-dimensional SDS-PAGE gels (Figs. 23-26). Again, it was not possible to tell whether a single protein species or several with same molecular weight was effected.

(e) Comment on IEF Gel Quality

Despite the fact that tests for differences between the subcellular fractions prepared in this work and Pena's experiments failed to show major differences, the profiles of my isoelectric focusing gels appeared more complex -- many more species were resolved. This may indicate that the ML-S fraction I prepared was different in its content from Pena's. Both the enrichment studies and the

cosedimentation observed with DAP-I verify the lysosomal nature of my ML-fraction. Two other factors may have contributed to the resolution of only a few major peaks: (i) inadvertant proteolysis; the sample that had undergone proteolysis (Fig. 15) displayed a broadening of peaks similar to profiles of Pena or (ii) merging of several focused bands during slicing of the low porosity gel; all of my gels were sliced at 4°C, while Pena's gels were sliced at room temperature. The advantage of slicing at 4°C was not proven experimentally.

3. NEED FOR GREATER RESOLUTION

Although one-dimensional gels did show an abnormality in Pompe disease fibroblasts, this was only the most significant of many of lesser differences between cell strains of different origin. In either IEF or SDS-PAGE, one can resolve at most 100-200 different species of cellular proteins in a single gel. This number is at least two orders of magnitude less than the number of protein species one would expect to find in a single cell (Anderson et al., 1979a). By combining these two separative techniques, the potential resolving power increases to within the range desirable for characterizing complete cellular protein synthesis. To date, no more than 1500 polypeptides have been observed in a combination of 2-D gel electrophoreses that separate both acidic and basic protein species (O'Farrell et al., 1977). This great improvement over one-dimensional gels provides the option of more

easily comparing patterns of protein synthesis in different strains without the use of dual-labeling. As opposed to the method of subcellular fractionation used in sample preparations for one-dimensional gels, particular areas of 2-D gels prepared from whole cell homogenates were scanned. In doing so, the potential for creating artifacts was avoided. In this system, the interference of proteins from other subcellular fractions is less critical.fp

C. TWO-DIMENSIONAL (2-D) GEL ELECTROPHORESIS

1. POLYMORPHISM IN 2-D ELECTROPHEROGRAMS

(a) Theoretical Considerations

By strict definition, non-identical nucleic acid sequences representing the same gene locus in a population of chromosomes constitutes genetic variation, and often genetic polymorphism if higher frequencies of variants obtain. The bulk of genetic variants defined so far, however, derive from structural genes encoding polypeptides, and primary structure of these polypeptides dictates recognizable physicochemical properties. Thus, genetic variation has been determined largely on the basis of electrophoretic and kinetic differences between similar proteins in different individuals. Aside from the assignment of restriction fragment length polymorphisms (Botstein et al., 1980), nucleic acid polymorphisms have been neglected because most either lie outside structural genes, or do not affect the sequence of amino acids designated within the gene. Further, it is suggested that many amino acid substitutions do not lead to protein

variations that can be qualitated by present techniques (Harris, 1980). Stochastically, only 30% of polypeptides with a single amino acid difference will result in a charge difference, and thus lead to an altered electrophoretic mobility (Harris, 1980). This determination has led to the prediction that nucleic acid polymorphism within structural genes aproaches a level three times that detected in proteins by standard electrophoretic techniques.

