PATHWAYS OF INTRACELLULAR PROTEIN DEGRADATION IN CULTURED MUSCLE CELLS

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University of Cape Town

TO MY HUSBAND

CHRIS

who, although having considerable
academic commitments himself,
continually supported and encouraged
me in the completion of this thesis.

ABSTRACT

To investigate mechanisms responsible for the turnover of endogenous muscle protein, lysosomotropic proteinase inhibitors have been employed to elucidate the relative contributions of lysosomal and non-lysosomal degradation pathways functioning under varying nutritional states and for different classes of intracellular proteins.

Proteolysis in cultured bovine aortic smooth muscle cells was measured as the percentage of ^{3}H-phenylalanine released per hour from pre-labelled cellular proteins. To reduce background radioactivity, the intracellular ^{3}H-phenylalanine pool was depleted by serial extraction at 37°C, effecting equilibration between the intracellular pool and the phenylalanine-free medium. Reutilization of labelled amino acids during subsequent incubation periods was minimized by the presence of excess non-labelled phenylalanine in the medium.

^{3}H-phenylalanine was released at a constant rate of 1,5% per hour for at least 4 h, from cells pre-labelled for 16 h ('long-lived' proteins). Leupeptin, an inhibitor of thiol proteinases including cathepsin B, inhibited degradation by 12%, whereas the general lysosomal inhibitors chloroquine and NH_4Cl inhibited degradation by 30%, presumably the contribution by the lysosomal pathway. In the case of 'short-lived' proteins (pre-labelled for 1 hour), the initial degradation rate was 6,5% per hour, which rapidly declined, reaching the basal rate of 1,5% after 4 h. Chloroquine and NH_4Cl reduced proteolysis by only 12-15% and leupeptin had no significant inhibition, consistent with the view that the majority of short-lived proteins are degraded by non-lysosomal pathways. Proteolysis rates of 'abnormal' proteins containing the arginine-analogue, canavanine, were found to be significantly elevated (80%) over controls. Leupeptin had no significant inhibition, and chloroquine and NH_4Cl only reduced degradation by 12-16%, showing that the rapid removal of 'abnormal' intracellular proteins proceeds mainly via extra-lysosomal mechanisms.

Incubation of the cells under nutritional step-down conditions, increased the average degradation rate of long-lived proteins to 3% per hour, and chloroquine and NH_4Cl inhibited degradation by 55-60%, indicating that the accelerated proteolytic condition is due to increased activity of the lysosomes. Nutritional deprivation did not increase the rate of degradation of short-lived proteins.

The results were clarified by the parallel use of the well-characterized LDL degradation system in this cell type, known to occur almost exclusively via lysosomes. This allowed the effectiveness of lysosomotropic inhibitors to

be tested. Chloroquine inhibited LDL degradation by over 90% and NH_4Cl inhibited by 80-95% in all cases.

Other proteinase inhibitors such as chymostatin, pepstatin and the chloromethyl ketones were also tested, and of these chymostatin seemed to be the most valuable because of its additivity to the effect of chloroquine, indicating its selective inhibition of non-lysosomal degradative mechanisms.

Incubations of smooth muscle cells under anoxic conditions or with metabolic inhibitors such as fluoride, azide and cyanide, resulted in an inhibition of protein degradation which was greater than, and partially additive to, the effect of chloroquine, i.e. both lysosomal and non-lysosomal degradation pathways have some energy-dependence.

The degradation of long-lived proteins appeared to be more sensitive to temperature than that of short-lived proteins, further indicating the activity of distinct proteolytic mechanisms for these two classes of intracellular proteins.

Preliminary studies have indicated a role for Ca^{++} in the regulation of proteolysis, since degradation rates were increased by elevated levels of Ca^{++} in the extracellular medium. Inhibition of this increased proteolysis by leupeptin has indicated a role for a thiol proteinase, possibly Ca^{++}-activated neutral proteinase.

In similar studies with cultured L8 skeletal muscle cells, an average proteolysis rate of 1,2% per hour was found, which was increased by 50% under nutritional step-down conditions. Once again, the lysosomal pathway was found to account for only about one-third of basal protein degradation but fully accounted for the increased proteolysis under nutrient deprivation.

The degradation characteristics of intracellular smooth and skeletal muscle cell proteins was examined using double isotope labelling. It was found that large molecular weight proteins and glycoproteins tended to be degraded more rapidly than small proteins and non-glycoproteins. In smooth muscle cells, these correlations were markedly reduced or absent under the accelerated proteolysis associated with nutrient deprivation, possibly confirming the increased activity of the non-selective autophagic lysosomal pathway under these conditions. A similar loss of correlations was not so clearly seen for skeletal muscle cell proteins.

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ABBREVIATIONS

ATP	-	adenosine triphosphate
C	-	Celcius
Ci	-	Curie (3,7 x 10^{10} disintegrations per second)
cpm	-	counts per minute
CQ	-	chloroquine
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
dpm	-	disintegrations per minute
EDTA	-	ethylene diamine tetraacetic acid
LDL	-	low density lipoprotein
leup	-	leupeptin
LPDS	-	lipoprotein-deficient serum
M	-	molar
MEM	-	Eagle's minimum essential medium
mg	-	milligram
ml	-	millilitre
mM	-	millimolar
MW	-	molecular weight
N	-	normal
PBS	-	phosphate-buffered saline
SDS	-	sodium dodecyl sulphate
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylene diamine
TPCK	-	N-α-Tosyl-L-phenylalanyl chloromethyl ketone
TLCK	-	N-α-Tosyl-L-lysyl chloromethyl ketone
Tris	-	Trishydroxymethyl aminomethane
μ (prefix)	-	micro (x 10^{-6})
v/v	-	volume by volume
w/v	-	weight by volume

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INTRODUCTION

There is at present a detailed knowledge of the molecular mechanisms involved in protein synthesis. Most intracellular protein is continually being broken down again to maintain the steady-state level of protein in the individual tissues, and it is now evident that modulation of the degradation process may play an important role in the overall control of intracellular protein concentration. The importance of degradation is revealed by calculations showing that more protein is broken down in body tissues than in the gastro-intestinal tract in any twenty-four hour period (Kay, 1978).

The term "intracellular protein degradation" describes a catabolic process whereby proteins are hydrolysed into their constituent amino acids and includes several different processes depending on the interpretation. It is most frequently used to describe the breakdown of endogenous proteins in the cell in which they were synthesized and performed their functions. However, the term may also include the proteolysis of exogenous proteins which are endocytosed into the cell for degradation as is the case for plasma proteins and endocytic cells in liver or macrophages, and for processes such as post-partum uterine involution and collagen degradation, which is generally initiated extracellularly but is completed within the cells. In addition, intracellular protein degradation necessitates a consideration of the turnover of subcellular organelles which in some cases appear to be degraded as whole units within the cell, yet in others are degraded by selective mechanisms within the organelle itself.

The main purpose of this section of the thesis is to provide a general outline of current knowledge on the degradation of endogenous proteins within mammalian cells, especially with regard to controlling factors and the various possible pathways involved. The degradation of exogenous endocytosed proteins will not be detailed here, except where mechanistic similarities to intracellular proteolysis exist and where specific reference is made to apolipoproteins in the section "Plan of Present Research".

A detailed discussion or survey of intracellular proteolytic enzymes and their activities is not included (for review see Barrett, 1977), and limited proteolysis is only referred to when applied to proteins which undergo complete subsequent breakdown within the cell. Protein degradation in procaryotic cells will not be specifically outlined here, although review articles covering this topic more fully have emphasized the considerable similarity between prokaryotic and eukaryotic degradation (Goldberg and Dice, 1974; Goldberg and St John, 1976). Other recent reviews on protein breakdown include those by Dean (1980) and Ballard (1978).

1. FUNCTIONS OF INTRACELLULAR PROTEIN DEGRADATION

Early workers investigating the metabolism of cellular proteins made a distinction between metabolically inert proteins and those derived from nutrient metabolism, considering only the latter proteins to be metabolically labile (Voit, 1866). A concept of breakdown of intracellular proteins resulting from "wear and tear" was introduced into the field by Folin in 1905,

but only in 1935 did Borsook and Keighley propose that virtually all proteins in mammalian tissues were in a continual state of synthesis and degradation (Borsook and Keighley, 1935). More definitive demonstrations of continual protein turnover were made in the late 1930's once radioactive tracer techniques became available, and the well-known phrase "dynamic state of body constituents" was coined by Schoenheimer in 1942 to describe the concept of a continual replacement of cellular constituents. Buchanan (1961) estimated that approximately 70% of protein of rat liver was replaced every 4-5 days from a dietary source, and since he showed the life-span of hepatic cells to be 160 - 400 days, it became clear that in liver, most of the protein degradation was occurring within intact cells and was not merely related to cell death and replacement.

1.1. Regulation of Metabolic Pathways

At first glance, the continual degradation of cell proteins appears highly wasteful, although it must obviously provide the organism with some selective advantage. On consulting a representative listing of the degradative rates of various enzymes of rat liver and other tissues (Goldberg and St John, 1976), a wide variation was seen, ranging from 10 minutes to 16 days. These marked differences in degradation rate appear to be important in the regulation of metabolic pathways. Thus, the rapid degradation of certain enzymes may have evolved in order that their intracellular levels can fluctuate rapidly in response to changes in the environment. A short half-life would allow the level of an enzyme to increase rapidly in response to enhanced synthesis and to fall very quickly on

decreased synthesis, which means that they can be altered more rapidly in response to environmental changes than enzymes which are degraded slowly. A survey of published half-lives for a series of rat liver proteins (Goldberg and St John, 1976) has shown that the sixteen proteins with short half-lives tend to be the rate-limiting enzymes in metabolic pathways such as polyamine biosynthesis, RNA synthesis, amino acid catabolism, gluconeogenesis, the hexose monophosphate shunt, heme synthesis and cholesterol production. In contrast, the fifteen proteins with the longest half-lives do not have key roles in metabolic control and are not known to be subject to allosteric regulation. These correlations strongly suggest that protein half-lives have evolved to regulate the flux of substrate through metabolic pathways and facilitate the adaptation of the organism to its environment.

The question of heterogeneity of degradation rates for individual proteins is also important in developing concepts of the mechanisms and regulation of specific protein degradation. Thus, if all constituents of a cell or organelle were degraded at the same rate, one could consider quite different mechanisms for such degradation than for a cell compartment in which the individual proteins were degraded at extremely varied rates. Generally, there have been no correlations found between half-lives and the cell fraction from which proteins have been isolated, which would argue against an all or none mechanism for degradation and rather favour a more selective proteolytic pathway.

1.2. Adaptation to a Poor Environment

In addition to adaptation where one group of intracellular proteins is replaced by a more appropriate group, the adaptation of an organism to a poor nutritional environment would require protein degradation simply to provide energy and sufficient amino acid precursors for the synthesis of essential proteins such as catabolic enzymes. This usually occurs at the expense of proteins required for "luxury" processes such as motility, an example being the catabolism of the large reserves of muscle protein during starvation. This type of degradation suggests a priority for each individual protein within the organism so that the activities of essential enzymes are preserved under suboptimal environmental conditions.

1.3. Removal of Aberrant Proteins

Another important function of protein degradation may be the removal of abnormal proteins which may arise due to exogenous (mutagens) or endogenous (e.g. biosynthetic errors) factors. All cells appear to have a mechanisms whereby incorrect proteins are recognized and rapidly degraded to prevent their accumulation, and to allow their constituent amino acids to be used for correct biosynthesis of other proteins.

2. GENERAL CHARACTERISTICS OF INTRACELLULAR PROTEIN DEGRADATION

2.1. First-order Kinetics?

The degradation of most isotopically-labelled proteins after single isotope administration follows first order exponential kinetics, but no definitive statements concerning the mechanisms

of protein degradation can be made from this observation because Fukuda and Yago (1976) have shown the data to be equally compatible with a random mechanism of degradation between molecules of a particular protein as well as with several other non-random kinetic interpretations.

2.2. Dependence on Physicochemical Properties of Proteins

The half-lives of intracellular proteins in steady-state conditions appear to be determined by their physicochemical properties, and general correlations have been shown to exist between protein degradation rates and polypeptide size, isoelectric points, surface hydrophobicities and carbohydrate content.

2.2.1. Degradation Rate Related to Subunit Size

Dehlinger and Schimke (1970) first showed that large subunit proteins have greater relative degradation rates than small subunit proteins as measured by the double-isotope technique of Arias et al. (1969). Further studies have shown that this relationship also applies to cytosol proteins in liver, kidney, testis, brain and muscle tissues (Dice, Dehlinger and Schimke, 1973) as well as to ribosomal proteins in liver (Dice and Goldberg, 1976), but does not apply to the extracellular protein in serum (Dice and Goldberg, 1975c). A lack of obvious correlation between total molecular weight and degradation rate in most tissues suggested that subunits rather than the multimeric proteins are substrates for the degradative mechanism (Dice, Dehlinger and Schimke, 1973) but Dice and

Goldberg (1975), based on their review of the literature, have found a weak but significant correlation between the multimeric size of a protein and its degradation rate. Such studies have led to the proposal that the correlation between size and relative degradation rate is based on the overall greater chance of a large protein or polypeptide being "hit" by a proteinase, resulting in an initial rate-limiting peptide bond cleavage, and a subsequent unfolding and rapid degradation of the polypeptide to its constituent amino acids. However, this relationship is only a general tendency and many exceptions have been shown to exist (Dice and Goldberg, 1975c).

2.2.2. Degradation Rate Related to Isoelectric Properties

Proteins having acidic isoelectric points also appear to be degraded more rapidly than neutral or basic ones. For twenty-two rat liver proteins, Dice and Goldberg (1975b) have found a highly significant relationship between relative degradation rates and isoelectric points, but the relationship appears to be distinct from that between subunit size and half-life (Dice, Hess and Goldberg, 1979). Thus size and charge appear to be two independent parameters determining degradation rates, so that if evolutionary selection would have favoured a low degradation rate for a specific protein, it would have been both both small and highly basic. It is noteworthy that one of the most stable groups of polypeptides in mammalian tissues is probably the histones, which fit these criteria exactly.

An important implication of this finding is that the net charge of a protein will affect its binding to other intracellular

molecules, a process that seems to be important in protein breakdown, but the precise manner through which charge may influence half-lives is unclear. The most rapidly degraded proteins are most negatively charged in the cytoplasm, but are probably least charged within the acidic environment of the lysosomes. Exactly which of these two environments is the site of the rate-limiting step of proteolysis remains to be established, but these studies would point to the extra-lysosomal compartment as the site for selectivity of protein degradation mechanisms.

2.2.3. Degradation Rate Related to Hydrophobicity

Another physicochemical property that may be relevant in the association of proteins with membranes is the extent of hydrophobic areas on the surface of the molecule. Bohley *et al.* (1977) have double-labelled proteins *in vivo* by the method of Arias *et al.* (1969) and have exposed them to various phase-partition systems. Partition between aqueous solutions and a number of organic solvents all showed a selective movement to the organic (hydrophobic) phase of those proteins which had the highest degradation rates *in vivo*.

2.2.4. Degradation Rate Related to Glycoprotein Content

Dice *et al.* (1978) have recently demonstrated a further factor influencing the relative degradation rate by showing that intracellular glycoproteins that bind to concanavalin A or to other immobilized lectins, tend generally to be degraded more rapidly than those proteins not binding to lectins, the non-

glycoproteins. This correlation has been consistently illustrated in a number of different tissues under optimal nutritional conditions.

2.3. Sensitivity to Proteinases *In Vitro*

Studies in the laboratories of Goldberg (1974, 1976), Dice (1973), Segal (1974) and Dean (1975) using both bacterial and mammalian cells, demonstrated a general correlation between protein half-lives *in vivo* and their inherent sensitivity to proteolytic enzymes *in vitro*. For example, 'abnormal' proteins which have been found to be degraded especially rapidly *in vivo* are particularly sensitive to a variety of proteolytic enzymes (Goldberg *et al*., 1975). Short-lived proteins are inherently more easily digested by various neutral endoproteases than more stable cell constituents (Dice and Schimke, 1973). These correlations have been extended for sensitivity to lysosomal enzymes (Segal, 1974; Dean, 1975). Since similar correlations can be demonstrated with proteases of very different specificity and activity, the results do not provide any insights into the selectivity of the responsible degradative enzymes, but instead suggest that degradation rates are determined by general conformational features that are recognized by all types of endoproteinases (Goldberg and St. John, 1976).

2.4. Dependence on Conformational State

Conformational changes in proteins such as denaturation, interaction with ligands such as cofactors, metals and substrates

and covalent modifications, can substantially affect their degradative rates. Denaturation generally increases the proteolytic rate whereas ligand binding generally reduces it. (See review of Goldberg and St. John, 1976). In some cases, the protecting ligands may be substrates such as NADP for glucose-6-phosphate dehydrogenase (Bonsignore et al., 1968) or tryptophan for tryptophan pyrrolase (Schimke et al., 1965), but it is likely that any substance which helps to maintain the correct conformation of a protein will protect it from inactivation and subsequent degradation. Dunaway and Segal (1974, 1976) have described a ligand whose prime function seems to be the control of degradation of phopsphofructokinase to which it binds in vivo. Osterlund and Bridger (1977) have shown that this factor also protects ATP citrate lyase against thermal and proteolytic degradation.

The relationship between conformation and degradation rate also probably underlies the selective degradation of artificially-produced "abnormal" proteins containing sequence errors or amino acid analogues (Goldberg and Dice, 1974; Knowles and Ballard, 1976) and of proteins synthesized in cells during amino acid depletion (Chandler and Ballard, 1978). Intracellular protein degradation appears to serve as a "cellular sanitation" mechanism to prevent the accumulation of abbrrant and potentially harmful proteins. E. Coli nonsense fragments in β-galactosidase are degraded with half-lives of a few minutes (Zabin et al., 1972); puromycin-containing polypeptides in mammalian cells are selectively hydrolyzed (Goldberg and St. John, 1976) as are analogue-containing proteins e.g.

canavanine-containing proteins are degraded up to twenty times faster than those containing arginine (Goldberg et al., 1978). Other amino acid analogues like azatryptophan, p-fluorophenylalanine, 6-fluorotryptophan have also been used to produce similar results, but canavanine is most effective in acceleration of degradation. The rapid degradation of aberrant protein appears to be a highly selective process since the degradation of normal protein within the same cells is not affected. This indicates the possible existence of two distinct pathways of proteolysis. Alternatively, normal intracellular proteins may share conformational features making them relatively resistant to proteinases and deviations from these structures might simply increase degradation rates.

While most ligands stabilize enzymes, the product of enzyme action may affect the turnover of at least one enzyme, e.g. glutamine synthetase, which has an accelerated degradation rate in the presence of glutamine (Milman et al., 1975).

2.5. Dependence on Physiological State

Dice and Walker (1978) have studied protein turnover in conditions of starvation and diabetes and have found that in liver and muscle tissue (but not in brain), the usual correlations between half-life and molecular properties of intracellular proteins are largely obliterated. This strongly suggests that a quantitative change in the contribution of two distinct degradative mechanisms with different selectivities may be occurring under conditions of starvation or diabetes.

3. REGULATION OF INTRACELLULAR PROTEIN DEGRADATION

3.1. Energy-Requirement for Degradation

It has been known for almost thirty years that protein degradation is an energy-dependent process (Simpson, 1953; Steinberg and Vaughan, 1956) which was inhibited in liver slices by agents which reduced ATP generation. This finding was particularly surprising on thermodynamic grounds since proteolytic cleavage reactions are exergonic and since no known intracellular proteinase requires high energy cofactors. It is also noteworthy that protein degradation appears to require a relatively low level of ATP since inhibition is seen only if the ATP level is reduced to below 10% of that found in growing cells (Goldberg and St. John, 1976).