The origin and role of protein polymorphism in evolution has been the subject of debate for many years, and are not the concern of this discussion. The nature and the degree of protein polymorphism are the two scientifically testable elements that form the basis of theories regarding the importance of genetic polymorphism, and those in which two-dimensional gels challenge classical electrophoresis. Although nearly all loci are presumed to have a few rare variant alleles, a locus is not considered polymorphic unless the most common allele has a frequency no greater than 0.99, and thus at least 2% of the population is heterozygous at the locus. Twenty-four of 104 enzyme loci (23%) tested in a European population displayed electrophoretic polymorphism in one dimensional gels stained histochemically (Harris, 1980). The chance that any individual displays genetic variability is dependent on the number of identifiable alleles at a locus and their individual frequencies. The proportion of heterozygotes therefore, is a good measure of polymorphism, and the sum of heterozygote frequencies over all loci examined provides the heterozygosity per locus, or simply heterozygosity, which can be used to estimate the number of polymorphic

loci in an individual. For the series examined by Harris and coworkers, the average degree of heterozygosity for enzymes was 0.063, and this level is commonly extended to all cellular proteins in man. However, Walton et al. (1979) found a heterozygosity of only 0.006 in 2-D analysis of five normal human fibroblast cell strains. Similarly, McConkey et al. (1979) in screening approximately 400 spots in 2-D gels from each of five different human cell strains found a heterozygosity of less than 0.001.

The reason for the lack of variation in proteins detected by 2-D gels is not known, but several hypothesis have been forwarded. The favoured explanation is that the electromorphs detected in 1-D gels represent a different class of proteins than those polypeptide spots nonspecifically represented in 2-D gels. It has been inferred that since the best 2-D gels reveal fewer than 10% of the expected number of polypeptides in a eukaryotic cell, the spots must represent structural or membrane proteins rather than those present only in catalytic amounts (Walton et al., 1979). The biological basis for the difference in degree of polymorphism between enzymes and structural proteins is uncertain, but some evidence contrasting enzymes and certain structural proteins is pertinent. The incidence of polymorphism is inversely related to the number of subunits in multimeric enzymes, and is greatest among monomeric molecules (Harris, 1980). Enzymes of the glycolytic pathway (Cohen et al., 1973) and skeletal muscle actin (Carsten and Katz, 1964) display very low levels of polymorphism, even between species. McConkey (1979) reasons that proteins which to a

large extent must interact with others as part of complex integrated units, either structural or functional, must be very sensitive to mutation, and that most altered polypeptides should be lost as dominant lethal events. Thus, extrapolating the level of polymorphism in soluble enzymes to all cellular proteins may have been inappropriate.

A second source for the reduction in observed polymorphism is the separation system itself. The ability to resolve so many proteins in an O'Farrell 2-D gel system no doubt relies on the denaturuing conditions of the electrophoreses, which dissociates protein aggregates into separate linear polypeptide chains. Such conditions would therefore not be expected to allow the distinction of native proteins homogenous in charge and size that had conformational differences manifested through determinants of secondary structure. The advantage of denaturing IEF in separating polypeptides with cryptic charge differences is challenged by its failure to discriminte conformational allozymes (conformers; Walton et al., 1979). Any single 2-D gel will exclude polypeptides outside a limited range of isoelectric points and molecular weights, especially those optimized for high spot numbers. Nevertheless, these differences probably cannot account for a reduction in polymorphism of such magnitude (Walton et al., 1979).

An interesting consequence of recent studies into the power of starch gel electrophoresis has questioned the necessity of elevating the frequency of polymorphs above that of starch gel electromorphs. In Drosophila hemoglobin, sequential one dimensional starch gel electrophoresis distinghished 90% of all chemically identical

substitutions at various chain positions, and 80% of charge equivalent polypeptides that were chemically different (Ramshaw and Eanes, 1978). This contradicts the "charge ladder" hypothesis that each electromorphic class is homogeneous with respect to nominal charge and that each class is separated by unit charges (Ramshaw et al., 1980). The level of polymorphism detected in the enzyme surveys may not be an underestimate. All of the above factors likely contribute to the inadequacy of 2-D gels in demonstrating high levels of protein polymorphism, and further work is needed to clarify the issue.

(b) Spot variability in the present study

The degree of qualitative variation displayed in Figs. 44-49 was judged to be very low as described in Table X. Quantitative variation was much more apparent, but this was difficult to evaluate because of differences in the levels of exposure between the gels. The assignment of qualitative differences was very subjective, since only spots that were clearly present in one autoradiogram and absent in the other were tallied. Several groups of spots were faded, but contained faint smudges that corresponded with clearly resolved spots on the opposite exposure. All such occurrences were judged as separation artifacts and not clear differences. The qualitative differences that were clear were among constellations of spots that were comparable in both autoradiographic exposures.

2. REPRODUCIBILITY OF 2-D ELECTROPHEROGRAMS

The merit of 2-D gel electrophoresis is realized only because the complex array of spots is reproducible with the same sample, and between experimental samples and their control samples. The patterns, however, are never similar enough to superimpose all spots on two different gels. The difficulty in producing identical patterns lies largely in the stages that precede the running of the second dimension, and the precision of these procedures determines the quality of the gel. Chrambach (1980) emphasized that the conditions for obtaining pattern constancy with time necessarily varies with each particular separation problem, and that even zone distribution and optimal resolution would not be obtained until such steady state conditions were met. Thus, the gradient established in the isoelectric focusing gel, which is most difficult to reproduce, may vary according to the protein species being compared.

The relative amounts of component proteins in different samples, which did vary among the strains tested, can strongly influence the loading of IEF gels (O'Farrell, 1975). The isoelectric gradient is also sensitive to the number of voltxhrs in electrophoresis, the concentration of electrolytes, and the presence of SDS in the sample preparation (Chrambach, 1980; O'Farrell, 1975). The strong influence of urea on the pK_a values of carboxyl groups, and the tendency for the urea to diffuse from the acid end of the isoelectric focusing gel introduces further difficulties in maintaining consistency

in the focusing procedure.

Aside from the above factors which influence spot pattern and distribution, the nature of the spots themselves is variable. Horizontal streaking arises from precipitation of protein in the IEF gel, which can occur if nucleic acids in the sample bind to proteins or if the urea concentration in the sample or IEF gel falls below 8M (O'Farrell, 1975). Posttranslational modifications such as deamidation or sialylation can lead to similar horizontal rows of spots representing charge heterogeneous secondary isoenzymes (Edwards et al., 1979; Harris, 1980). Vertical streaking can likewise occur from varying degrees of carbohydrate added posttranslationally, and this phenomenon is apparent in protein concentrates from urine (Anderson et al., 1979a). Since posttranslational modifications can occur both in vivo and in vitro, precautions to prevent such modifications during sample preparation are necessary to ensure reproducibility (Garrels, 1979). Carbohydrate modifications are especially important to minimize, since sugar residues bind poorly to SDS while causing physical retardation in molecular weight separation (Anderson, 1979a). We have noticed in our laboratory that excess SDS in the running buffer of the second dimension, incurred by using either "high-SDS" electrophoresis buffer or too much agarose overlay solution, that low molecular weight spots tend to diffuse.

Since 2-D gels resolve presumably only 10% of the expected number of spots, one might question whether as in one-dimensional gels,

different polypeptides comigrate. In an artificial context, where protein overloading might be expected to obscure some spots by expansion of the overloaded species, O'Farrell claims that the overloaded species will displace smaller spots rather than obscuring them. Further, a theoretical consideration of the percent abundance of spots in contaminating levels suggests that the probability a spot containing 0.1% of the total protein is more than 90% pure is 95%. The spots that remain undetected, therefore, must be those that are excluded from the ranges resolved by the gel and those in only minute amounts that lie in blank regions of the gel or comigrate with other proteins.

The quality of gels obtained with original Garrels (1979) sample preparations could not be maintained, but the reason for this difficulty is not known. Several experimental samples were prepared subsequent to the success on the trial strain, SPF, and two Hamster cell strains, CHW and 1103 (See Figs. 37,38). Cells were regrown and samples reprepared on suspicion of improper sample preparation. The horizontal streaking persisted and when the original SPF sample was electrophoresed a second time, the gel suffered from an apparent failure in isoelectric focusing.