The biochemical basis for this energy requirement remains an important unsolved problem. Further, the energy requirement is generally lost when tissues are homogenized and incubated *in vitro*, and the degradation is markedly reduced unless a low pH is maintained (Steinberg and Vaughan, 1956; Bromstrom and Jeffay, 1970). These requirements have been equated with intralysosomal proteolysis where there is both a low internal pH and the capacity for the bulk of cellular proteolytic activity (Dean and Barrett, 1976). The possibility of an ATP-driven proton pump in lysosomes may explain the energy requirement (Mego, 1973 & 1975; Henning, 1975; Dean and Barrett, 1976). However, the ATP requirement is not only observed in mammalian cells (De Martino and Goldberg, 1976), but also in bacteria (Murakami and Goldberg, 1979) where

there is an absence of lysosomes. ATP could also act as an energy source for the membrane transport of other compounds or to drive reactions against unfavourable equilibria (Ballard, 1978). Studies showing that various protein synthesis inhibitors such as puromycin, cycloheximide and pactamycin, which all act at different sites in protein synthesis, inhibit protein degradation, have resulted in the proposal that the ATP-requirement results from protein synthesis (Epstein _et al_., 1975).

More recent experiments show that intralysosomal proteolysis, studied using substrates already internalized by the lysosomes, is energy-dependent (Mego and Farb, 1978). Cathepsin L, a major lysosomal thiol proteinase, is also activated by ATP. (Barrett, 1977).

An ATP requirement was first demonstrated in a soluble protein degradation system from rabbit reticulocytes (Etlinger and Goldberg, 1977). This extralysosomal system degrades abnormal globin at a pH optimum of 7,8, and appears to involve a proteinase of the thiol class. More recent evidence suggests that ATP may be required at or before the initial cleavage of the polypeptide molecule (Hershko _et al_., 1978). This group of workers have resolved the ATP-dependent proteolytic system into several required components: a heat-stable, relatively small polypeptide designated APF-I (ATP-dependent proteolysis factor I) which has no proteolytic activity itself but stimulates ATP-dependent proteolysis by a crude protein fraction eluted from DEAE-cellulose, termed fraction II. APF-I has

been recognized as ubiquitin (a highly conserved, heat-stable polypeptide found in all mammalian cells) and may thus be the essential component of the ATP-dependent degradation system in all tissues (Ciechanover *et al*., 1980; Wilkinson *et al*., 1980). Fraction II has been further resolved into 2 subfractions, both required for protein degradation by the ATP-APF-I system (Hershko *et al*., 1979). APF-I has been shown to bind covalently (probably via an isopeptidic linkage) with substrate protein in fraction II, the conjugation requiring Mg^{++} and ATP (Ciechanover *et al*., 1980), and more recently an enzyme in fraction II, believed to activate APF-I for subsequent trasnfer to substrate protein, has been described (Hershko *et al*., 1981). A sequence of reactions in which the linkage of one or more APF-I molecules to the substrate is followed by the proteolytic breakdown of the substrate has been proposed to explain the role of ATP (Hershko *et al*., 1980).

3.2. Regulation by Hormones and Nutrients

It is now clearly established that the rate of protein degradation in organs and cultured cells is sensitive to changes in the hormonal and nutritional environment. Proteolysis is markedly accelerated by the removal of amino acids, i.e. nutritional step-down conditions, and is lowered by the addition of enriched media. In general, insulin and the branched-chain amino acids suppress proteolysis rates (Fulks, Li and Goldberg, 1975). In perfused liver and isolated hepatocytes, glucagon accelerates proteolysis (Mortimore and Ward, 1976), and thyroid hormones appear to accelerate muscle protein degradation

(Flaim *et al.*, 1978). Physiological activity, for example in muscle, may also play a role in the control of protein degradative rates with overall degradation being reduced during contractile activity (Goldberg and St. John, 1976). Such changes in degradation can make important contributions to the control of intracellular levels of particular proteins.

Intracellular protein degradation in muscle and liver is markedly affected by nutrient deprivation. The levels of various hormones and intermediates of carbohydrate, fat and protein metabolism in the environment seem to influence these responses, but clear inter-relationships have not been established. Distinct proteolytic rates can be obtained in perfused organs and cultured cells according to nutrient and hormonal supply, with "basal proteolysis" occurring in the presence of 10% serum and plentiful amino acids, and an "accelerated proteolysis" occurring in their absence. As previously mentioned, the experiments of Dice and Walker (1978) have shown that accelerated proteolysis in liver and muscle associated with diabetes and starvation differs fundamentally from basal degradation. Protein degradation in brain, however, retained its normal characteristics under accelerated conditions; they are also apparently retained in the accelerated protein degradation occurring in dystrophic muscle.

3.3. Regulation of Degradation during Growth and Differentiation

Selective changes in the levels and degradative rates of individual proteins often occur during differentiation and growth

(Mayer, 1978) but this may involve control of the degradative machinery itself as well as ligand stabilization. Low bulk turnover is sometimes found during developmental organ growth (Conde and Scornik, 1977) and in regenerative processes. Tauber and Reutter (1978) have shown that normal turnover of liver plasma membranes is heterogeneous, but is more homogeneous in the regenerating liver. This may indicate that a basal lysosomal mechanism of unit degradation is retained during regeneration, but is accompanied by other mechanisms in normal liver. In the same way, protein degradation increases in non-growing cell cultures, i.e. when they reach confluence, due to serum deprivation (Bradley, 1977) or the removal of certain hormones (Robinson _et al_., 1976). The accelerated degradation usually only involves the more stable intracellular proteins, but may also involve some short-lived proteins (Tanaka and Ichihara, 1976). This regulation probably involves alteration in half-lives of only certain cellular proteins. (Yamasaki and Ichihara, 1976). Serum deprivation of rat fibroblasts is associated with accelerated proteolysis of long-lived proteins (Poole and Wibo, 1973) and it seems that a factor in fresh serum is responsible.

However, these observations are by no means universal and certain cell types show no dependence of degradation on growth state (Bradley, 1977). Some transformed cells also have an altered basal protein degradation rate and sensitivity to serum and hormones (Bradley, 1977; Castor, 1977). Gunn _et al_. (1977) have found that protein turnover in SV-40 transformed 3T3 fibroblasts is slower than in untransformed cells, although

contrary evidence also exists (Bradley, 1977).

Varied responses of the lysosomal system to growth conditions have also been found. Lockwood and Shier (1977) have demonstrated elevated proteinase levels in normal cells during serum deprivation and confluent quiescence, but not in transformed cells whereas Wang and Touster (1976) have shown that in Hela cells, lysosomal proteinase levels are raised by inc increased serum levels. In general, therefore, there are no definite correlations between lysosomal enzyme levels, degradative rates and growth control, and it also remains to be seen whether derangement of control of degradation characterizes transformed cells. Increased protein degradation seems to characterize wasting muscles, both in muscular dystrophy (Goldspink, 1976), while protein synthesis is increased in the former and decreased in the latter.

3.4. Degradation during Cellular Senescence

Protein degradation has also been studied with respect to cellular senescence, because the "error catastrophy" hypotheses of Orgel (1963, 1973) proposed that senescence occurred as a result of progressive accumulation of error-containing proteins. It has been shown that WI-38 human fibroblasts, a widely used model of the ageing process (Hayflick, 1965), retain the capacity for degradation of analogue-containing proteins throughout their life span (Bradley *et al*., 1976; Dean and Riley, 1978), but a progressively increased proportion

of short-lived proteins was evident, resulting in an increased average turnover rate (Shakespeare and Buchanan, 1976; Bradley et al., 1976; Dean and Riley, 1978). This increase in short-lived proteins may also be related to the observed increases in the proportions of thermo-labile or altered enzyme molecules in ageing cells (Cutler, 1976; Linn et al., 1976). Thus the degradative machinery of ageing cells seems to remain intact, able to accommodate for the rapid removal of increased numbers of error-containing proteins.

3.5. Regulation of Degradation by Ca^{++}

Various reports have indicated a possible role for Ca^{++} in regulation of protein breakdown in the frog cutaneous pectoris and mouse diaphragm muscles (Stratham et al., 1976; Publicover et al., 1978). These authors have shown that treatment of muscle with the divalent-cation ionophore A23187 (Reed and Lardy, 1972) leads to rapid dissolution of the myofilaments. It is known that muscle tissues contain a Ca^{++}-activated proteinase (Okitani et al., 1974; Dayton et al., 1976) which is active at neutral pH and appears to be cytoplasmic, and several authors have proposed that this enzyme may be important in the turnover of muscle proteins.

Etlinger et al. (1980) have investigated the roles of Ca^{++} and tension in regulating protein turnover in rat muscles incubated *in vitro*, and found that proteolysis was stimulated by the

addition of Ca^{++} to the incubation medium and was further enhanced by A23187. The overall effect of Ca^{++} in the absence of applied tension appears to favour a net catabolic state. This finding may relate to dystrophic muscle because of the characteristically increased permeability of dystrophic plasma membranes to the high concentrations of Ca^{++} normally present in the extracellular fluid. However, in the presence of tension, Ca^{++} produces effects which may be related to hypertrophy since Ca^{++}-dependent degradation is reduced or reversed while synthesis is further stimulated.

Sudgen (1980) has proposed that the effects of Ca^{++} in muscle proteolysis are unlikely to be of importance in steady-state protein turnover _in vivo_, but may be important in the degradation of necrotic tissue.

4. MECHANISMS OF INTRACELLULAR PROTEIN DEGRADATION

4.1. Rate-Limiting Steps

It has often been suggested that enzyme inactivation may precede extensive proteolysis. Some workers have shown inactivation to be a proteolytic event while others argue that denaturation may precede proteolysis (Bond, 1976). Ballard (1978) has characterized a labile, membrane-localized system which inactivates thiol-containing proteins resulting in attachment of the protein to the membrane followed by limited localized proteolysis. Efforts to demonstrate partially degraded intermediates of protein breakdown have mostly failed, although some have been observed in bacterial proteolysis (Goldberg _et al_., 1978).

Some "group-specific" and other less specific proteinases which inactivate native enzymes by limited proteolysis have been described by Katanuma (1977) and Beynon and Kay (1978), but care must be exercised in the interpretation of this data because it has been recently demonstrated that some of these enzymes are probably derived from contaminating mast cell proteinases. For example, Woodbury and Newath (1978) have illustrated that one chymotrypsin-like serine proteinase isolated from rat intestinal muscle, actually originates from mast cells, and that this "group-specific" enzyme is not likely to control the degradation of the pyridoxal enzymes which it specifically inactivates _in vitro_. The susceptibility of native proteins to these proteinases can be modified by ligand attachment (Kay _et al._, 1977; Carney _et al._, 1978) or by covalent modification such as phoshporylation (Hales, 1978), which may be related to their degradation rates _in vivo_.

It is not known whether inactivation mechanisms are proteolytic or non-proteolytic or where these mechanisms are located within the cell. It is also not clear whether inactivation constitutes a rate-limiting step in proteolysis or whether complete degradation of inactivated proteins can occur at these sites. It seems likely that the production of a pool of free subunits of any protein precedes the rate-limiting step in its breakdown (Dean, 1980) which in itself suggests that the enzyme is already inactivated before the rate-limiting step. However, some association-dissociation reactions might become rate-limiting in cases where specific ligands alter the stability of a protein.

Three possible rate-limiting events are conformational changes, limited proteolysis and membrane attachment. Denaturation renders many proteins more susceptible to degradation *in vitro*, thus any unfolding of a protein may be a signal for its rapid catabolism (McLendon and Radany, 1978). Short half-life proteins are always found to be preferentially degraded *in vitro* by both lysosomal proteinases (Dean, 1976) or non-lysosomal proteinases (Goldberg and Dice, 1974). This may reflect inherent rate-limiting differences in denaturability or their large subunit size, making more sites available for initial cleavage. Membrane attachment may effect a selective turnover of proteins which are degraded on membranes or intra-lysosomally (Bohley, 1968). There is evidence that proteins bind differentially to hydrophobic interfaces including lysosomal membranes (Dean, 1975c) and this selectivity corresponds well with their rates of turnover.

Segal (1976) has argued that intralysosomal proteolysis may be rate-limiting. His theory requires a non-specific mechanism to allow the return of internalized material back into the extralysosomal compartmentwhich is inconsistent with most of the current literature on lysosomes (Dean and Barrett, 1976). Short-lived proteins would then be those that are more easily denatured and degraded within the acidic conditions and high proteinase concentrations of the lysosome. However, little evidence in support of such a mechanism has yet been obtained.

4.2. Pathways of Intracellular Proteolysis

4.2.1. Single Mechanism Unlikely

A single mechanism or pathway of protein degradation could clearly not account for all of the following observations: degradation of normal and "abnormal" proteins with their selective controls, specific proteolytic reactions such as those involved in the cleavage of N-terminal methionine or the processing of proteins synthesized for export from the cell, heterogeneous degradation rates of intraorganellar protiens, especially those which turnover more rapidly than the organelle in which they are localized, e.g. α-amino-laevulinic acid synthetase (Goldberg and Dice, 1974); and the degradation of endocytosed proteins. However, portions of the proteolytic sequence could be common to different overall processes. For example, lysosomes appear to contribute to the degradation of extracellular endocytosed protein, those targeted for release from the cell and the autophagic degradation of bulk cellular protein (Dean and Barrett, 1976).

Organellar proteins showing heterogeneous rates of turnover despite being excluded from direct contact with the cell cytosol, are most likely to be degraded by intraorganellar enzyme systems. Such systems have been described in mitochondria (Banno et al., 1978) and chromatin (Suzuki and Murachi, 1978). On the other hand, organellar proteins exposed to the cytosol and to other cellular membranes e.g. proteins of the external mitochondrial membrane, may undergo

direct interaction with these membranes and be degraded by different mechanisms to those of the inner mitochondrial membrane (Russell, Burgess and Mayer, 1980). Turnover of some organelles may thus be purely internal or lysosomal, but most mechanisms probably involve a combination of internal and external degradation. In general, organelles showing unit turnover are probably degraded by lysosomal sequestration of the whole organelle (Dean and Barrett, 1976), whereas organelles showing heterogeneous turnover of internal proteins have an internal degradative machinery which is at least partly responsible for their turnover.

4.2.2. Main Experimental Approaches

Two general approaches have been adopted to investigate degradation pathways and their specificities. A genetic search for mutations which are non-lethal but lead to an absence of one aspect of protein degradation has been attempted, and Zipser has described bacterial strains with differential loss of the ability to catabolize separate classes of abnormal proteins (Bukhari and Zipser, 1973; Apte, Rhodes and Zipser, 1975), but the necessity for a mutation to be non-lethal has been difficult in multicellular organisms and no deletions of lysosomal cathepsins have been found amongst the thirty or more genetic defects of lysosomes in man (Neufield et al., 1975). The second approach was initially adopted by Dean (1975d) where rates of protein degradation are measured after specific inhibitors of intracellular proteinases are directed to lysosomes or other cell compartments. Difficulties encountered using

this approach are mainly due to the fact that these inhibitors rarely show complete specificity and some cannot rapidly penetrate the cell to the site of proteolysis. Many experiments of this nature have involved very brief periods of exposure of cells to the inhibitor: in cases where the inhibitor (such as pepstatin) enters cells by the relatively slow process of pinocytosis rather than by the rapid process of permeation, this period may be insufficient to allow maximal inhibition. The data of Knowles and Ballard (1976) and Dean (1979) on macrophages indicate that inhibition does increase with time for several of these inhibitors. These problems are amplified for studies on short-lived proteins because the periods of exposure to any inhibitor are necessarily brief. Furthermore, the observed inhibition cannot be simply equated with the quantitative significance of the lysosomal mechanism in untreated cells since inhibition of one degradative route might well accelerate another. A further problem of interpretation arises if the inhibitor is not totally specific to a particular proteinase, but has other non-specific actions on the cell and its normal metabolism. The large body of literature on the inhibition of proteolytic activities has been excellently reviewed by Dean (1980). Of the proteinase inhibitors used, pepstatin and Z-phenylalanine-alanine-diazomethylketone have appeared to be the most diagnostic for lysosomal activity, because of their well-established endocytotic mode of entry into cells, and in the case of pepstatin, because there is only one known intracellular carboxyl proteinase in mammalian cells, namely cathepsin D (Umezawa and Aoyagi, 1977). The actions of agents like

ammonia, chloroquine, colchicine, vinblastine and cytochalasin are less specific, and they have many known effects in addition to lysosomal actions. For example, ammonia inhibits protein secretion in rat hepatocytes (Seglen and Reith, 1977) and chloroquine reduces ATP levels (Goldberg and St. John, 1976).

4.2.3. Evidence for a Lysosomal Mechanisms

For many years, lysosomes have been implicated as the sites of intracellular protein degradation primarily because they are the only well-characterized organelles with substantial proteolytic capacity as well as a known mechanism for acquiring cellular substrates for degradation, namely autophagy (Dean and Barrett, 1976). A number of studies have established that the degradation of exogenous, pinocytosed proteins probably occurs exclusively within lysosomes (Goldstein and Brown, 1977; Von Figura *et al.*, 1978; Goldstein *et al.*, 1975), but the extent to which lysosomal proteinases function in the degradation of intracellular proteins is still controversial.

Dean (1975d) provided some of the most direct evidence from studies of perfused liver, showing that the degradation of long-lived proteins can be inhibited by pepstatin, a small peptide derivative isolated from actinomycetes and a specific inhibitor of lysosomal cathepsin D. Pepstatin was entrapped within liposomes to facilitate its entry into the cells and a substantial degree of inhibition (50%) of protein degradation was obtained. The observation was confirmed by studies with isolated parenchymal cells (Bohley *et al.*, 1977) and with perfused liver in which dimethylsulphoxide (DMSO) was used to

facilitate the entry of pepstatin (Ward et al., 1979). Pepstatin also has a marked effect on the morphology of cell cultures from dystrophic animals (McGowan et al., 1976) and inhibits basal proteolysis in rat hepatocytes (Hopgood et al., 1977).

Leupeptin, a less selective inhibitory peptide derivative from actinomycetes, which acts most effectively against thiol proteinases such as cathepsins B, H and L, as well as being inhibitory to the action of some serine proteinases, has been shown to inhibit protein degradation in perfused liver (Ward et al., 1976), isolated hepatocytes (Seglen et al., 1979), skeletal muscle (Libby and Goldberg, 1978) and cardiac muscle (Ward et al., 1979; Wildenthal and Crie, 1980) without affecting protein synthesis. However, it has not yet been clearly established which enzymes are affected by leupeptin and because it permeates cell membranes, could act anywhere inside the cell, whereas pepstatin selectively enters the lysosomes by endocytosis.

Recently, Dean (1980) has successfully used a more selective inhibitor of thiol proteinases, Z-Phenylalanine-alanine-diazomethylketone (Leary and Shaw, 1977) to inhibit the degradation of long-lived proteins in cultured mouse peritoneal macrophages and because it enters the cell by pinocytosis, its effects are likely to be exclusively intralysosomal.

Weak bases such as ammonia, chloroquine and methylamine accumulate inside lysosomes by being trapped within the organelle by protonation (Dean and Barrett, 1977), thus reducing the

activity of the acidic lysosomal proteinases, have been found to have diverse inhibitory effects. In conditions of basal degradation, these agents produce a maximal inhibition of 30-50% in cultured cells (Livesey _et al_., 1980; Amenta and Brocher, 1980) and 70-80% in isolated rat hepatocytes (Seglen _et al_., 1979; Grinde and Seglen, 1980).

The investigation of protein degradation in perfused organs has revealed extensive correlations between the state of the lysosomal system and the proteolysis rate (Mortimore and Ward, 1976). When normal rat livers are perfused without added amino acids, protein degradation rises by about 75% and lysosomes become enlarged, greater in number, increased in density, more sensitive to osmotic shock and their protein content rises, which may well represent increased sequestration of substrate protein. Insulin and amino acids can suppress this rise in the proteolytic rate and the observed lysosomal changes. This work has led to the characterization of an intralysosomal amino acid pool derived from protein degradation (Ward and Mortimore, 1978), providing further direct evidence for lysosomal involvement in protein degradative mechanisms. Some cytoplasmic proteins such as ferritin and tryptophan pyrrolase, have been observed within lysosomes under electron microscopy, and further studies have revealed the presence of glycogen and probably endoplasmic reticulum proteins within lysosomes (Mortimore _et al_., 1978).