After stringent inspection of all other variables, two differences between the O'Farrell system and that originally employed remained. The internal diameter of the IEF gel tubes was originally 1mm greater than that recommended by O'Farrell, which was maintained to enhance the protein loading capacity of the gel. This could

conceivably alter electrofocusing by increasing the current, owing to the decreased resistance of the wider gel (constant voltage was applied). The second was the sample preparation, notably different in its content of SDS, which was expected to disaggregate polypeptides that might be resistant to nonionic detergents and urea (Garrels, 1979). The feasibility of using SDS in IEF is contingent upon the formation of micelles between NP-40 and SDS, and the migration of these micelles to the acidic terminus of the IEF gel. The micelle formation and the pH range of the gradient in the gel are both sensitive to the ratio of NP-40:SDS, and failure to achieve this minimal ratio (8:1 by weight) has been shown to lead to horizontal streaking of the same nature I experienced (Ames and Nikaido, 1976). Although the ratio used to test sample was within the lower prescribed range of Garrels (1979), the ratio was elevated to the recommended level in one experiment, without any effect.

The overwhelming difficulty in considering either of these factors as contributing to failure is that they were successfully used on the same sample preparation earlier. One could imagine that the original SPF sample had degraded after thawing and refreezing, but this is unlikely under such stringent conditions (Garrels, 1979), and none of the freshly prepared samples were better before freezing and rethawing. A final comparative experiment employed the cell strain MCH40 prepared by either the method of Garrels (1979) or of O'Farrell (1975) in the larger size IEF gel tube. This showed that tube size was irrelevant, but that the sample without SDS gave better resolution and

lacked the great smears and horizontal streaking seen in the SDS-containing sample. In the interest of time, the use of SDS in sample preparation was forgone, and new samples prepared in its absence.

As is presented in the results, the best gels were produced when proteins were labeled with ^{35}S-methionine and electrophoresed strictly by the method of O'Farrell. The quality could be compromised slightly for the purpose of quantitating observed deficiencies in silver stained gels of double label samples. The latter method was developed on a DMD model and proven on a Gaucher strain, but found no precedent in the study of Pompe strains. It appears that mere macromolecular mapping does not hold the promise of easily solving problems of molecular biology.

D. THE FUTURE OF STUDYING HEREDITARY LYSOSOMAL DISEASES

Progress in the study of acid maltase deficiency has been rather slow and awkward, probably because the simple techniques of biochemical genetics that were enlightening in the past have found little success with the lysosomal disorders. It may be that the diseases for which solutions are still required need more than just inferences based on single laboratory tests and gross clinical phenotypes. This is especially apparent when lysosomal enzyme activity consistently varies in the absence of known variation in experimental conditions (Harris, 1981; the development of defined cell culture media, [eg. Bettger *et al.*, 1981] may help to solve this problem). Both clinical and basic research must be intensified. The lysosomal

enzymes from many different tissues should be purified, their structures analyzed, and their kinetics established on a variety of natural and artificial substrates. Understanding the molecular basis of lysosomal enzyme processing and coordinate regulation may be aided by the study of patients with multiple lysosomal enzyme deficiencies (Yutaka, et al., 1981), or individuals with apparently unrelated disorders that cause wholesale disruption of lysosomal enzyme functioning (Calvo et al., 1982; Kraaijenhagen et al., 1982).

The method developed by Pena for studying double labeled samples after separation in one dimension does not appear to be a viable technique for the detection of inconspicuous protein deficiencies in heterogeneous samples. I believe this technique served better in its original applications where the populations of protein species were limited (Mattieu et al., 1975; Philips et al., 1975). The refinement of two-dimensional electrophoresis was a tremendous improvement in terms of resolving proteins in heterogeneous mixtures, and yet this method too has found little success in the characterization of abnormalities when applied to genetic disorders (eg. Comings, 1979; Willers, et al., 1981). It has appeared inferior in detecting genetic variants at the level displayed by unidimensional electrophoresis. Edwards and Hopkinson (1980) suggest that introduction of modifications to the more or less standard method for 2-D gel electrophoresis may improve its success in detecting heterozygosity. Such improvements include the development of a nondenaturing second dimension compatible with the first dimension

electrofocusing gel, such that charge identical conformational isozymes could be distinguished, or histochemical methods could be applied. The tangible benefits of 2-D gel electrophoresis rely in its strategic use as a much improved method of protein separation, i.e. in applications that could be performed, albeit with more difficulty, by unidimensional methods. Thus, it has been used to study selectable gene mutations (Milman et al.. 1976), to characterize antibody/antigen complexes (Pearson and Anderson, 1980) to establish precursor-product relationships (Anderson, 1981), and to monitor effectors of protein synthesis (Willard nd Anderson, 1981). The excision of dual labeled protein spots from 2-D gels has been used to measure rates of protein synthesis through the cell cycle of yeast (Elliot and McLaughlin, 1978). The more monumental task of developing a data base cataloguing all proteins of every human tissue is not yet practical, but offers the potential of screening a wide range of genetic loci in parallel, for the study of development, protein synthesis, protein turnover and modification, and environmental mutagenesis (Edwards and Swallow, 1978; Garrels, 1980, Wade, 1980).

The new frontier of molecular biology in the form of recombinant DNA methodology holds no special promise for studying lysosomal enzyme deficiencies. With any protein that can be purified and partially sequenced, probes for unique gene segments can be isolated from total genomic libraries via synthetic oligonucleotides (Suggs et al., 1981). These may then be used to find restriction fragment length polymorphisms. The technical problems beyond the

purification of the protein will necessarily vary for each endeavor. A more serious limitation is the expected insight to be derived from a knowledge of gene structure, and the practicality of establishing restriction fragment length polymorphism for a particular gene.

I am uncertain that the fundamental biology required to understand these heterogeneous disorders is in hand, but confident that an important contribution will be made by the time they are solved.

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APPENDIX A

2-D GEL ELECTROPHORESES SOLUTIONS

The following solutions are not defined in the text of the described methods.

METHODS OF GARRELS (1979)

1. Staphylococcal nuclease solution - 50ug/ml micrococcal nuclease, 2mM $CaCl_2$, 20mM Tris-HCl, pH 8.8.

2. DNAse I-RNAse A - 1mg/ml DNAse I, 500ug/ml RNase A, 50mM $MgCl_2$, 500mM Tris-HCl, pH 7.0.

3. Sample buffer - 9.95M urea, 4% NP-40, 2% ampholytes (pH range 5-7), 100mM DTT.

4. IEF overlay solution - sample buffer plus 0.05 volumes of water, 0.05 volumes of 2-mercaptoethanol.

METHODS OF O'FARRELL (1975)

1. Lysis buffer - 9.5M urea, 2% Nonidet P-40 (NP-40), 1.6% pH range 5-7 ampholytes, 0.4% pH range 3.5-10 ampholytes, 5% 2-mercaptoethanol.

2. Sample overlay solution - 9M urea, 0.8% pH range 5-7 ampholytes, 0.2% pH range 3.5-10 ampholytes.

3. SDS sample buffer - 10% (w:v) glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5mM Tris-HCl, pH 6.8.

4. *SDS running buffer - 25mM Tris, 192mM glycine, 0.1% SDS

5. *High SDS running buffer - 25mM Tris, 192mM glycine, 2.0% SDS

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* All above solutions except these were stored in small aliquots at -20°C.