The studies outlined above indicate that lysosomes are important in the degradation of long-lived proteins (labelled for 16 hours

or more), and there is also some evidence implicating them in the degradation of short-lived proteins (labelled for ½ to 4 hours), although their contribution is generally presumed to be substantially lower in this case. It also remains to be satisfactorily established whether lysosomes participate substantially in the rapid degradation of proteins containing errors or amino acid analogues. Generally, it appears that their role, if any, will be quantitatively small (Knowles and Ballard, 1976), although Dean (1980) maintains that there is relatively little basis for proposing any unique mechanism for the degradation of analogue-containing proteins and that lysosomes are probably involved to some extent in all these rapid-turnover processes, but that other pathways may be involved as intermediate or additional alternatives.

The proteins of some membranes such as plasma membranes and the endoplasmic reticulum generally show a correlation between subunit size and half-life, while those of lysosomal membranes do not (Dean, 1975; Wang and Touster, 1975). However, short-lived ER proteins are not hydrolyzed more rapidly than long-lived proteins by proteinases *in vitro*, so the simplest explanation may be that selectivity is conferred by a discriminating uptake into lysosomes or selective protein clustering in areas of membrane to be taken into the lysosomes (Dean and Barrett, 1976). This proposal, however, remains to be substantiated.

Lysosomes have been clearly implicated in accelerated proteolysis in perfused liver (Dean, 1975), cultured hepatocytes (Seglen and Sollheim, 1977) and hepatoma cells (Ballard, 1978).

Using these experimental systems, various lysosomatropic agents result in a 50-80% inhibition of the degradation rate of long-lived proteins, whereas there is only a 30-40% reduction in degradation under basal (or nutritionally optimal) conditions. Thus, lysosomes appear to participate in both basal and accelerated proteolysis but more substantially in the latter (Amenta _et al_., 1978).

The degradative capacity of the lysosomal system does not usually appear to be a factor limiting proteolysis. It is noteworthy, however, that endocytosis by macrophages suppresses endogenous turnover (Poole _et al_., 1977; Dean, 1979), although it appears that endocytosed proteins are degraded in a lysosome population distinct in some ways from lysosomes degrading endogenous proteins. In this regard, lysosomotropic agents like chloroquine inhibit the degradation of endocytosed proteins far more than that of cellular proteins (Poole _et al_., 1977).

Microtubule agents such as colchicine and vinblastine have inhibitory effects mainly under conditions of accelerated proteolysis (Amenta _et al_., 1977b, 1978c) and the microfilament agent cytochalasin B also has some inhibitory effect on proteolysis (Dean, 1977c, 1978b; Amenta _et al_., 1978c) and a possible interpretation currently under direct assessment is that microfilaments are involved in autophagy.

Quantitation of the lysosomal autophagic uptake of molecules such as sucrose or polyvinylpyrrolidone (which exert a minimal disturbance on the steady state), have indicated that the

minimum rate of autophagic protein degradation in cultured cells is probably about 1% per hour (Dean, 1977; Dean and Barrett, 1976), which is very similar to the proteolysis rate of 1-2% per hour found in cultured cells. However, in order to strengthen the arguments for lysosomal involvement in most degradative processes, it needs to be shown whether the rate of autophagy is controlled by the various physiological and pharmacological agents which modulate protein degradation rates.

4.2.4. Evidence for Extralysosomal Mechanism(s)

There is an increasing amount of evidence that more than one intracellular pathway may exist for protein degradation. The presence of a variety of non-lysosomal proteinases, such as the alkaline proteinases (Katanuma, 1977; Reville *et al.*, 1976; Katanuma *et al.*, 1975), the previously mentioned ATP-dependent enzymes (Etlinger and Goldberg, 1977; Hershko *et al.*, 1980) and calcium-activated proteinase, suggest cytoplasmic protein breakdown.

If a single proteolytic process were responsible for the degradation of all cellular proteins, then the half-lives would be expected to change similarly under different experimental conditions. However, under nutritional "step-down" conditions, more stable cell proteins are generally degraded at accelerated rates while the degradation of proteins with shorter half-lives does not change (Poole and Wibo, 1973; Warburton and Poole, 1977; Hershko and Tomkins, 1971). In addition, protein synthesis inhibitors and microtubular poisons prevent the accelerated breakdown of stable proteins without affecting the

basal rate of proteolysis seen under optimal nutritional conditions (Hershko and Tomkins, 1971; Amenta et al., 1976, 1977, 1978).

Additional evidence for the existence of multiple degradative pathways comes from the observation by Knowles and Ballard (1976) that conditions which alter the rates of degradation of stable proteins such as addition of insulin, NH_4Cl, inhibitors of lysosomal proteins or the removal of amino acids, have either no or only slight effects on the rapid degradation of proteins containing amino acid analogues. A major goal for future research remains the definitive demonstration of a role for cytoplasmic systems and subsequent quantitation of extralysosomal and lysosomal roles, possibly by developing selective proteinase inhibitors to cytosolic enzymes.

4.2.5. Models Proposed for Intracellular Protein Degradation

Two basic models for intracellular protein degradation have been recently proposed. Firstly, the single, largely lysosomal model of Dean with the lysosomes involved at some stage of most degradative processes. Selectivity and regulation would then operate on several possible discrete steps as discussed above. In contrast, Ballard (1978) has proposed a dual pathway model which assigns the lysosomes about 30% of the cell's basal proteolytic activity via its system-limited non-selective route. This degradative pathway can be completely suppressed by serum, insulin, cycloheximide, lysosomotropic weak bases and proteinase inhibitors used singly or in combination, and is activated by glucagon or nutritional step-down conditions.

This system is considered mainly relevant to the turnover of long half-life proteins. The second pathway which is considered to account for approximately 70% of basal protein turnover is mainly concerned with short half-life proteins. It is substrate-limited, in that the degradation rates are functions of the molecular properties and ligand binding of each protein, and the capacity of the system is not limiting. Denaturability of proteins plays an important role in their inherent susceptibility to this second pathway, but the known accumulation of "abnormal" proteins in cells suggests that it is the hydrolysis of these proteins which is rate-limiting in their turnover.

The dual pathway model is largely based on the evidence obtained using various inhibitors. Other morphological evidence for this model is that under the conditions of nutritional and hormonal deprivation, when the lysosomal pathway appears to predominate, autophagic vacuoles are very pronounced (Amenta et al., 1977b, 1978c). Also, some inhibitors which have comparatively little action on basal proteolysis become effective under these accelerated proteolytic conditions, e.g. NH_4Cl, chloroquine.

Amenta et al. (1978c) have proposed another version of the dual pathway, involving lysosomes in both pathways. Some intracellular proteins are envisaged as being degraded entirely within the lysosomal compartment, whereas others are presumed to be partially degraded extralysosomally and to have their degradation completed within the lysosomes.

A major unresolved question concerns the specificity of the lysosomal and non-lysosomal systems under basal or step-down conditions. Dice and Walker (1978) have shown that under step-down conditions, the normal characteristics of degradation are altered and have correlated this with increased autophagy (presumed to be the non-selective pathway). Jenkins *et al.* (1979), however, have reported that accelerated proteolysis under step-down conditions is non-lysosomal in origin. However, selectivity is only a requirement of single-pathway models if there is no preliminary rate-limiting modification preceding lysosomal degradation.

OUTLINE OF PROPOSED RESEARCH

The aim of the present study was to investigate the pathways whereby intracellular muscle proteins are degraded. Certain basic characteristics of intracellular proteolysis have been elucidated, but as yet there is no clarity concerning the intracellular pathways operating for different classes of proteins under varying physiological conditions.

The experimental model system chosen for smooth muscle tissue was cultured foetal bovine aortic cells. Generally, cultured cells offer great advantages for experimental manipulation over whole animal or whole muscle studies. However, the main advantage of this model system lay in the fact that the degradation of an exogenous protein, namely the apoprotein B moiety of low density lipoprotein, by these cells had been extensively studied and could thus be used as a convenient system for comparison to the degradative mechanisms operating on intracellular proteins. Apoprotein B is known to be hydrolyzed intralysosomally following its uptake into cells by a receptor-mediated endocytotic pathway (Goldstein and Brown, 1977), and its degradation is inhibited by general inhibitors of lysosomal function such as chloroquine and ammonia.

In order to investigate the effect of various proteinase inhibitors on intracellular proteolysis, it was necessary to establish a rapid and accurate method for measuring proteolysis rates in these cell cultures by choosing an appropriate amino acid for radioactive labelling of intracellular protein, i.e.

one that is not metabolized by the cells, and measuring the percentage of that amino acid released per hour from pre-labelled protein under conditions which minimize intracellular re-utilization of the amino acid. This approach was then adapted to characterize the proteolysis rates of long-lived, short-lived and "abnormal" polypeptides in these cells. In addition, similar studies were undertaken using cultured L8 cells as a model system for skeletal muscle.

The effect of lysosomotropic inhibitors on the degradation of labelled endogenous protein was then measured in order to illustrate any possible involvement of lysosomes in the turn-over of short half-life and long half-life proteins. Since apoprotein-B degradation is known to be intralysosomal, the effectiveness of the inhibitors used within the degradation periods chosen (which in some cases are necessarily short) could be effectively analyzed. However, an important pre-requisite for this type of comparison was to show that the presence of the inhibitors only affectedthe degradation of apoprotein B and not the receptor-mediated endocytosis. It was thus necessary to measure the binding, uptake and degra-dation of ^{125}I-apo-B under all experimental conditions.

Secondly, a characterization of protein turnover rates in relation to molecular structure was carried out using both smooth and skeletal muscle cells in culture. The double-isotope technique of Arias _et al_. (1969) was used to label intracellular proteins, and their relative rates of degradation were measured after fractionation by SDS-electrophoresis and

affinity chromatography. The approach was to characterize degradation processes under different nutritional environments in order to gain an understanding of the mechanisms operating under these conditions.

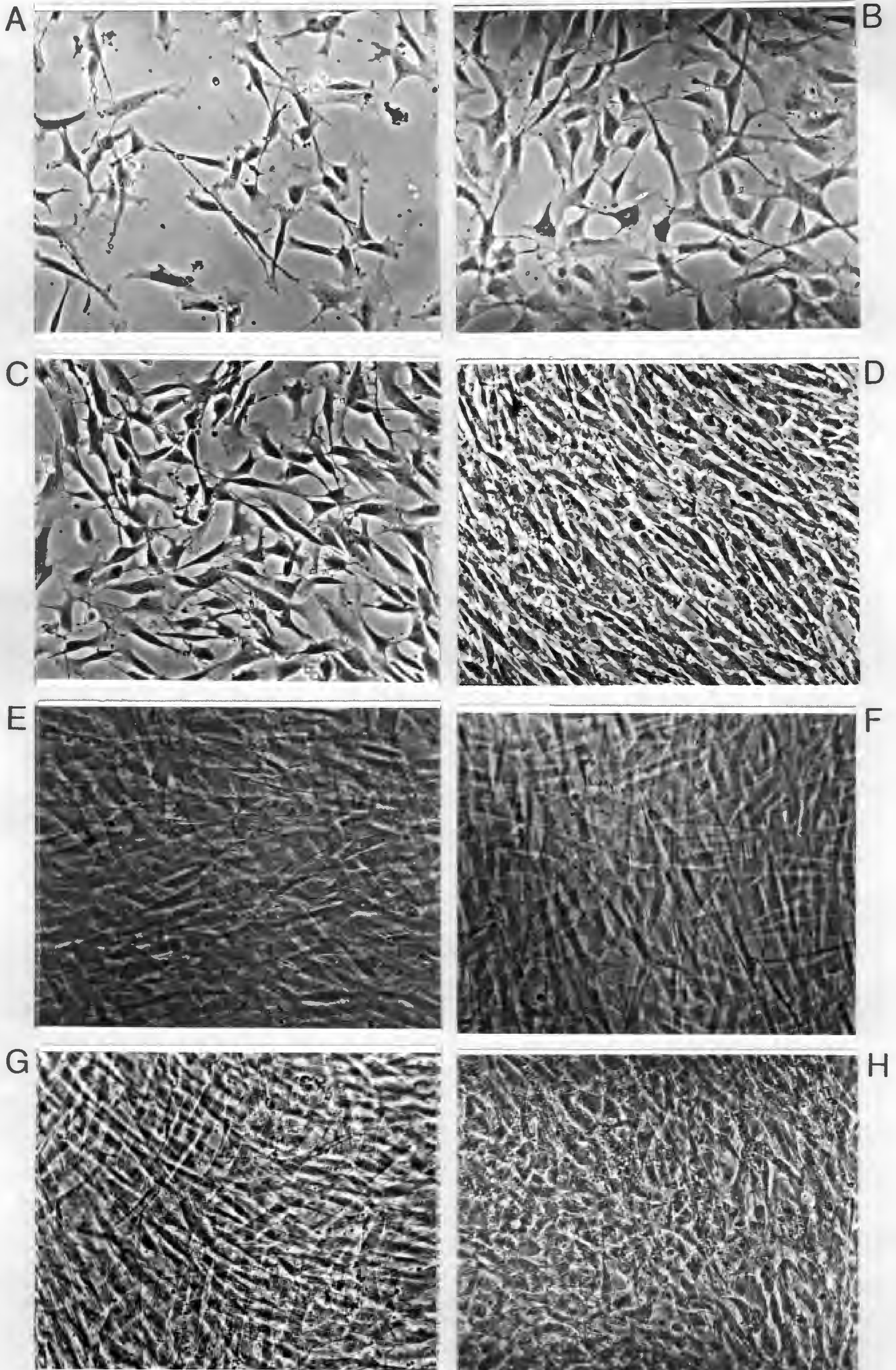
EXPERIMENTAL PROCEDURES

1. CELL CULTURE

1.1. Smooth Muscle Cells

Smooth muscle cells were obtained from an uncloned mass culture isolated from foetal bovine aortic medial explants. The cultured cells exhibited the characteristic morphology of modulated smooth muscle cells under electron microscopy (Chamley-Campbell *et al.*, 1979), namely an abundance of thin filament bundles containing many dense bodies and a large number of pinocytotic vesicles below the plasma membrane, and showed prominent bands of myofibrillar proteins when cell extracts were run on SDS polyacrylamide separating gels. The cells grow to form a thick multilayer with characteristic "hills and valleys" on the plastic culture dishes visible under a light microscope. Cells were cultured in Eagle's Minimum Essential Medium (MEM) buffered with Earle's salts and containing 10% tryptose phosphate broth, 10% heat-inactivated foetal calf serum, 60 μg/ml of penicillin G and 100 μg/ml streptomycin sulphate. The cells were maintained in a humidified incubator (5% CO_2) at 37°C in 75 cm^2 stock flasks (Corningware) containing 10 ml of growth medium. Confluent monolayers of cells from stock flasks were dissociated with 0,05% trypsin - 0,02% EDTA solution and 10^5 cells were seeded into 35 mm petri-dishes containing 2 ml of growth medium, which was replaced every three days. Cells were used between fifth and tenth passages in culture, and all experiments were initiated on post-confluent cultures grown for 6-8 days.

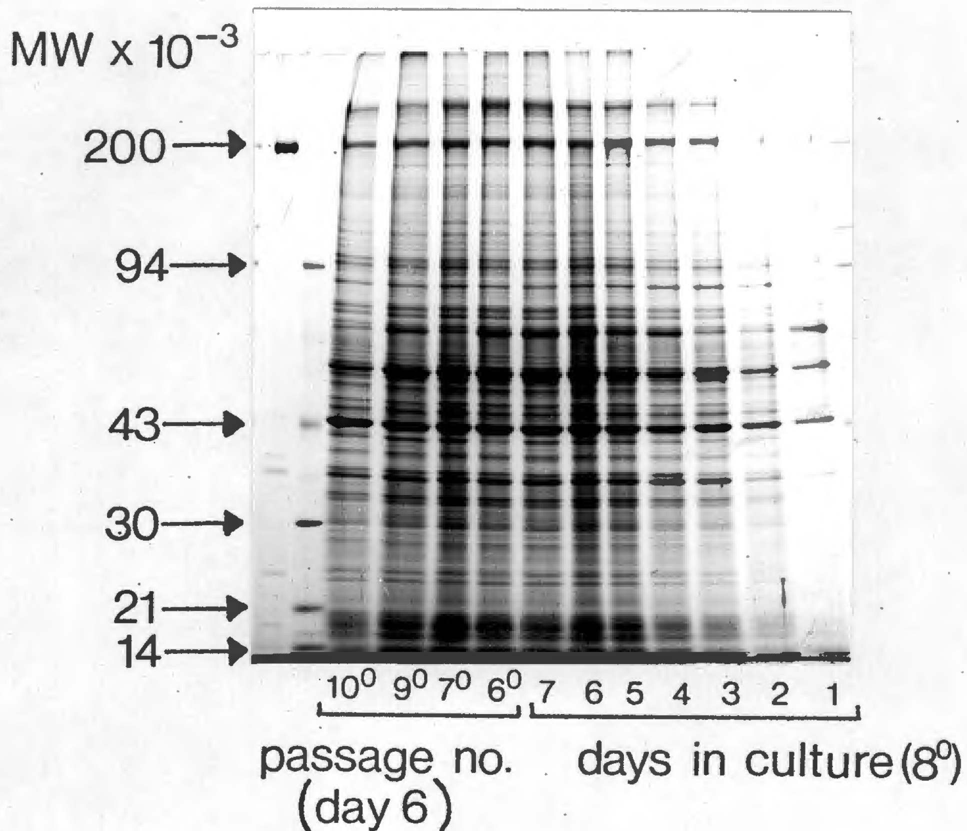
SMOOTH MUSCLE CELLS



KEY TO PHASE CONTRAST MICROGRAPHS OF BOVINE AORTIC SMOOTH MUSCLE CELLS IN CULTURE

(Magnification 600x)

A: 24 hours in culture (Day 1) Cells were seeded at 10^5 per 35 mm culture dish.
B: Day 2, Rapid proliferation to confluence
C: Day 3, subconfluent layer
D: Day 4, Cells form confluent monolayer
E: Day 5, further proliferation producing multilayers
F: Day 6, several layers clearly visible.
G: Day 7, thick multilayer with characteristic "hills and valleys". Experiments initiated at this stage in culture.
H: Postconfluent layers exposed to 70 μM chloroquine for 4 hours, showing extensive vacuolation arising from lysosomes.



SDS-polyacrylamide gel analysis of smooth muscle cell proteins according to passage number and days in culture. Cell layers were harvested and run on 7-14% acrylamide gradient gels as described under "Experimental Procedures, 13". Molecular Weight standards included were:- myosin heavy chain (200 000), phosphorylase B (94 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000) and lysozyme (14 300).

1.2. Skeletal Muscle Cells

The established myoblast cell line L8 was originally isolated from rat skeletal muscle (Richler & Yaffe, 1970; Yaffe & Saxel, 1977). Cells thawed from storage in liquid nitrogen were grown to confluence in 75 cm^2 stock flasks in a 4:1 mixture of Dulbecco's Modified Eagle's Medium and Medium 199 containing 10% horse serum, 1% chick embryo extract (Yaffe, 1971), 60 µg/ml of penicillin G and 100 µg/ml streptomycin sulphate. Cells were grown at 37°C in a humidified incubator with 5% CO_2 in air. After myoblast proliferation to confluence in the stock flask, the cells were dissociated with Trypsin-EDTA solution and seeded at 10^5 cells per 35 mm petri-dish containing 2 ml of growth medium which was changed every three days. Once confluent, the cells progressively fused to form large multinucleate syncytia (myotubes) which synthesized muscle-specific proteins and contracted spontaneously after 7-8 days' growth. Cells were used between fourth and tenth passage in culture, and all experiments were initiated on dishes showing at least 80% of nuclei in myotubes, i.e. 80% fusion (7-8 days' growth). Cell numbers and percentage fusion were determined by counting nuclei in cultures fixed in methanol and stained with 0,25% May-Grunwald for 10 minutes followed by 10% Giemsa for 20 minutes.