APPENDIX B

DIPEPTIDYL AMINOPEPTIDASE I (DAP-I) IN CULTURED SKIN FIBROBLASTS FROM PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY

Short title: DAP-I Activity in Dystrophic Fibrolasts

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APPENDIX B

Abstract

The activity of the lysosomal enzyme dipeptidyl aminopeptidase-I (DAP-I) on the artificial substrate glycyl-L-phenylalanine-B-napthylamide was studied in cultured skin fibroblasts from patients with Duchenne muscular dystrophy and in age and sex-matched normal control strains. Experiments were set up in a blind fashion. The mean specific activity of DAP-I was not significantly different in whole cell homogenates from seven normal and seven dystrophic strains. A strong inverse correlation between donor age and DAP-I specific activity at cell confluency ($b \neq 0, p < 0.2$)may explain the variability in control activities, but this trend was not apparent in dystrophic strains. DAP-I activity was significantly higher in confluent cells compared to those undergoing logarithmic growth ($p < 0.025$).

Key Words: Ageing; biochemical genetics; cultured cells; dipeptidyl aminopeptidase-I; enzymes; inborn errors of metabolism; lysosomes; Duchenne muscular dystrophy.

APPENDIX B

Introduction

For many reasons cultured skin fibroblasts are an ideal tissue source to search for the primary defect in Duchenne muscular dystrophy (DMD) and to develop a specific test for the disease. Fibroblasts do not exhibit the gross pathological changes of skeletal muscle, where numerous secondary changes tend to obscure the underlying primary defect. They can be obtained with minimal discomfort to the patient, can be propagated in the laboratory, and if the defect can be detected in fibroblasts, prenatal diagnosis of the disease is possible. To date, the numerous abnormalities reported in DMD fibroblasts suggest that the DMD gene is expressed in cultured skin fibroblasts. However, none of the reported abnormalities have been confirmed by other laboratories and some (WYATT and COX, 1977; IONASESCU et al., 1977; SHAY and FUSELER, 1979) have indeed been contradicted (CULLEN and PARSONS, 1977; STEPHENS et al., 1980; RUNGGER-BRANDLE et al., 1980).

Recently Gelman et al. (1980) found a 70% reduction of dipeptidyl aminopeptidase I (DAP-I, E.C. 3.4.14.1, also known as "cathepsin C") activity in all subcellular fractions of dystrophic fibroblasts compared to their control strains. A subsequent study revealed a reduction in the structure-linked latency of DAP-I in dystrophic lysosomes, and ultrastructural examinations revealed an excess of lamellar bodies in DMD cells suggesting a defect in the lysosomal membrane of DMD fibroblasts (Gelman et al. 1981).

We attempted to reproduce the basic observation by Gelman

et al. (1980, 1981) of decreased DAP-I activity in DMD fibroblasts by determining the DAP-I activity in whole cell homogenates of randomly coded pairs of strains. The results were inconclusive because the mean DAP-I activity was not significantly different between normal and dystrophic cell strains from seven matched pairs.

Materials and Methods

Seven strains of fibroblasts from DMD patients and seven age and sex matched normal control strains were obtained from the sources indicated in Table 1. Cells were routinely cultured in 100 x 20 mm Falcon plastic culture dishes (Falcon Labware, Oxnard, Calif.) in a humidified incubator at 37° and 5% CO_2. The growth medium (10 ml/dish) consisted of McCoy's 5A modified medium (Gibco Laboratories, Grand Island, N.Y.) plus 90 U/ml penicillin, 90 ug/ml streptomycin sulfate, 10% (vol/vol) fetal calf serum (Flow Laboratories, Mississauga, Ont.), 23 mM $NaHCO_3$, and had a final pH of 7.5. Cells were passaged with a 0.05% trypsin solution, and received fresh medium every 3 days.

For experiments, cells were seeded at a density of 5 x 10^5 cells/100 mm dish, and received fresh medium every three days. All cell lines were between passages 10 and 18 during the course of these experiments, and members of matched pairs were no more than 3 passages apart.