2. MEASUREMENT OF TOTAL DNA AND PROTEIN

Total DNA was measured by the method of Burton (1956). Skeletal muscle cells were harvested from the dish by scraping into phosphate-buffered saline (PBS) with a rubber policeman, the

cell pellet was spun down and stored at -20°C until analysis. Cell samples were then homogenized in PBS and an equal volume of 20% trichloroacetic acid was added. After 10 minutes at 4°C, the samples were centrifuged at 2000 rpm for 10 minutes, and the pellets washed once with a 1:1 ethanol/ether mixture and then twice with ether alone. The DNA was then extracted by incubation of each dry pellet with 1 ml 10% perchloric acid for 30 minutes at 70°C, then 1 ml 4% diphenylamine and 50 µl of 0,16% acetaldehyde were added, followed by a further incubation of 1 hour at 56°C. The O.D. values at 595 nm were then determined and compared to a range of standard values obtained using calf thymus DNA dissolved in 5 mM KOH.

Total protein was determined by the method of Lowry _et al_. (1951) using bovine serum albumin as a standard. The cell layers were washed twice with PBS and solubilized in 1 N sodium hydroxide for use in the protein determination.

3. RADIOACTIVE LABELLING OF CELL PROTEINS AND MEASUREMENT OF BASAL INTRACELLULAR PROTEOLYSIS

Cell cultures were grown for 16 hours in growth medium containing 1 µCi/ml of [^{3}H]-phenylalanine. Proteins labelled in this way are referred to as "long-lived" proteins, as this labelling period, followed by the washing procedure outlined below, results in the preferential labelling of proteins having relatively long half-lives. After 16 hours, the radioactive medium was removed and the cell layers were washed rapidly four times with ice-cold medium in order to remove all extracellular [^{3}H]-phenylalanine.

In order to reduce the large intracellular acid-soluble pool of radioactivity to an acceptable background level before the net release of radioactivity from intracellular labelled protein can be measured, the cells were then incubated under conditions allowing carrier-mediated transport of [^{3}H]-phenylalanine out of the cells. A series of four consecutive 30-minute incubations at 37°C, adding 1 ml fresh growth medium after each one, were performed to wash out intracellular soluble radioactivity. Subsequently, 1 ml of fresh chase medium containing 1 mM excess non-labelled phenylalanine was added to the cell layers and the release of labelled amino acid into this medium was used to measure intracellular proteolysis. Cells were incubated in this medium at 37°C for the indicated time intervals, after which the medium was withdrawn and assayed for trichloroacetic acid-soluble radioactivity by precipitation with an equal volume of 20% TCA for an hour at 0°C. The cell layers were washed twice with PBS, 1 ml 1% sodium dodecyl sulphate (SDS) was added to solubilize the cells, which were then removed from the culture dish using a rubber policeman. The SDS lysate was briefly sonicated, a 500 µl aliquot was counted to determine the total radioactivity within the cells and the remaining 500 µl was supplemented with 1 mg albumin as carrier, precipitated with an equal volume of 20% TCA at 0°C for 1 hour and then spun at 2000 rpm for 15 minutes. The supernatant was counted to obtain the residual amount of intracellular TCA-soluble radioactivity. All countings were performed in a Beckman scintillation counter with Instagel solubilizer. All samples were initially checked for chemiluminescense by recounting after 24 hours storage in the dark,

but this was never found to be significant due to the acidic nature of the samples. Intracellular protein degradation in each case was expressed as the percentage of initial total protein radioactivity that was released into the chase medium, i.e.

$$\% \text{ Proteolysis} = \frac{\text{dpm } [^3H]\text{-phe in TCA-soluble medium}}{\text{dpm in medium + dpm TCA-ppt. cell fraction}}.$$

4. ACCELERATED INTRACELLULAR PROTEOLYSIS

Cells were grown as described and labelled for 16 hours as for basal proteolysis, but during the 2 hour step-wise extraction incubations and the following chase, the cell layers were incubated in a medium which contained no foetal calf serum or tryptose phosphate broth (minimal medium), i.e. nutritional step-down conditions. The proteolysis rate ("accelerated proteolysis") was measured in the same way as described above except that 1 mg albumin was added to the medium samples before assaying for TCA-soluble radioactivity.

5. PROTEOLYSIS OF RAPIDLY-DEGRADED PROTEINS

In this case, the cell layers were pre-labelled with 5 μCi/ml $[^3H]$-phenylalanine for one hour in order to preferentially incorporate label into "short-lived" proteins. The cells were washed rapidly four times with ice-cold medium as described above, and then subjected to 4 consecutive 15-minute incubations, each with fresh medium, at 37°C to reduce the intracellular acid-soluble radioactivity. Chase medium containing 1 mM excess non-labelled phenylalanine was then added to the cell layers and proteolysis measurements during a further hour incubation at 37°C were carried out as described above.

The accelerated degradation rate of "short-lived" proteins was also measured in a poor nutritional environment, i.e. in minimal medium. In this case, the cell layers were incubated in minimal medium for the hour of the four pre-chase extraction incubations, and then proteolysis measurements were made at the end of the following hour incubation at 37°C in minimal medium containing 1 mM excess non-labelled phenylalanine.

The degradation of "abnormal" proteins was studied by incubating the cell layers for 3 hours in the presence of 2 µCi/ml [^{3}H]-phenylalanine in the presence or absence of an arginine analogue, L-canavanine (added at a concentration of 10 mM in the extracellular medium). The cells were washed with ice-cold medium, subjected to four fifteen-minute extraction incubations at 37°C and then the subsequent proteolysis rate was measured in fresh chase medium containing 1 mM excess non-labelled phenylalanine after a further hour of incubation.

6. INHIBITION OF PROTEOLYSIS

A series of proteolytic and lysosomotropic inhibitors have been used to study the pathways of intracellular protein degradation. In most cases, the cell layers were pre-treated with the inhibitor during the 2-hour serial extraction incubations at 37°C prior to the addition of chase medium also containing the inhibitor at the concentration indicated. In the case of rapidly-degraded proteins, the pre-treatment time with inhibitor was 1 hour to coincide with the four 15-minute extraction incubations performed in these experiments. Leupeptin (Protein

Research Foundation, Osaka, Japan) was routinely used at a concentration of 100 μg/ml in the growth medium and was stored at $4^{o}C$ as a 4 mg/ml stock solution which retained full activity over a period of several months. Chloroquine (Sigma Chemical Company) was used at a final concentration of 70 μM and was stored frozen in 2 ml aliquots as a 2 mM solution in minimal medium. This inhibitor was temperature- and light-sensitive so a fresh frozen sample was used each day, and then discarded to ensure no loss of activity. Ammonium chloride (Merck) was stored at $4^{o}C$ as a 2 M solution in phosphate-buffered saline made freshly each week and used at a concentration of 10 mM in the growth medium to inhibit proteolysis. Chymostatin (Protein Research Foundation, Japan) and Pepstatin A (Sigma) were both dissolved in dimethyl sulphoxide (DMSO), stored at $4^{o}C$ for several weeks as 4 mg/ml stock solutions and were both used at a concentration of 100 μg/ml in the degradation medium. In each case where these two latter inhibitors were used, a control value of degradation was determined with DMSO alone in the incubation medium.

Two chloromethyl-ketone protease inhibitors, N-α-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK) and N-α-Tosyl-L-lysyl chloromethyl ketone (TLCK) (both obtained from Sigma), were also used to inhibit protein degradation and these were made up freshly each day due to their high instability in solution, e.g. TLCK has a half-life of only a few hours in aqueous solution (Shaw et al., 1965). TPCK and TLCK were dissolved in PBS and used at a level of 100 μg/ml in the growth medium unless otherwise indicated.

7. ESTIMATION OF RADIOACTIVITY IN PHENYLALANINE IN MUSCLE CELL PROTEIN

Phenylalanine is known not to be metabolized in cardiac or skeletal muscle (Morgan *et al.*, 1971) and phenylalanine hydroxylase has been reported to be lacking in muscle tissue (Udenfriend and Cooper, 1952). In order to show that there is no conversion of phenylalanine into other amino acids or by-products in the smooth muscle cell layers used in this study, cell layers were exposed to 1 μCi/ml [^{3}H]-phenylalanine for 16 hours, washed in PBS and then harvested. Cells were prepared for total amino acid analysis by hydrolysis in 6 N HCl in a boiling water bath for 2 hours. The hydrolyzed extract was then lyophilized and the residue dissolved in lithium citrate buffer pH 2.2, to a final concentration of 125 μM, for amino acid analysis, using a Beckman Amino Acid Analyzer. Fractions were collected directly from the analyzer column into scintillation vials, counted, and the radioactivity appearing in the elution position corresponding to phenylalanine was expressed as a percentage of the total counts obtained from all cell fractions.

8. LIPOPROTEINS

Human low density lipoprotein (density = 1,019 - 1,063 g/ml) and lipoprotein-deficient serum (density > 1,25 g/ml) were prepared from the plasma of healthy subjects by differential ultracentrifugation (Havel *et al.*, 1955). The lipoproteins were washed at the same density and dialyzed exhaustively against 0,9% NaCl - 0,01% EDTA solution, pH 7.4. The

concentration of low density lipoprotein (LDL) was expressed in terms of its protein content.

9. IODINATION OF HUMAN LOW DENSITY LIPOPROTEIN

Iodination of LDL was by a modified iodine monochloride method (Bilheimer *et al.*, 1972). A 4,2 mg/ml solution of iodine monochloride (ICl) was diluted 1:5 with 2 M NaCl and 0,4 ml of this solution was syringed with a quick, forceful action into a mixture containing 10 mg LDL, sufficient 2 M glycine to give a final concentration of 0,4 M, and the radioactive iodine (^{125}I). The resulting solution was dialyzed for 24 hours at $4^{o}C$ against five litres of 0,9% NaCl - 0,01% EDTA, pH 7.0, which was changed four times in the 24 hour period. The final protein concentration was calculated by the method of Lowry, using bovine serum albumin as a standard, and the specific activity of the iodinated LDL was expressed as cpm per nanogram of protein. Values obtained were usually between 100 - 300 cpm/ng protein. When desired, the ^{125}I-LDL was diluted with unlabelled LDL.

During the above procedure, all iodinated solutions were kept on ice and all operations were carried out under sterile conditions.

10. MEASUREMENT OF SURFACE-BOUND ^{125}I-LDL, INTRACELLULAR ^{125}I-LDL AND DEGRADED ^{125}I-LDL IN SMOOTH MUSCLE CELL LAYERS

Smooth muscle cell multilayers in 35 mm culture dishes were incubated with 20 µg ^{125}I-LDL in 1 ml of growth medium (Eagle's

Minimum Essential medium containing lipoprotein-deficient serum (LPDS) at a final concentration of 5 mg/ml, instead of foetal calf serum. After the indicated time intervals, the medium was removed, treated with 0,3 ml 50% trichloroacetic acid (TCA) to give a final concentration of 10% TCA, left for one hour at $0^{\circ}C$ and spun at 2000 rpm for 15 minutes. 1 ml of the acid-soluble fraction was treated with 20 µl of 40% potassium iodide, 50 µl of hydrogen peroxide and then extracted with 2 ml of chloroform to remove free iodine (Bierman, Stein and Stein, 1974). An aliquot of the resultant aqueous phase was counted in a Packard gamma counter to determine the amount of ^{125}I-labelled acid-soluble material released by the smooth muscle cells into the extracellular medium, most of which is in the form of ^{125}I-monoiodotyrosine (Goldstein and Brown, 1974). A blank value, due to the presence of small amounts of acid-soluble material in the ^{125}I-LDL preparation, was routinely determined at the appropriate LDL concentration by simultaneous incubation of the ^{125}I-LDL at $37^{\circ}C$ in the lipoprotein-deficient medium containing no cells.

To determine the amount of ^{125}I-LDL bound to the cell surface and the amount that had entered the cells, each dish of cells was rapidly chilled to $4^{\circ}C$ to stop the uptake process. After removal of the radioactive medium, the cell layers were washed four times with 2 ml phosphate-buffered saline (PBS) containing 0,2% albumin, followed by four washes with PBS alone to remove any non-specifically bound ^{125}I-LDL. 2 ml of an incubation medium containing 4 mg/ml sodium dextran sulphate, 50 mM NaCl buffered with 10 mM HEPES pH 7.4, was then added to each dish

and incubated for one hour at $4^{o}C$. The dextran sulphate medium was removed and counted to determine the amount of ^{125}I-LDL that had been bound to the cell surface and released by dextran sulphate into the medium (Goldstein and Brown, 1977). The cell layers were then scraped off the dish into 50% methanol using a rubber policeman, extracted with a 40/60% chloroform/methanol mixture, and spun at 2000 rpm for 20 minutes. 1 ml of 1 N NaOH was added to solubilize each dry pellet. The NaOH solution was incubated overnight at $37^{o}C$ and then counted to determine the total amount of ^{125}I-LDL present in the cells. An aliquot of this NaOH solution was also analyzed for protein concentration by the method of Lowry, using bovine serum albumin as a standard.

11. INHIBITION OF ^{125}I-LDL DEGRADATION

The same series of proteolytic inhibitors used to study the degradation of endogenous proteins, namely leupeptin, chloroquine, ammonium chloride, chymostatin, pepstatin A, TLCK and TPCK, was tested on the degradation of endocytosed ^{125}I-LDL in order to evaluate their effectiveness against the lysosomal pathway of protein degradation. In each case, the various inhibitors were used at the same concentrations as employed for endogenous proteolysis and were present during the incubation period with ^{125}I-LDL, and also during the 2 hr period preceding the addition of ^{125}I-LDL. Experiments measuring LDL-degradation and intracellular proteolysis rates were always run concurrently in each experiment, using the same batch of cells and the same stock solutions of inhibitors.

To coincide with experiments measuring accelerated intracellular proteolysis, duplicate cell layers were first incubated in a minimal medium in the presence or absence of inhibitors for 2 hours, then 20 μg/ml ^{125}I-LDL was added for a further 4 hours whilst maintaining the cells on the minimal medium.

To compare ^{125}I-LDL degradation with the degradation of "short-lived" intracellular proteins, ^{125}I-LDL in LPDS-containing growth medium was initially incubated with duplicate cell layers for 2 hours, the inhibitors were then added for the pre-incubation wash period of one hour, then fresh ^{125}I-LDL and inhibitors were added to the dish for a further 1 hour incubation before analysis of ^{125}I-LDL degradation.

12. DOUBLE-ISOTOPE LABELLING OF INTRACELLULAR PROTEINS

12.1. Outline of Double-Isotope Method

Relative degradation rates of intracellular proteins were compared by the double-isotope technique first described by Arias *et al.* (1969), and subsequently modified by Dice and Walker (1978), to enable the technique to be applied to animals undergoing physiological changes.

In the double-label procedure, two isotopic forms of an amino acid, in this case [^{3}H]- and [^{14}C]-phenylalanine, are used to establish two time points on the curve describing the degradation of any protein. This method bypasses the need for measurements of specific radioactivities of the precursor and product. The consecutive administration of two radioisomers

of an amino acid allows one to compare the turnover of several proteins having the same intracellular origin. The rationale of the experimental protocol is shown schematically in Figure 1. Tracer administration is chosen so that the cells are exposed to the first isotope (^{14}C-phenylalanine) over the time necessary for the protein specific radioactivity to pass the cross-over point, and should thus reflect the descending limb of the curve representing protein specific radioactivity. The second isotope (^{3}H-phenylalanine) is administered just before the experiment is terminated so that this reflects the ascending limb of the protein specific radioactivity. By expressing the incorporation data as a ratio of the activity of the two isotopes, one can obtain a more sensitive index of relative rates of protein turnover without measurements of protein specific activity, than by comparing the two levels of labelling with one isotope only. The ^{3}H to ^{14}C ratio of a protein fraction therefore reflects its turnover rate. When originally described, this technique was only applicable to animals under "steady-state" conditions. The major modification of the experimental design by Dice _et al_. (1978) involved using two animals instead of one, in order to apply the procedure to animals undergoing physiological change. In this investigation, I have followed the same experimental approach as Dice _et al_. (1978), and have employed separate culture dishes in order to compare the turnover rates of proteins of cultured muscle cells exposed to various environmental conditions. As an example, in an experiment to study relative turnover rates in basal and nutritional "step-down"

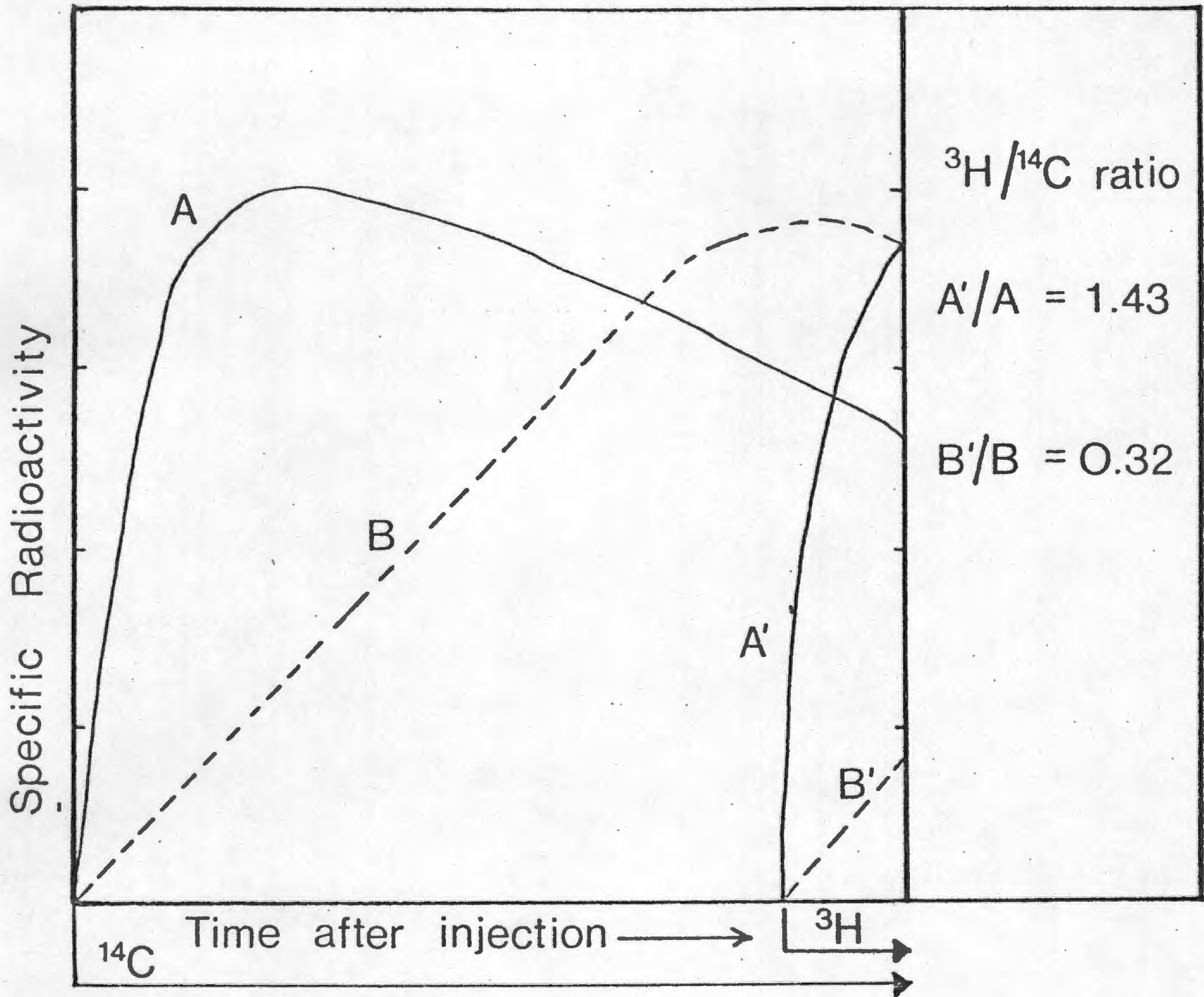


FIGURE 1. SCHEMATIC REPRESENTATION OF PRINCIPLE OF DOUBLE-ISOTOPE METHOD.

Injection of one radioisomer of a given amino acid is followed by administration of a second radioisomer at some later interval. Experiment is terminated shortly afterwards and the ratio of the 2 radioisomers is determined in 2 isolated proteins, A and B. (Adapted from Zak *et al.*, 1979).

conditions, two dishes of cells were labelled with [^{14}C]-phenylalanine for a period of 16 hours, and the radioactive medium was then removed. The chase medium subsequently added was complete growth medium for one dish and minimal medium alone for the other. After a chase period of 24 hours, [^{3}H]-phenylalanine was added to two new cell dishes (which had been maintained continuously under normal conditions of growth) for a further two hours, then all four dishes were harvested. The cells of each of the [^{14}C]-labelled dishes were pooled with the cells of one of the [^{3}H]-labelled dishes to yield the appropriate "basal" and "step-down" groups, and the proteins of each group were then analyzed for the two isotopes.

According to certain assumptions discussed by Dice and Walker (1978), the ^{14}C in a protein fraction was assumed to reflect the amount of degradation during the 24 hour chase in complete or minimal medium, while the ^{3}H was taken to reflect an initial point on the curve. Therefore, proteins that are degraded rapidly had high ^{3}H:^{14}C ratios. A control experiment was also run using proteins prepared from muscle cells which had been labelled with both [^{14}C]-phenylalanine and [^{3}H]-phenylalanine for 16 hours, pooled and analyzed in the same manner.

The usefulness of the double-isotope method for discrimination between protein half-lives depends to a large extent on the injection schedule for the two isotopes (Zak _et al_., 1979) and to a lesser extent on the magnitude of the protein half-life. Some general statements about the technique can be made:-

1) The isotope ratio for a given labelling period decreases

rapidly with increasing protein half-life. However, although the sensitivity of the method decreases, the ratios can still indicate nonidentity even among proteins turning over at relatively slow rates.

2) The length of exposure to the first isotope should be selected so that all proteins being compared are in the phase of declining specific radioactivity.

3) The most important factor determining the sensitivity of the double-isotope method is the time of exposure to the second isotope. The longer the exposure, the lower the sensitivity of the protocol.

4) Due to the complexity of protein-labelling kinetics, there is no single injection schedule by which the double-isotope method is able to adequately discriminate between proteins over the full range of protein half-lives.

In some experiments, therefore, the labelling protocol was modified in an attempt to preferentially label those proteins having either relatively short or long half-lives, and to increase the sensitivity of the procedure for these groups. In each case, the exposure to the second isotope, [^{3}H]-phenylalanine was 2 hours, but the exposure to [^{14}C]-phenylalanine was altered to 8 hours, followed by a 12 hour chase period for one experiment, and to 30 hours, followed by a 24 hour chase period, for another.

12.2. Double-labelling of Proteins in Cultured Cells

Muscle cell layers were incubated in the presence of 3 µCi/ml

$[^{14}C]$-phenylalanine for 16 hours. The radioactive medium was then removed, the cells washed three times with sterile PBS and 2 mls of fresh medium added, containing 1 mM excess non-radioactive phenylalanine to minimize any reutilization of released radioactive phenylalanine. Half the cell cultures received complete growth medium for the following 24-hour chase and the others received minimal medium. After this 24-hour period had elapsed, 10 μCi/ml of $[^{3}H]$-phenylalanine was added to a corresponding number of new culture dishes for 2 hours, after which all the dishes were harvested. Other labelling protocols used will be outlined in the appropriate legends to figures in Chapter 2 of "Results". In some experiments, the length of exposure to 3 μCi/ml $[^{14}C]$-phenylalanine was changed to 8 hours or 30 hours, and the chase time to 12 hours and 24 hours, respectively, but the length of exposure to 10 μCi/ml $[^{3}H]$-phenylalanine was always 2 hours.

12.3. <u>Preparation of Double-labelled Proteins</u>

Cell layers were washed several times with phosphate-buffered saline. For the preparation of total cellular protein, cells were harvested into sodium dodecyl sulphate "sample buffer" (see Methods, section 14) in preparation for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoretic analysis.

To prepare soluble cytosolic proteins, the cell layers were washed as before with PBS, harvested by scraping with a rubber policeman into PBS, and the cell pellet obtained by centrifugation was kept frozen at -20°C until analysis. The $[^{3}H]$- and $[^{14}C]$-labelled cells were mixed and 1,5 volumes of hypotonic

medium (0,01 M Tris buffer, pH 7.35; 0,1 mM EDTA) were added. Soluble proteins were prepared by homogenizing the cells with 20 strokes of a tight-fitting plunger in a 1 ml Dounce homogenizer on ice. The homogenate was made isotonic by adding exactly 0,1 volumes of hypertonic medium (0,11 M Tris buffer pH 7.35, 1,43 M NaCl, 0,11 M KCl and 0,033 M $MgCl_2$) and the nuclei were removed by centrifugation at 2000 g for 10 minutes. The resulting supernatant was centrifuged at 100000 g for 2 hours in an airfuge (Beckman), the soluble protein collected and kept frozen at -20°C. A sample of soluble protein was precipitated with sufficient 50% trichloroacetic acid to give a final concentration of 10% TCA, and the pellet was resuspended in SDS "sample buffer" prior to electrophoresis.

13. SDS POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis was carried out in 110 mm x 150 mm x 2 mm slabs after the method of Laemmli (1970), using a linear 7-14% acrylamide gradient. Large width wells (8 mm) were moulded into the gel to ensure that enough protein could be applied to obtain sufficient radioactivity to count ^{3}H and ^{14}C accurately in each protein fraction.

Gel solutions containing 5% (stacking gel), 7% and 14% acrylamide were prepared from a stock solution of 30% (w/v) acrylamide and 0,8% (w/v) N,N'-bis-methylene acrylamide. The gels were polymerized chemically by the addition of 0,025% (v/v) tetramethylethylenediamine (TEMED) and 0,5 mg/ml ammonium persulphate. Final solutions also contained 0,375 M Tris-HCl, pH 8.8 and 0,1% SDS. The ammonium persulphate was prepared fresh daily

(50 mg/ml) and the solid was kept dessicated in small aliquots at room temperature. After polymerization of the separation gel (about 40 minutes), the stacking gel of 5% acrylamide containing 0,125 M Tris-HCl pH 6.8, 0,1% SDS was polymerized in the same way as the separating gel, with the well comb in place for about 20 minutes. The electrode buffer (pH 8.3) contained 0,025 Tris, 0,192 M glycine and 0,1% SDS. Protein samples were prepared in "sample buffer" containing final concentrations of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol. Prior to electrophoresis, β-mercaptoethanol was added to the samples (final concentration of 5%), and each sample was immersed in a boiling water bath for three minutes. Up to 50 µg of protein in a maximum volume of 80 µl could be applied to the large well, using a Hamilton syringe. Bromophenol blue was added to some wells as the tracking dye. Electrophoresis was carried out using a current of 25 mA per slab for the first hour, then at 30 mA until the tracking dye reached the bottom of the gel (about 4 hours). The gel was then removed from the chamber, stained overnight at room temperature with 0,025% Coomassie brilliant blue solution made up freshly in 25% isopropanol and 10% acetic acid, restained for 3-4 hours in fresh stain and then destained by repeated washing in 10% acetic acid.

Myosin and "Low Molecular Weight" standards obtained from Bio-Rad were routinely used as molecular weight markers.

14. COUNTING OF DOUBLE-LABELLED SEPARATED PROTEINS

Destained gels were sliced evenly into 2 mm sections, and slices were then incubated overnight at 45°C in 300 µl of 30% (v/v)

hydrogen peroxide to dissolve the acrylamide gel. 5 ml of Instagel scintillation fluid (Packard) was added to each vial before counting. Ratios were not plotted for fractions that contained less than ten times the background count for either isotope, i.e. less than 80 dpm for ^{14}C and less than 320 dpm for ^{3}H.

15. SEPARATION OF GLYCOPROTEINS FROM NON-GLYCOPROTEINS

Glycoproteins were isolated by affinity chromatography using concanavalin A (ConA) linked to agarose (Miles Laboratories). Double-labelled soluble proteins (1-2 mg) were applied to a column containing 2 ml of packed ConA-agarose. The column was then washed with 30 ml of a solution containing 75 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.0), to collect proteins that did not bind to the column. Glycoproteins that bound to the ConA-agarose were eluted with 20 ml of 5% α-methyl-D-mannoside in the salt solution described above. The two protein fractions were precipitated in 10% trichloroacetic acid (final concentration) and their ^{3}H:^{14}C ratios were determined and compared.

RESULTS

CHAPTER 1 : INVESTIGATION OF PATHWAYS OF PROTEIN DEGRADATION

1.1. VALIDATION OF EXPERIMENTAL APPROACH

1.1.1. Pre-labelling of Muscle Protein with ^{3}H-Phenylalanine

Protein degradation can be measured as the release of a radioactive amino acid, i.e. trichloroacetic acid soluble radioactivity, into the medium of cultured cells from prelabelled intracellular proteins. In order for this to be an accurate measure of protein degradation, several conditions must be met. Firstly, the rate of release of TCA-soluble radioactivity from the cells into the medium must be at least as rapid as its rate of release from the protein by degradation. Secondly, conditions must be used which maximally prevent the re-utilization of the released radioactive amino acid.

[^{3}H]-phenylalanine was chosen as a marker for proteolysis since this amino acid is largely non-metabolized in muscle (Manchester, 1965; McGee _et al._, 1972) due to the absence of the enzyme phenylalanine hydroxylase in this tissue (Morgan _et al._, 1971). Figure 1.1 shows the results from a radioactive analysis of fractions collected from an amino acid analyzer after the application of an acid hydrosylate of muscle cells labelled for 16 hours in the presence of [^{3}H]-phenylalanine. This type of experiment was repeated several times for both smooth and skeletal muscle cells. Over 94% of the total radioactivity of the hydrosylate appeared in the phenylalanine peak, showing that it undergoes no conversion into other amino acids.

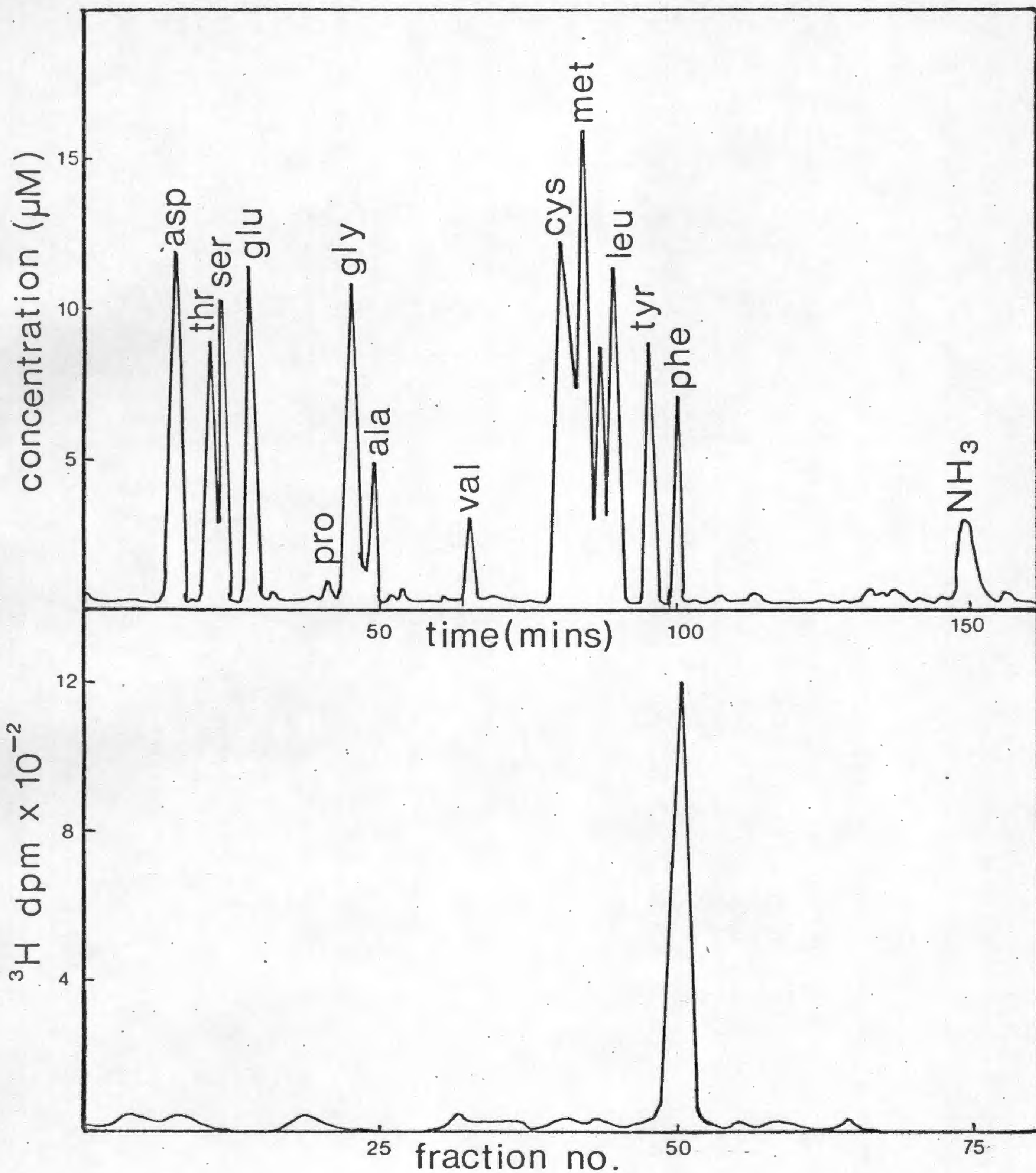


FIGURE 1.1. ESTIMATION OF RADIOACTIVITY OF PHENYLALANINE IN CULTURED MUSCLE CELL PROTEIN.

Smooth muscle cells were labelled with ^{3}H-phenylalanine as described under "Experimental Procedures, 7", the cellular proteins hydrolyzed and analyzed for amino acids and radioactivity. The results shown above are those of a representative experiment using smooth muscle cells.

Furthermore, the presence of chase concentrations of phenylalanine in the extracellular medium was found to have no effect on the release of [^{3}H]-leucine from prelabelled muscle proteins (Figure 1.2.), indicating that phenylalanine itself does not have any direct effect on the rate of degradation of intracellular proteins in this experimental system.

Since one objective of the prelabelling period was to incorporate as much radioactivity as possible into protein during a relatively short time, a high, rather than constant, specific radioactivity of [^{3}H]-phenylalanine, i.e. a tracer dose, was used. Under such conditions, the rate of incorporation decreased markedly during the labelling period (Figure 1.3), presumably due to isotope dilution by unlabelled phenylalanine derived from protein degradation. The decrease in acid-soluble radioactivity (intracellular and extracellular) could be quantitatively accounted for by the corresponding incorporation of radioactivity into protein (intracellular acid-insoluble radioactivity), thus showing that free and protein-incorporated [^{3}H]-phenylalanine was measured with the same efficiency. Similar results to those represented in Figure 1.3. were also obtained using skeletal muscle cells. Therefore, in all subsequent protein degradation measurements, [^{3}H]-phenylalanine release was more conveniently expressed as a percentage of the initial protein radioactivity.

16 hours was chosen as the standard time period for pre-labelling to obtain measurements of the average proteolysis rate of proteins that have been termed "long-lived" proteins (Knowles

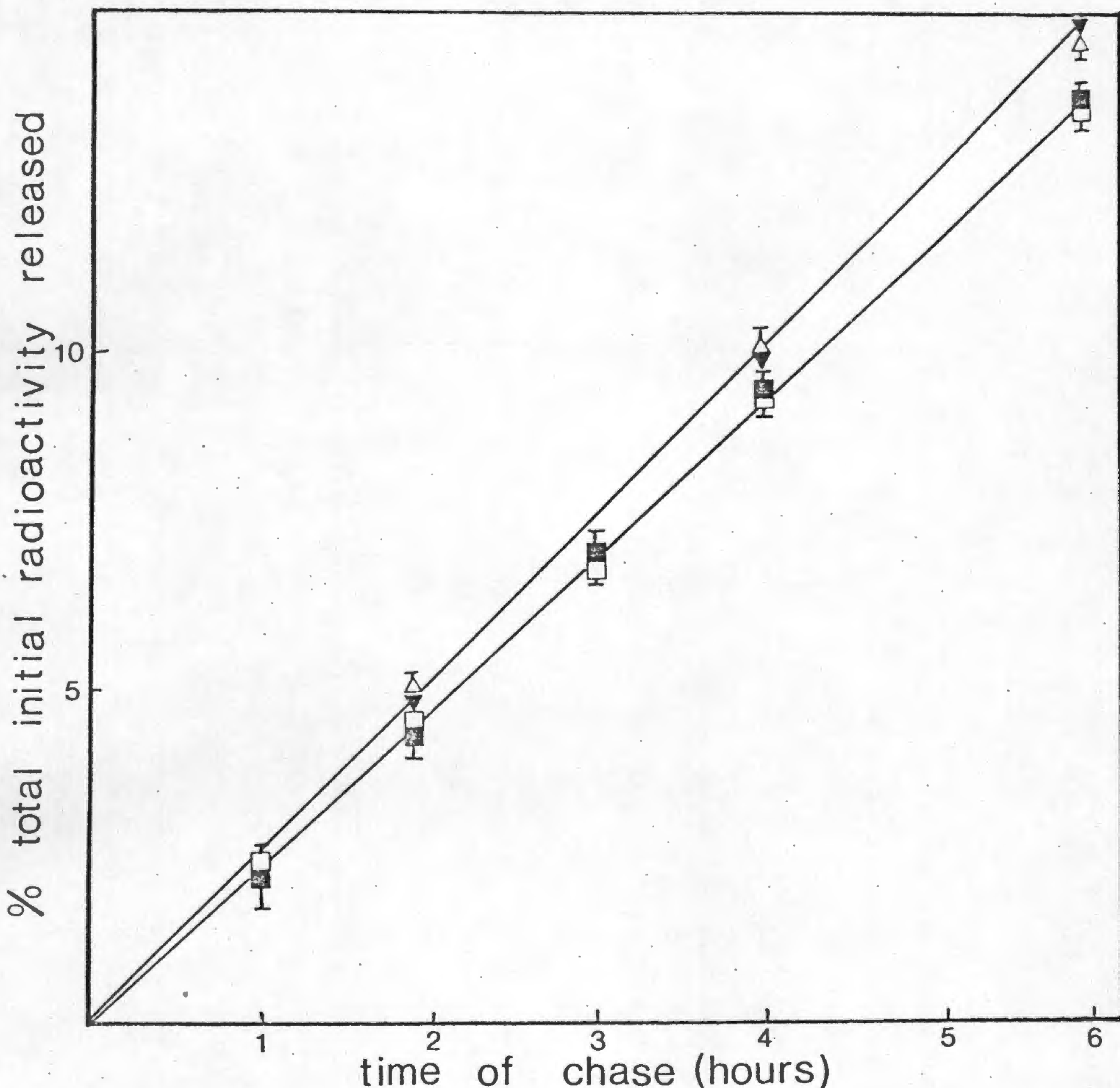


FIGURE 1.2. EFFECT OF MEDIUM PHENYLALANINE ON RELEASE OF ^{3}H-LEUCINE FROM PRELABELLED PROTEINS.