Two dishes of each strain were selected both at 3 and 10 days after seeding and examined microscopically. No strains were

confluent at day 3, but all were confluent by day 10. Each was rinsed twice with ice-cold 0.25 M sucrose, and scraped with a rubber policeman in 1.5 ml/dish of ice-cold 0.25 M sucrose. The suspensions from the two dishes were pooled and homogenized with 30 strokes of a Kontes 21 Teflon-glass tissue homogenizer (Kontes Co., Vineland, N.J.) with the pestle rotating at 200 rpm. After removal of an aliquot for enzyme and protein determinations, the crude cell homogenate from confluent dishes was centrifuged at 750 x g for 10 min, separating the nuclei and debris (fraction N) from the post-nuclear supernatant (fraction PNS). The supernatant (PNS) was centrifuged at 10,000 x g for 20 min, to produce a crude lysosomal pellet (fraction L) and the post-lysosomal supernatant (PLS). Pellet fractions (N,L) were suspended in .25 M sucrose for assays.

DAP-I activity was measured by the method of Gelman et al. (1980). The release of B-napthylamine from the substrate glycyl-L-phenylalanine-B-napthylamide (Sigma Chemical Co., St. Louis, Mo.) was measured fluorometrically at an excitation wavelength of 365 nm and an emission wavelength of 460 on an Aminco-Bowman spectrofluorometer (Aminco, Silver Spring, Md.). Commercial B-napthylamine (Sigma Chemical Co.) was used as a standard. The reaction mixture contained 0.1 mM substrate, 1.0 mM dithiothreitol (DTT), 30 mM sodium chloride, 1.0 mM ethylenediaminetetraacetic acid (EDTA) 0.02% TRITON X-100, 50 mM sodium acetate buffer, pH 5.0, and 50 ul of sample in a final volume of 200 µl. The reaction was terminated after 30 min at

37° by adding 2.0 ml of cold 50 mM glycine NaOH, pH 10.4, 5 mM EDTA. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine plasma albumin as standard. Specific activity was expressed as nanomoles of substrate hydrolyzed per hour per mg cell protein.

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Results

To reduce any experimental bias, normal and dystrophic cell strains were analyzed in matched pairs, the distinction of which was unknown to the experimenter. For each pair of strains, one member showed greater specific activity in whole cells (Table 2) and all subcellular fractions (data not shown). According to the observation of Gelman et al. (1980), the pair member with higher specific activity was predicted to be the normal cell strain. In the final analysis, the predictions proved to be correct in only four of seven pairs, and the mean activity among control strains at either stage of growth was not significantly different from the mean dystrophic activity for whole cells or any subcellular fraction. Closer inspection of the data, however, revealed that dystrophic strains with above control specific activity were those not from the Montreal repository. Each of these dystrophic strains was derived from local (Winnipeg) patients, and was a member of the same family. We noted a significant regression of day 10 DAP-I specific activity on donor age (b [coefficient of regression] $\neq 0$, $p<.02$) among the normal strains (Fig. 1). The significance of the regression was improved when dystrophic strains from Montreal were included ($p<.005$), but the dystrophic strains as a whole did not show this trend. Despite the possible effect of donor age on activity, the incorrect designation of dystrophic strains as normal could not be explained by imperfect age-matching. So far, we have found no

electrophoretic difference between DAP-I of different strains on starch gels (data not shown). As noted by Gelman et al. (1980), specific activity of DAP-I was higher in confluent cells than in cells undergoing rapid growth (paired t-test, $p<.025$).

Discussion

DAP-I specific activity is not a reliable marker for the expression of DMD in fibroblasts, displaying wide variability of activity in both normal and dystrophic cell strains. Much of the variability between strains may be accounted for by the highly significant regression of optimal specific activity on age, when the analysis excludes the related dystrophic strains obtained in Winnipeg. Since three of these last strains show specific activities higher than any other normal or dystrophic strain, a familial variant enzyme could be responsible. The DAP-I molecule from the local strains appears qualitatively similar to DAP-I of other strains, but further experiments are in progress.

Another possible explanation is that the enzyme may vary according to source, i.e. the repository or laboratory from which the cells were biopsied and grown (Goetz et al., 1981). Many enzyme levels are sensitive to slight variation in culture conditions, and lysosomal enzymes are particularly sensitive to the condition of the cell (Shapiro, 1981). We originally noted a significantly higher specific activity in confluent normal Montreal strains as opposed to other normal strains ($p<.02$), but the former strains were also from younger donors, and the

difference could be accounted for by the regression on age. Further, all cells were grown under identical culture regimes for several generations prior to the start of the experiment.

The association of donor age and DAP-I specific activity is very similar to that seen in fibroblast alkaline phosphatase (Vanneuville et al., 1978) for the range of ages in our experiment. Likewise, we are unable to distinguish between an aging phenomenon and the possibility that different cell populations may be unequally represented in strains derived from different biopsy specimens. Several enzymes purified from fibroblasts in late passages show lower specific activities than those purified in early passages (Rothstein, 1977), but our strains were of similar in vitro age. Further, the above reduction in activity was seen in cytosolic but not in lysosomal enzymes (Houben and Remacle 1976, 1978). It is not known whether the strains from older donors contain less total enzyme or less functional enzyme.

Our intention in this study was to confirm one of the better documented abnormalities in DMD fibroblast. Results from the few strains that are common to this and published data (the Montreal strains of Gelman et al., 1980) are not in disagreement but we provide exception to the generalization that DAP-I activity is reduced in all cultured fibroblasts and that this effect is secondary to DMD expression. Our report thus adds to the growing list of contradictory findings in DMD fibroblasts a situation reminiscent of that in fibroblasts from other major single gene

disorders, like cystic fibrosis and Huntington's disease (Comings, 1981).

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Table 1. SOURCES OF CULTURED HUMAN FIBROBLAST STRAINS*

PR. NO.	NORMAL STRAIN	AGE YEARS	SOURCE	DYSTROPHIC STRAIN	AGE YEARS	SOURCE
1	MCH 48	5	M[1]	WG 502	5.5	M
2	GM 2987	19	C[2]	R.C.	13	W[3]
3	J.W.	11	W	J.B.	11	W
4	GM 499	8	M	D.C.	13	M
5	GM 316	12	C	C.A.	12	W
6	MCH 35	3	M	WG 280	7	M
7	MCH 40	6	M	WG 448	6	M

* All strains were of male origin.
1. Repository for Mutant Human Cell Strains, Montreal, Canada.
2. Institute for Medical Research, Camden, N.J.
3. Department of Pediatrics (Genetics), University of Manitoba, Winnipeg, Canada.

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Table 2. SPECIFIC ACTIVITY OF DAP-I IN HOMOGENATES OF WHOLE CELLS IN DIFFERENT STAGES OF GROWTH

PR.	NO.	SPECIFIC ACTIVITY* LOG PHASE GROWTH	CONFLUENCY
1.	N	512	768
	D	180	444
2.	N	88	180
	D	63	100
3.	N	24	192
	D	256	1236
4.	N	240	451
	D	173	1117
5.	N	438	136
	D	537	959
6.	N	764	825
	D	202	181
7.	N	420	595
	D	280	344
Mean + SE	N	355 + 97	450 + 109
	D	241 + 56	625 + 177

* Specific activity is expressed as nmoles of substrate hydrolyzed x mg cell protein^{-1} x hr^{-1}. N = normal D = dystrophic.

APPENDIX B

Fig. 1 Plot of DAP-I specific activity in homogenates of confluent fibroblasts (ordinate) versus donor age (abscissa). Line represents regression of activity on donor age for normal strains only (b = 0, p<.02) ● = Control ○ = DMD.