Cells were labelled with 1 μCi/ml [^{3}H]leucine for 16 hours, and percent proteolysis measured at each time as described under "Experimental Procedures, 3". Each value is the mean ± S.E. of three cell dishes.

■——■ Smooth muscle cells, medium containing no excess phenylalanine.

□——□ Smooth muscle cells, medium containing 1 mM excess phenylalanine.

▼——▼ Skeletal muscle cells, no excess phenylalanine.

△——△ Skeletal muscle cells, 1 mM excess phenylalanine.

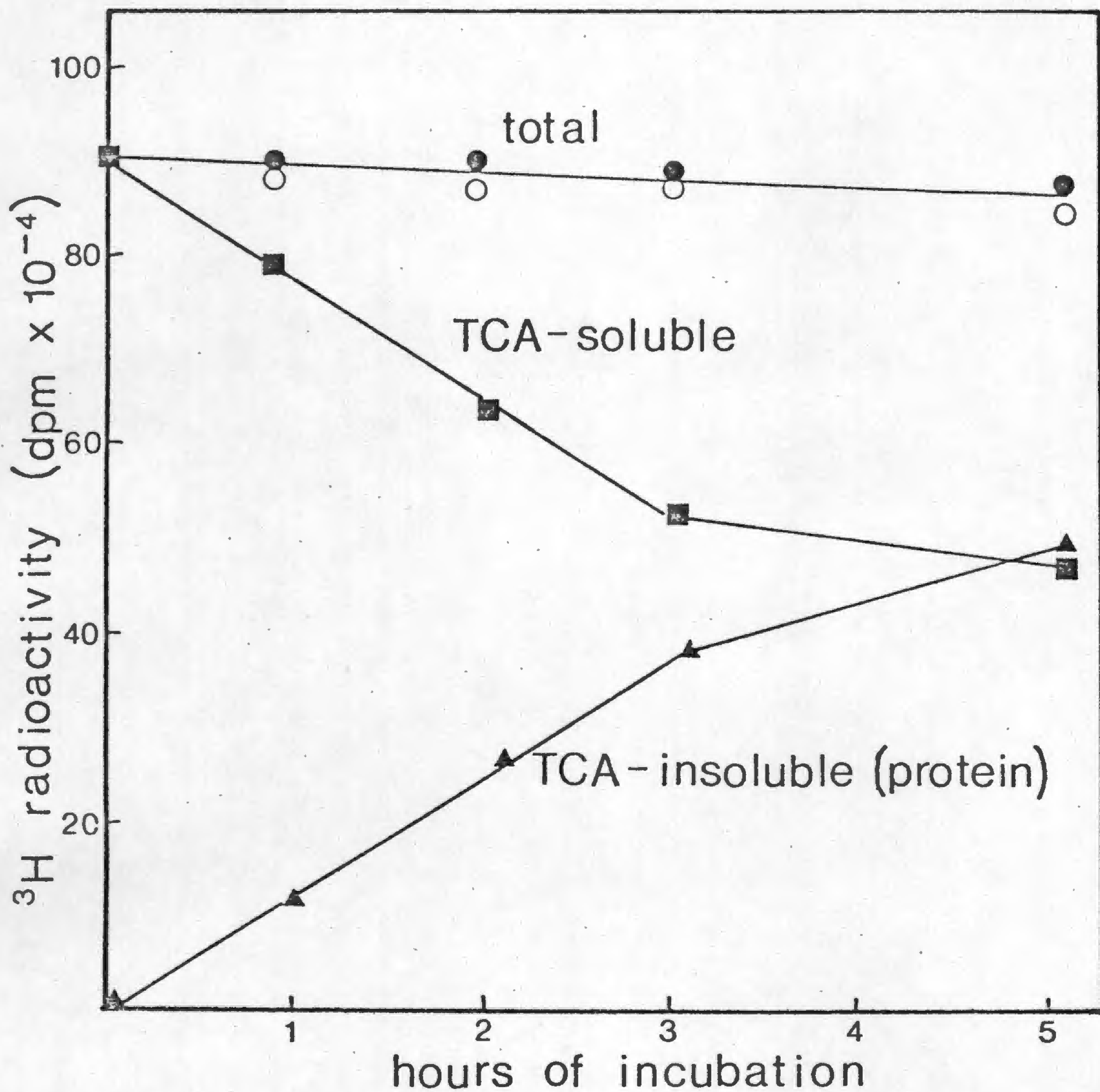


FIGURE 1.3. TIME COURSE OF CONVERSION OF TCA-SOLUBLE ^{3}H-PHENYLALANINE INTO PROTEIN.

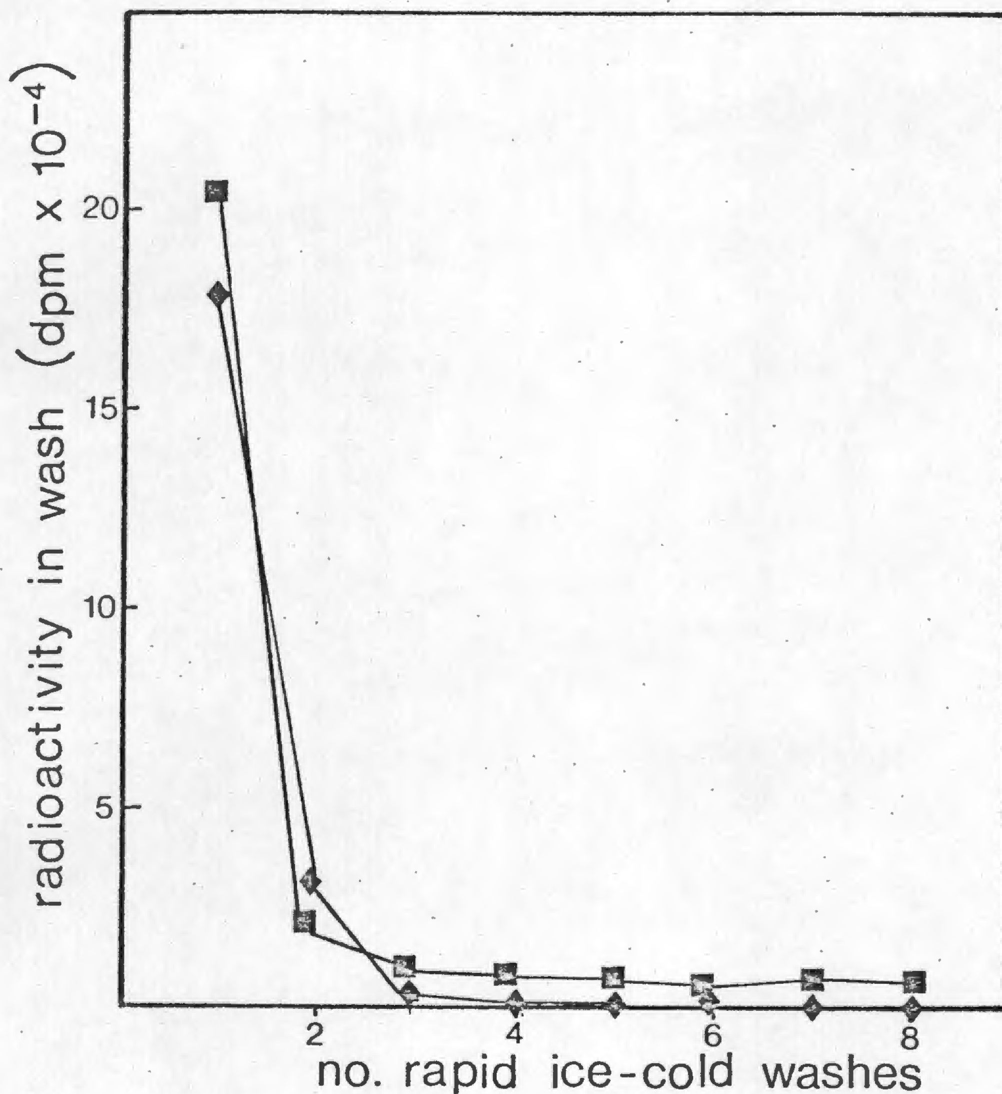
Smooth muscle cell layers were incubated for 5 hours at 37°C in the presence of 1 μCi/ml ^{3}H-phenylalanine. At each time interval, the acid-soluble, acid-insoluble and total radioactivity in the system was measured. Total radioactivity measured separately ●, or as the sum of acid-soluble and acid-insoluble radioactivity ○. Each point is a mean of three cell dishes.

and Ballard, 1976). This period was reduced to 1 hour to obtain measurements for the proteolysis of "short-lived" proteins which are assumed to be preferentially labelled in such a short incorporation period.

1.1.2. Depletion of the Intracellular [^{3}H]-Phenylalanine Pool

In order to remove any free [^{3}H]-phenylalanine at the end of the labelling period, cells were first washed at 4°C with repeated changes of growth medium. Extracellular [^{3}H]-phenylalanine was rapidly removed and Figure 1.4. shows that four such washes were sufficient. However, the intracellular pool could only be depleted under conditions which allowed carrier-mediated transport of [^{3}H]-phenylalanine out of the cells. Phenylalanine transport in muscle cells is fortunately of the facilitated diffusion type, functioning equally well in both directions and thus serves to equilibrate phenylalanine between the intracellular and extracellular compartments. This is illustrated in Figure 1.5., which shows that the intracellular concentration of [^{3}H]-phenylalanine is directly proportional to the extracellular concentration after 30 minutes at 37°C, and also shows that chloroquine and NH_4Cl, two of the inhibitors to be used in most of the subsequent experiments, do not significantly alter this equilibrium distribution or affect the transport rate of phenylalanine across these cell membranes. Similar results were obtained using L8 skeletal muscle cells.

Since phenylalanine transport in muscle cells was found to be relatively rapid, a 30 minute period of incubation at 37°C



<u>FIGURE 1.4.</u> REMOVAL OF EXTRACELLULAR ^{3}H-PHENYLALANINE AFTER LABELLING.

Cell layers were incubated with 1 μCi/ml ^{3}H-phenylalanine for 16 hours, then placed on ice and washed repeatedly with growth medium at 4^oC. The TCA-soluble radioactivity in each wash was measured. Each point is the mean of two cell dishes.

◆—◆ Smooth muscle cells.

■—■ Skeletal muscle cells.

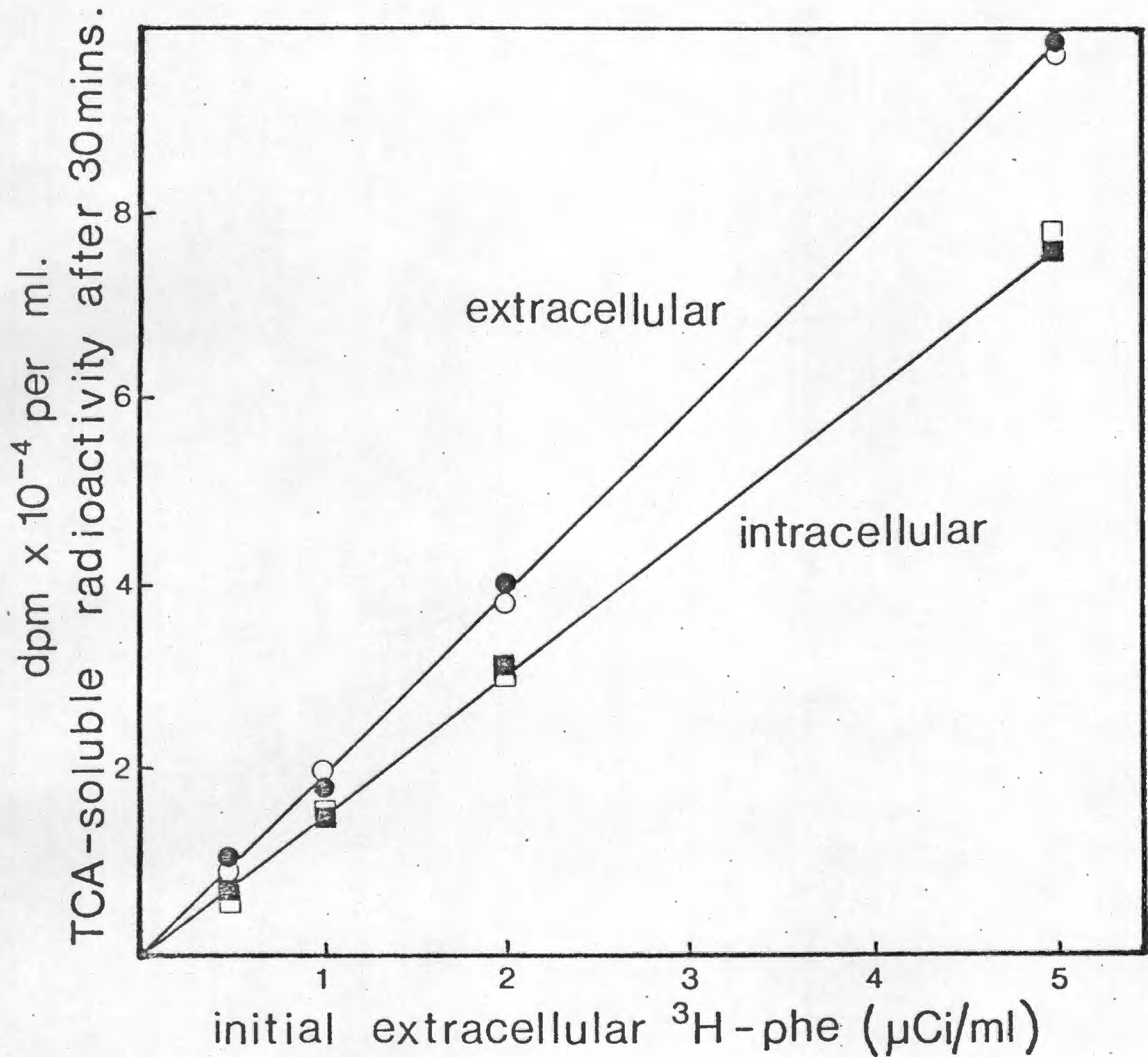


FIGURE 1.5. EQUILIBRATION OF ^{3}H-PHENYLALANINE ACROSS MUSCLE CELL MEMBRANES.

Smooth muscle cell layers were incubated at 37°C for 30 mins with various concentrations of ^{3}H-phenylalanine (constant specific activity of 37 Ci/mmol) in the presence or absence of chloroquine (70 µM) and NH_4Cl (10 mM). At the end of the incubation, TCA-soluble radioactivity was measured intracellularly (■ control; □ with inhibitors) and extracellularly (● control; O with inhibitors). Each point is the mean of two cell dishes.

effected complete equilibration between the intracellular and extracellular compartments. A series of consecutive 30-minute incubations at 37°C, with fresh isotope-free medium each time, resulted in a stepwise extraction of the intracellular [^{3}H]-phenylalanine (Figure 1.6.). After four such extractions, a minimum level of intracellular [^{3}H]-phenylalanine was reached, which remained constant throughout any subsequent incubations, while the [^{3}H]-phenylalanine continuously released from degraded protein rapidly reached levels which exceeded the intracellular background radioactivity of the non-equilibrating intracellular pool (Figure 1.7.). In the degradation experiments, [^{3}H]-phenylalanine release was measured as the net increase in acid-soluble radioactivity of the whole experimental system, i.e. intracellular and medium.

In the case of the "short-lived" proteins, where it was necessary for the degradation rate to be measured over much shorter time periods (See 1.2.2.1.), the length of each of the four stepwise extraction incubations at 37°C was reduced to 15 minutes so that the level of intracellular TCA-soluble [^{3}H]-phenylalanine was brought to its minimum in a period of one hour. This procedure was found to be equally satisfactory in washing out the large intracellular pool of soluble radioactivity.

1.1.3. Prevention of Intracellular Re-utilization of Degradation-Derived [^{3}H]-Phenylalanine

A major problem encountered in measurements of intracellular protein degradation has been the fact that amino acids derived from protein degradation are re-utilized to a considerable

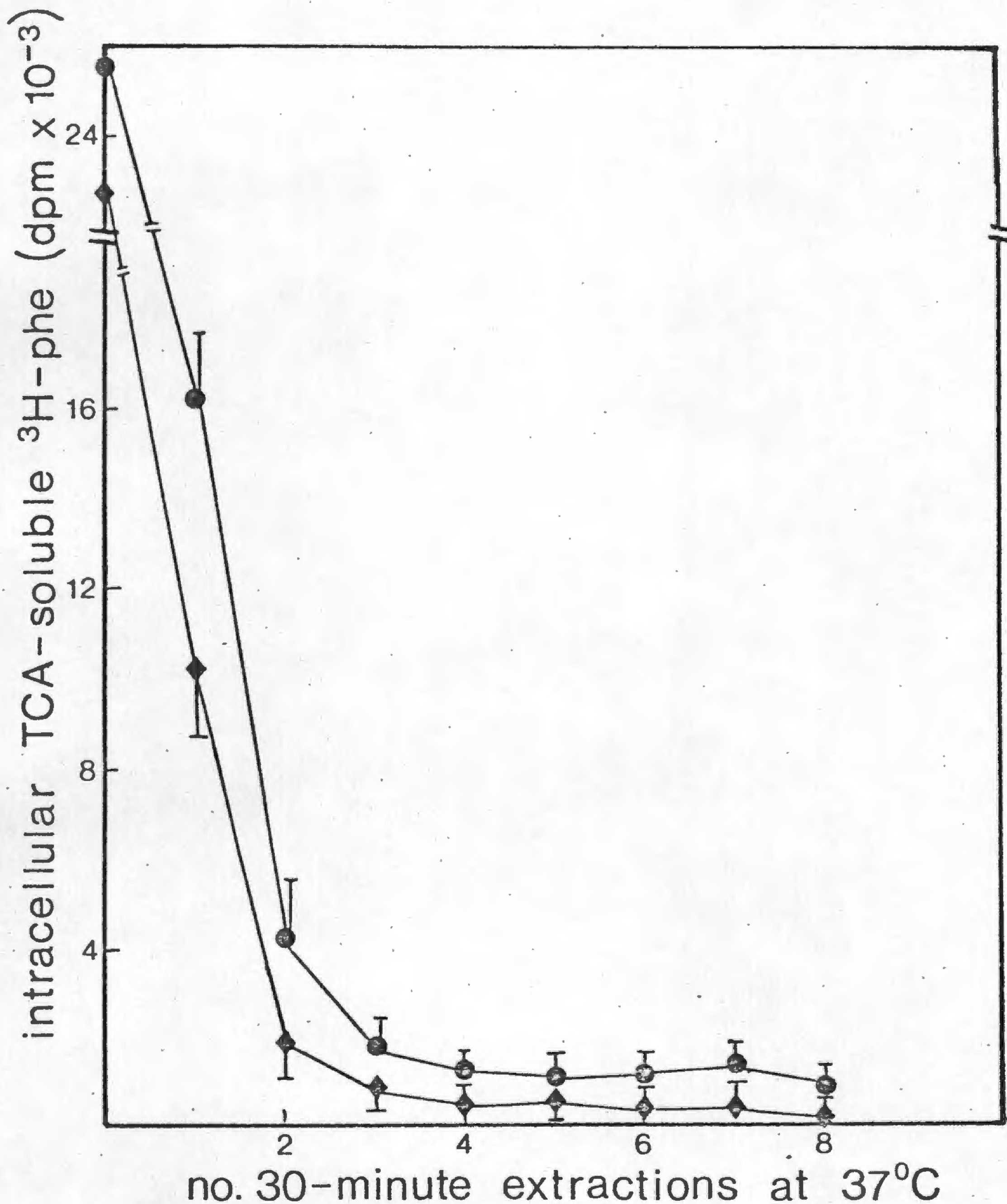


FIGURE 1.6. EXTRACTION OF INTRACELLULAR TCA-SOLUBLE RADIOACTIVITY BY REPEATED CHANGES OF MEDIUM.

Cell layers were labelled for 16 hours in the presence of 1 μCi/ml ^{3}H-phenylalanine, washed four times at 4°C, and reincubated at 37°C for 8 consecutive 30-minute periods. After each incubation, the intracellular acid-soluble radioactivity was measured. Each point is the mean ± S.E. of 5 cell dishes.

◆—◆ Smooth muscle cells.

●—● Skeletal muscle cells.

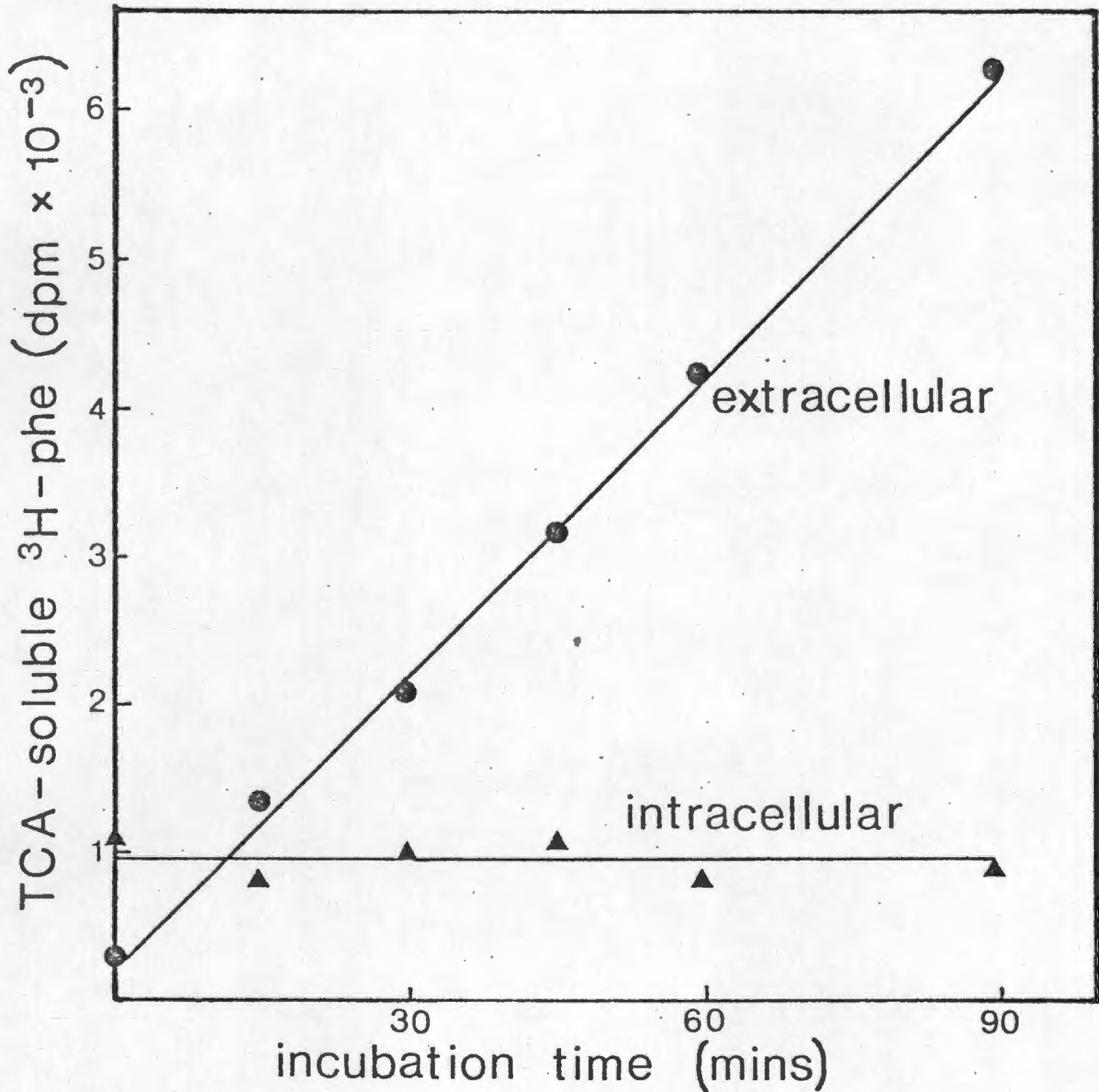


FIGURE 1.7. RELEASE OF ^{3}H-PHENYLALANINE FROM PRE-LABELLED SMOOTH MUSCLE CELLS.

Cells were prelabelled for 16 hours with 1 μCi/ml ^{3}H-phenylalanine, washed as described in "Experimental Procedures, 3", then incubated at 37°C in the presence of 1 mM excess phenylalanine for the times indicated. Each point is the mean of 3 cell dishes.

NOTE: In the case of cells labelled with 5 μCi/ml ^{3}H-phenylalanine for 1 hour, the intracellular TCA-soluble radioactivity remained at approx. 500 dpm with the extracellular levels rising to approx. 3000 dpm after 60 mins incubation.

extent for ongoing intracellular protein synthesis, thus reducing the apparent rate of proteolysis measured experimentally. Re-utilization can be prevented by expanding the intracellular amino acid pool with nonlabelled amino acid, thereby effectively diluting the released isotope; or by blocking protein synthesis with an inhibitor such as cycloheximide. The results in Figure 1.8. show that the presence of nonlabelled phenylalanine at ten times (i.e. 1 mM) the normal concentration in medium maximally increased the rate of release of [^{3}H]-phenylalanine, presumably by decreasing isotope re-utilization. Addition of a higher excess (up to 10 mM) of nonlabelled phenylalanine to the medium did not further increase the apparent rate of release over that noted at 1 mM phenylalanine; thus degradation incubations were routinely performed in the presence of 1.1 mM phenylalanine (Vandenburgh and Kaufman, 1980).

The presence of 1 mM cycloheximide in the chase incubations did not increase the rate of release of [^{3}H]-phenylalanine into the medium, and in fact inhibited protein degradation slightly, a result which has been commonly reported for intracellular protein degradation (Seglen and Solheim, 1979; Amenta _et al_., 1978).

Thus the measurement of release of labelled phenylalanine in the presence of a 1.1mM chase concentration of unlabelled phenylalanine seems to afford a reliable estimate of the net intracellular protein degradation by minimizing the problem of re-utilization of labelled phenylalanine.

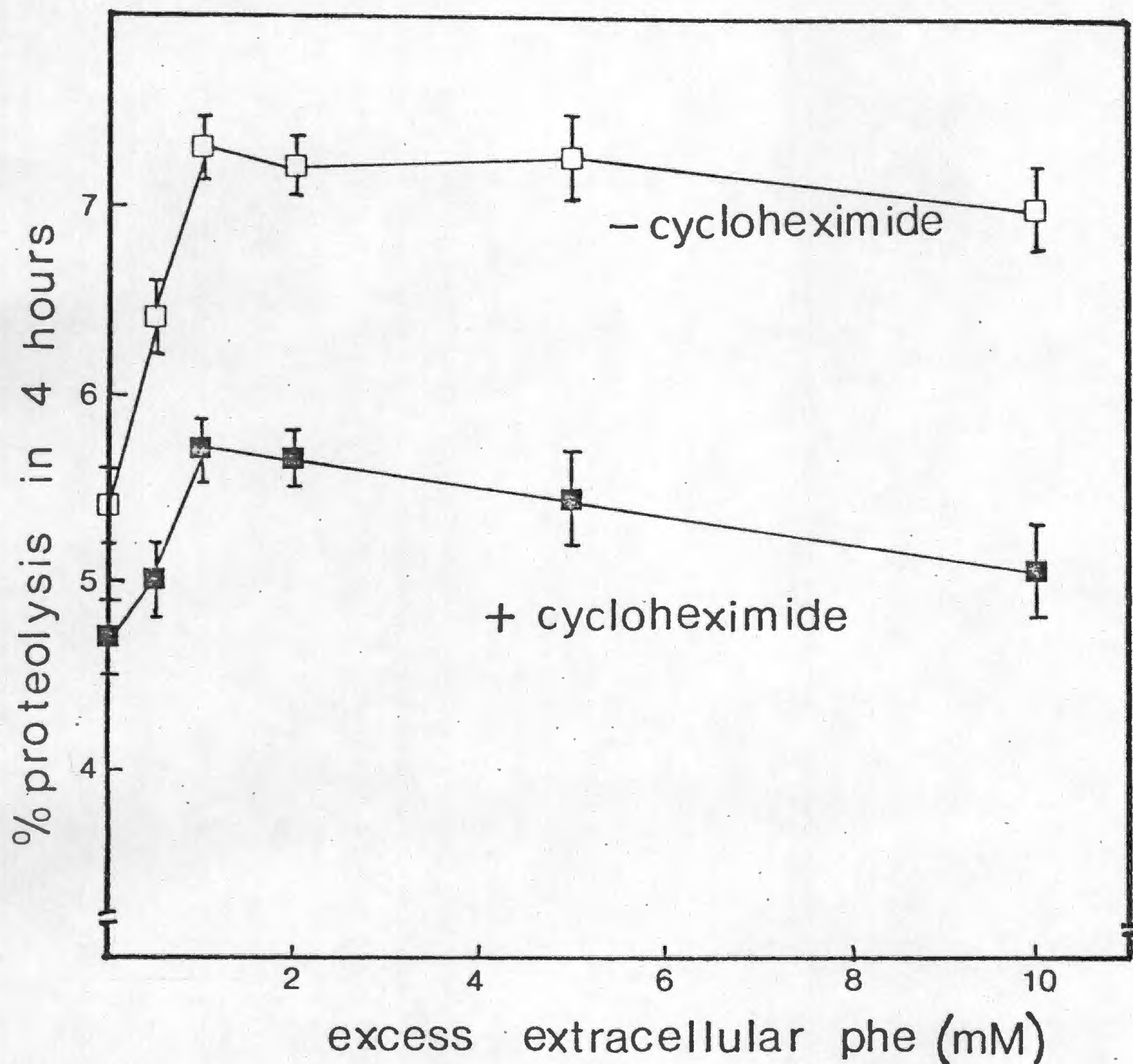


FIGURE 1.8. EFFECT OF VARIOUS CONCENTRATIONS OF MEDIUM PHENYLALANINE ON RELEASE OF ^{3}H-PHENYLALANINE FROM PROTEINS PRELABELLED FOR 16 HOURS WITH 1 μCi/ml ^{3}H-PHENYLALANINE.

Smooth muscle cell layers were washed as described under "Experimental Procedures, 3", then incubated for 4 hours in the presence or absence of 1 mM cycloheximide at the indicated concentrations of excess unlabelled phenylalanine.
Each point is the mean ± S.E. of four cell dishes.

1.2. PROTEIN DEGRADATION IN SMOOTH MUSCLE CELLS

1.2.1. Long-Lived Proteins

1.2.1.1. Basal Proteolysis

Under the normal nutritional conditions, i.e. 10% serum, the average rate of degradation of intracellular proteins in smooth muscle cells prelabelled with [^{3}H]-phenylalanine for 16 hours ("long-lived" proteins), was found to be 1,5% per hour (1,54 ± 0,11), a rate which is similar to values reported for other cultured cell types in the steady state, and which has been termed the basal rate of degradation (Dean, 1980). The degradation rate proceeded linearly for at least 4 hours, then declined as illustrated in Figure 1.9. Rates of intracellular protein degradation were measured both in the presence of foetal calf serum and lipoprotein deficient serum (LPDS), and no significant differences were found (Table 1.1.).

Action of Inhibitors:

The inhibitory action of chloroquine, a lysosomotropic proteinase inhibitor which, due to its weak base properties, accumulates inside lysosomes and neutralizes their contents as well as being a specific inhibitor of the lysosomal proteinase cathepsin B (Wibo and Poole, 1974), was found to be a maximum of 30% (see Figure 1.10). NH_4Cl, another lysosomotropic inhibitor (Seglen and Reith, 1976; Amenta and Brocher, 1980), was found to have a similar inhibitory action to chloroquine but was generally slightly less effective. When these inhibitors were used in combination, they showed no significant

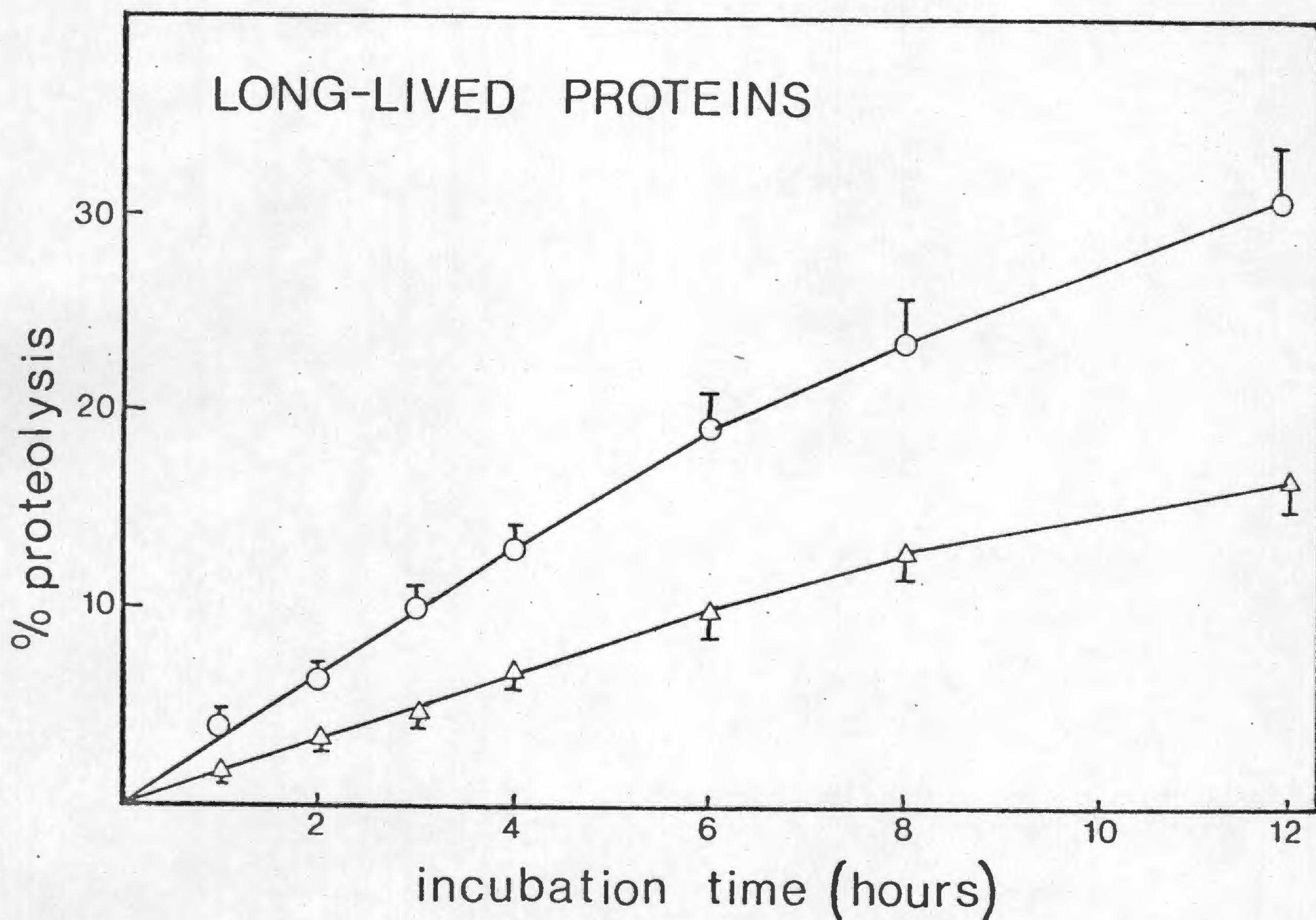


FIGURE 1.9. TIME COURSE OF PROTEIN DEGRADATION IN SMOOTH MUSCLE CELLS.

Cell layers were labelled for 16 hours with 1 μCi/ml ^{3}H-phenylalanine, washed and proteolysis measured at the indicated times as described under "Experimental Procedures, 3". Each point is the mean ± S.E. of 5 cell dishes.
△——△ Basal proteolysis.
○——○ Accelerated proteolysis.

"LONG-LIVED" PROTEINS

CONDITIONS	% PROTEOLYSIS IN 4 HR	% INHIBITION
(a) GROWTH MEDIUM CONTAINING FOETAL CALF SERUM:	(n=5)	
NO ADDITIONS	7,12 ± 0,15	-
70 μM CHLOROQUINE	5,19 ± 0,08	27%
CQ + 10 mM NH_4Cl + 100 μg/ml LEUPEPTIN	5,08 ± 0,09	29%
(b) GROWTH MEDIUM CONTAINING LPDS		
NO ADDITIONS	7,16 ± 0,11	-
+ CQ	5,25 ± 0,09	27%
+ CQ + NH_4Cl + LEUP	5,17 ± 0,13	28%

"SHORT-LIVED" PROTEINS

CONDITIONS	% PROTEOLYSIS IN 1 HR	% INHIBITION
(a) GROWTH MEDIUM CONTAINING FOETAL CALF SERUM:	(n=5)	
NO ADDITIONS	6,26 ± 0,08	-
+ CQ	5,14 ± 0,04	18%
+ CQ + NH_4Cl + LEUP	5,07 ± 0,07	19%
(b) GROWTH MEDIUM CONTAINING LPDS:		
NO ADDITIONS	6,22 ± 0,10	-
+ CQ	5,13 ± 0,04	17%
+ CQ + NH_4Cl + LEUP	4,92 ± 0,08	20%

TABLE 1.1. PROTEOLYSIS RATES IN THE PRESENCE OF FOETAL CALF SERUM OR LPDS.

Smooth muscle cells were labelled as described for "long-lived" and "short-lived" proteins. During the wash and degradation incubations, the cells were incubated in growth medium containing either foetal calf serum or lipoprotein-deficient serum (LPDS), and proteolysis measurements made. Values are means ± S.E. for 3 different experiments.

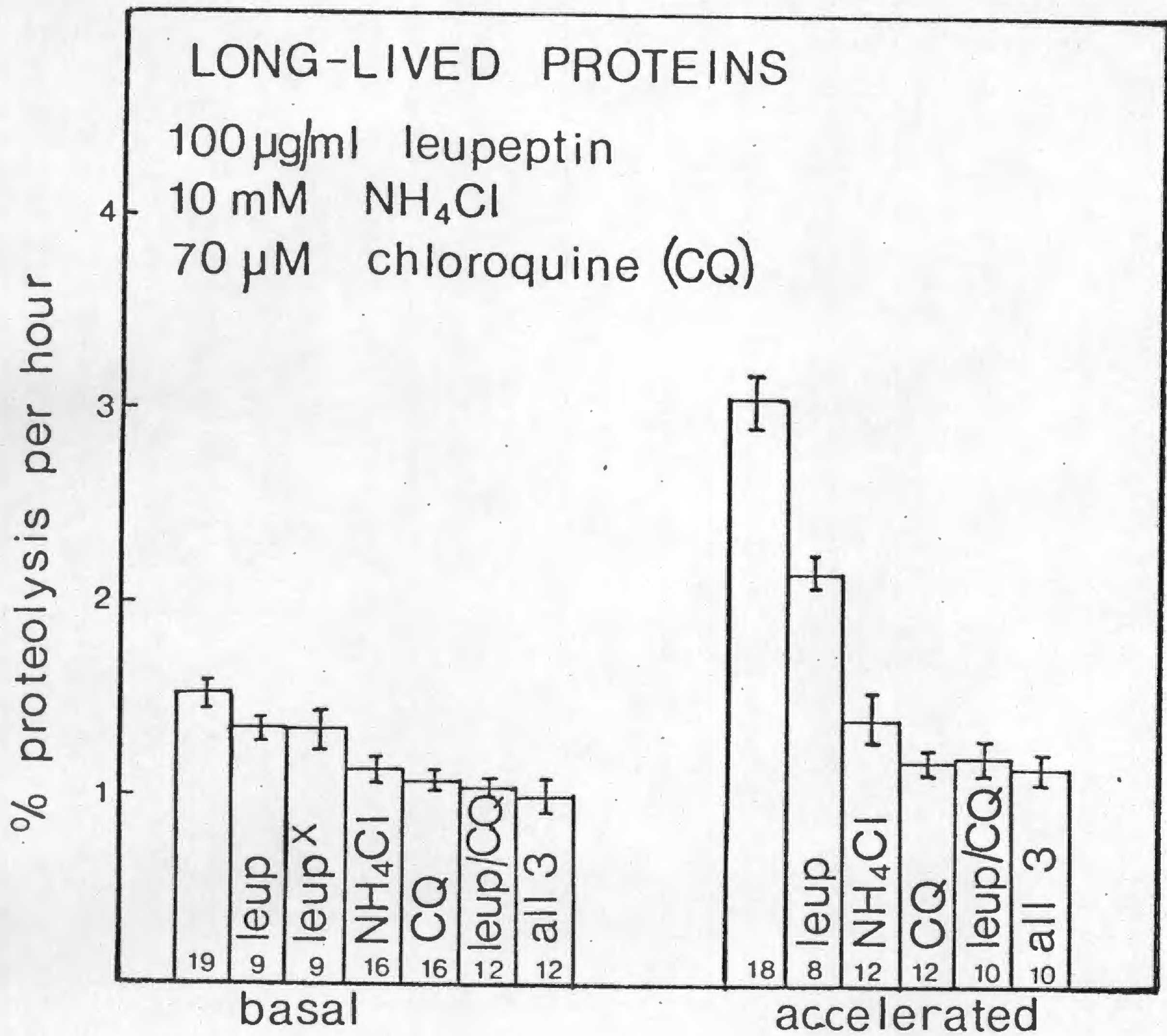


FIGURE 1.10. INHIBITION OF PROTEIN DEGRADATION IN SMOOTH MUSCLE CELLS.

All measurements were made after 4 hours of degradation in normal growth medium (basal) or "step-down" minimal medium (accelerated), and the inhibitors were pre-incubated with the cells for 2 hours as described under "Experimental Procedures, 6". Numbers indicate the amount of celldishes analyzed in at least 5 different experiments.

x indicates 18-hour preincubation of leupeptin with the cell layers.

additivity, indicating that chloroquine and NH_4Cl at these conditions each block the lysosomal pathway of protein degradation almost completely.

Leupeptin, which is a modified tetrapeptide isolated from actinomycetes (Aoyagi and Umezawa, 1975) specifically binds to and inhibits the lysosomal thiol proteinases, cathepsins B, H and L (Kirsche et al., 1977), as well as inhibiting the action of some extralysosomal proteinases such as calcium-activated neutral proteinase, trypsin and papain. Leupeptin only decreased the basal degradation of long-lived protein by about 12%. This level of inhibition was very rapidly reached and no significant further enhancement could be obtained by prolonged pre-incubation of leupeptin with the cell layers for 18 hours. The effect of leupeptin was also not found to be significantly additive to the effects of chloroquine, and NH_4Cl, and even when all three inhibitors were incubated together with the cell layers, the maximum inhibition obtained was rarely over 30% (Figure 1.10). The inhibitory effects of chloroquine, NH_4Cl and leupeptin were found to be the same in the presence of foetal calf serum or LPDS (Table 1.1).

1.2.1.2. Accelerated Proteolysis

Incubation of cells labelled for 16 hours with [^{3}H]-phenylalanine in a minimal medium only, i.e. nutritional step-down conditions, accelerated degradation by about 90% to a rate of 2,9% per hour (2,92 $\pm$ 0,18), which remained constant over the four-hour degradation period tested (Figure 1.9). The

increased degradation was inhibited by chloroquine or NH_4Cl to a rate below the basal rate of degradation. The effect of leupeptin was also far more significant in these conditions than under basal proteolytic conditions, the percentage inhibition increasing from 12% to about 30%. No significant additive effect was obtained by using these inhibitors in combination (Figure 1.10), and chloroquine and NH_4Cl each appeared to inhibit near maximally when added alone.

1.2.2. Short-Lived Proteins

1.2.2.1. Basal Proteolysis of Short-lived Proteins

The rate of degradation of short-lived proteins, routinely measured over the first hour of the degradation period, was found to be 6,5% per hour (6,49 $\pm$ 0.61), was linear over the one-hour period tested (Figure 1.11) and then gradually declined, reaching the average basal rate of 1,5% per hour after 4-5 hours. Degradation of these short-lived proteins was much less sensitive to the inhibitors tested, with chloroquine and NH_4Cl reducing the degradation rate by only 12-15% and the effect of leupeptin being even smaller ($< 6\%$). Under these conditions, however, the effect of adding the inhibitors together did slightly increase the degree of inhibition obtained, although the effects were not totally additive (Figure 1.12).

1.2.2.2. Accelerated Proteolysis of Short-lived Proteins

The incubation of cell layers prelabelled for 1 hour with $[^3H]$-phenylalanine in a minimal medium in place of complete

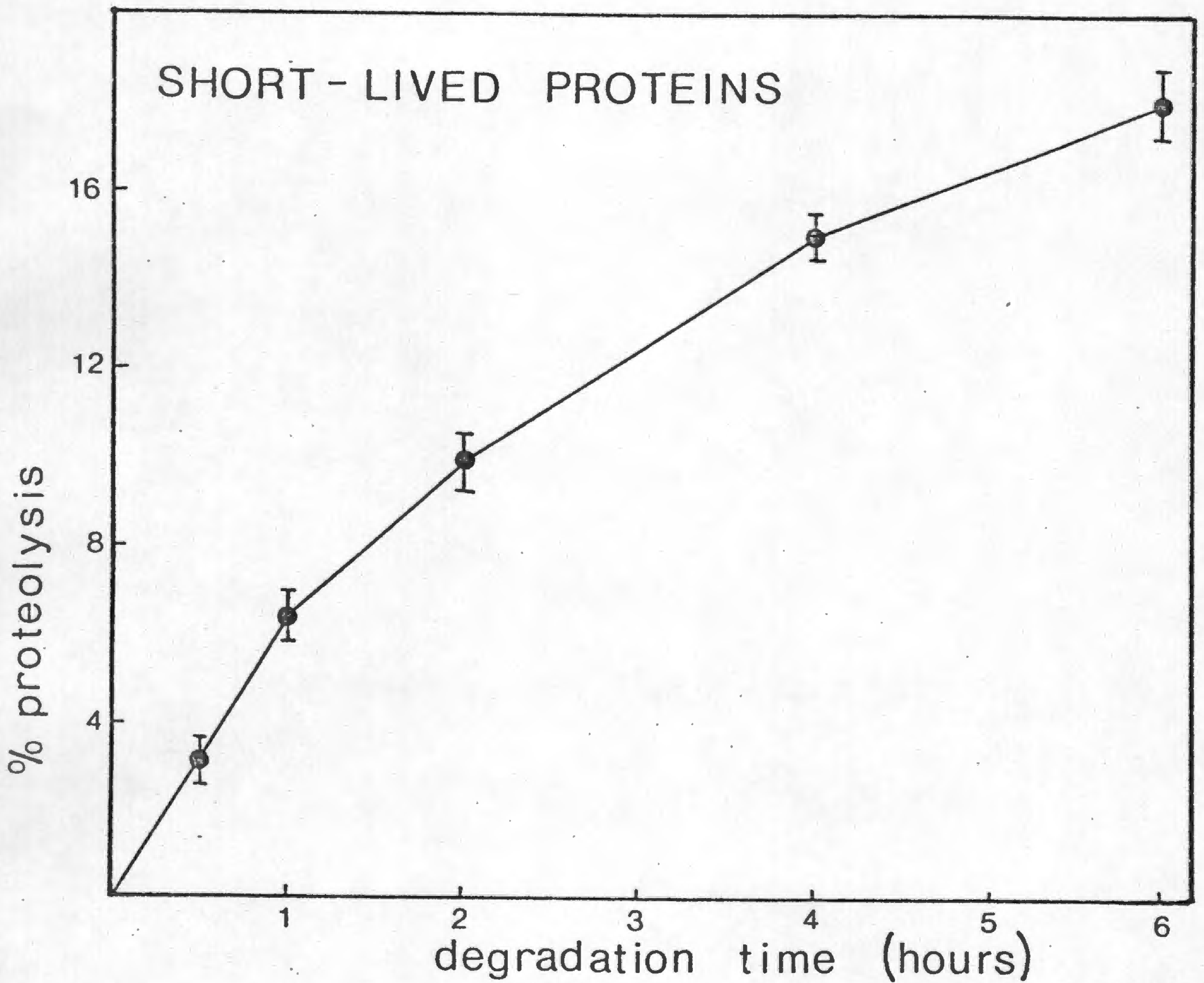


FIGURE 1.11. TIME COURSE OF DEGRADATION OF SHORT-LIVED PROTEINS. Smooth muscle cells were labelled with 5 µCi/ml ^{3}H-phenylalanine for 1 hour, then washed, and proteolysis measurements made as described under "Experimental Procedures, 5". Each point is the mean ± S.E. of five cell dishes.

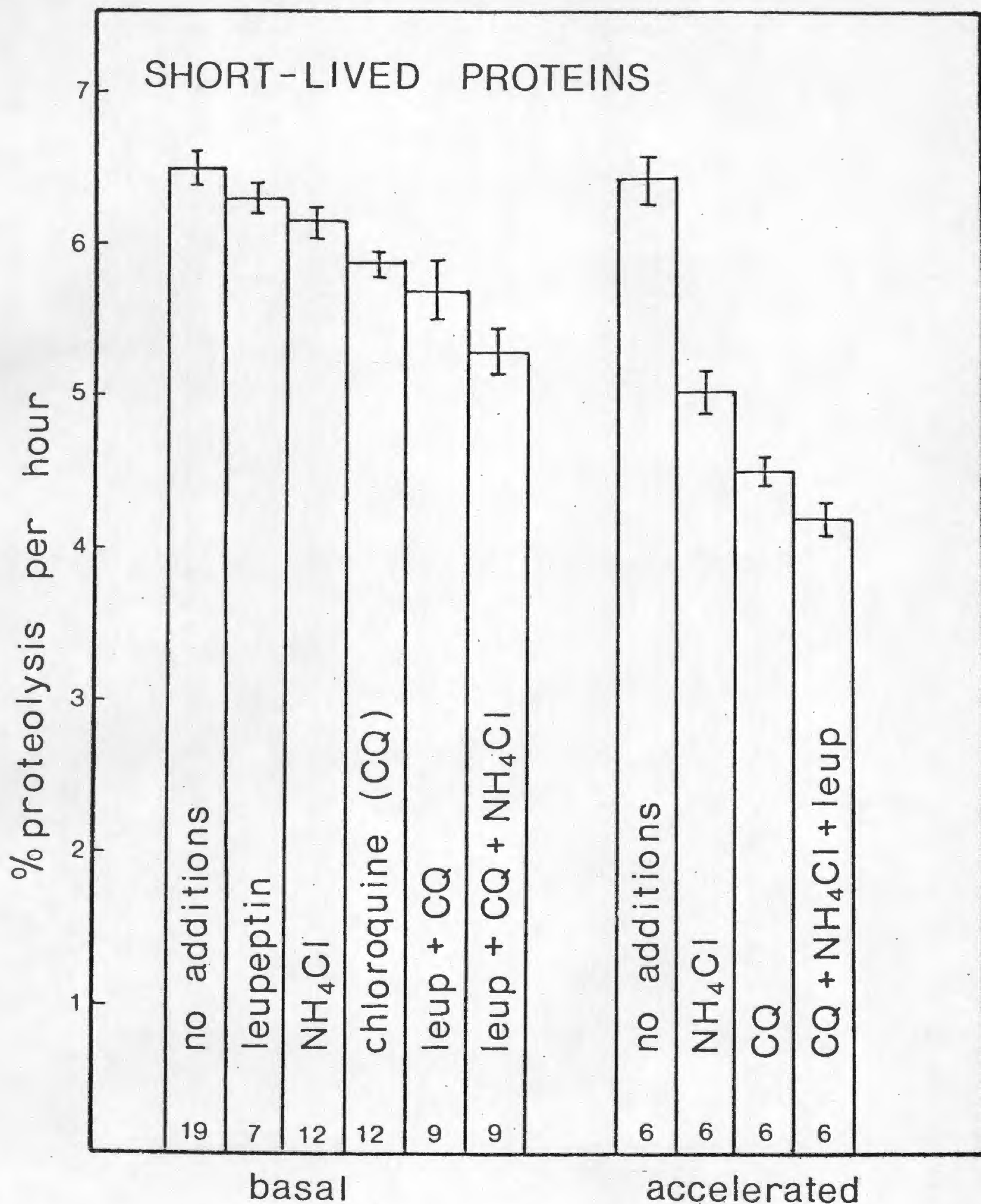


FIGURE 1.12. EFFECT OF INHIBITORS ON PROTEOLYSIS OF SHORT-LIVED PROTEIN UNDER BASAL AND ACCELERATED CONDITIONS.

Smooth muscle cells were labelled for 1 hour, and basal and accelerated proteolysis was measured after 1 hour as described under "Experimental Procedures, 5". The inhibitors were all pre-incubated with the cells for 1 hour, and used at the concentrations given in Figure 1.10. Numbers indicate the amount of cell dishes analyzed in at least 3 different experiments.

growth medium, did not significantly alter the degradation rate of short-lived proteins (Figure 1.12). However, the inhibitory effects of leupeptin, NH_4Cl and chloroquine were significantly increased under these conditions.

1.2.3. Degradation of "Abnormal" Proteins

Cells were incubated with [^{3}H]-phenylalanine in growth medium which also contained L-canavanine, an arginine analogue. High concentrations of the analogue were employed (10 mM) to achieve incorporation of the analogue into proteins, since it is known that the respective tRNA-acylating enzyme has a much lower affinity for L-canavanine than for arginine (Knowles and Ballard, 1976).

Over the first hour of the degradation period, canavanine-containing proteins were degraded at approximately double the rate of normal proteins, in cells labelled for 3 hours with [^{3}H]-phenylalanine in the presence or absence of canavanine (Figure 1.13). The rate (about 7,5% per hour) was five times the basal degradation rate of long-lived proteins. This very rapid removal of "abnormal" intracellular proteins was relatively insensitive to chloroquine and NH_4Cl, and these inhibitors only reduced the proteolytic rate by 12-16%, while leupeptin exerted no significant effect.

1.2.4. Degradation of Extracellular Apoprotein-B of Low Density Lipoprotein (LDL)

The degradation of ^{125}I-labelled apolipoprotein by smooth muscle cells was measured during an incubation of cell layers with

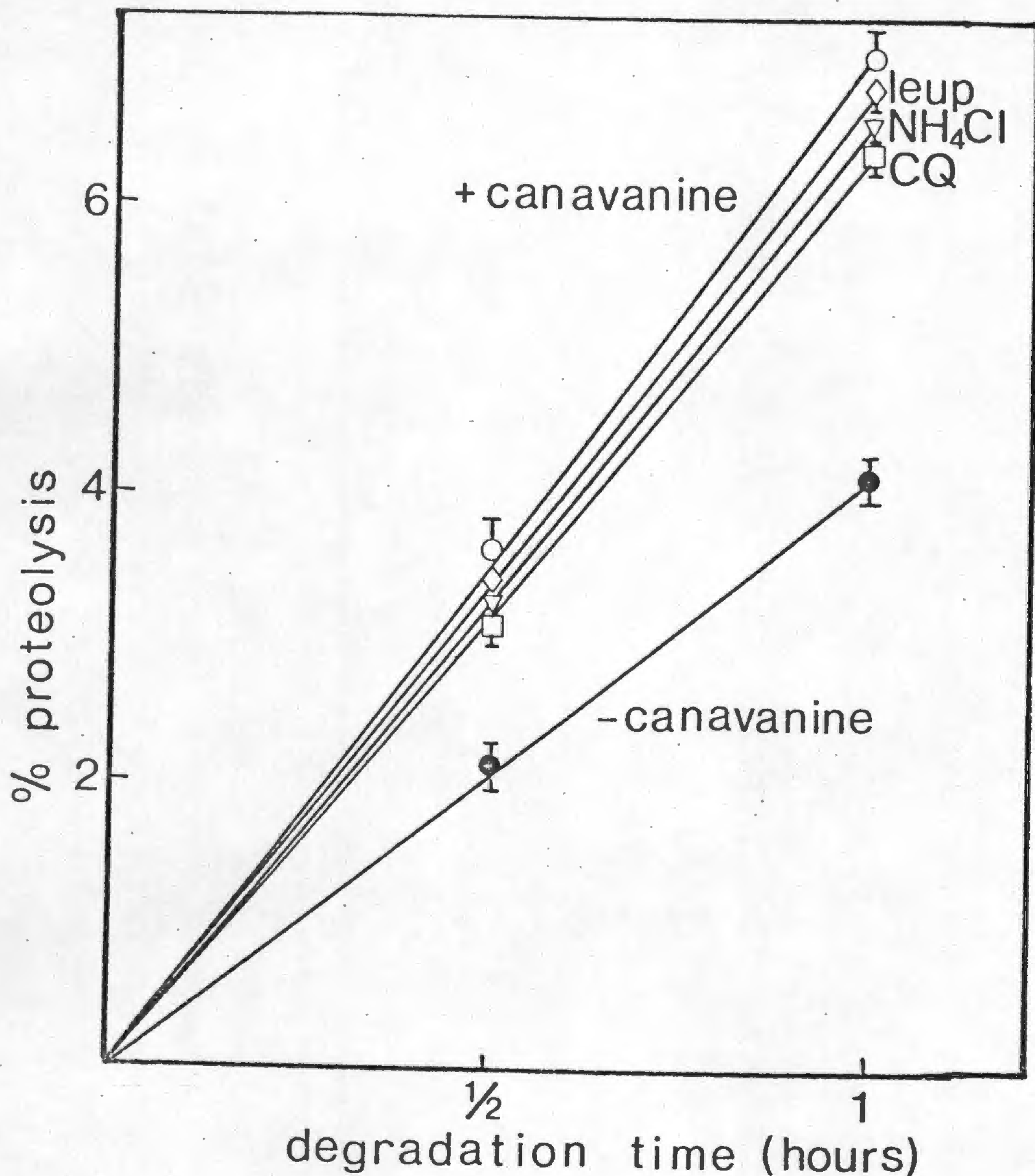


FIGURE 1.13. DEGRADATION OF ANALOGUE-CONTAINING PROTEINS AND ACTION OF INHIBITORS.

Smooth muscle cells were incubated for 3 hours with 3 μCi/ml ^{3}H-phenylalanine in the presence or absence of 10 mM L-canavanine, washed for 1 hour, then incubated for a further hour in the presence of 1.1 mM nonlabelled phenylalanine. The inhibitors were present from the beginning of the wash period at the following concentrations: 100 μg/ml leupeptin, 10 mM NH_4Cl and 70 μM chloroquine. Each point is the mean ± S.E. of 8 cell dishes analyzed in 3 different experiments.

^{125}I-LDL. The degradation was found to be linear during the 4-hour period tested following an initial lag of approximately 30 minutes (Figure 1.14). The metabolism of ^{125}I-LDL by cells was also tested in the presence of chloroquine and NH_4Cl, added either singly or in combination, and the results are shown in Table 1.2. The amount of ^{125}I-LDL bound and subsequently internalized by the cells in the presence or absence of inhibitors is not significantly altered. Thus any effect of the inhibitors on the amount of ^{125}I-LDL degraded by the cell layers can be attributed to a direct effect on the degradative mechanism (lysosomal system) rather than a secondary manifestation of a reduced uptake of ^{125}I-LDL by the cells. Results obtained from ^{125}I-LDL degradation experiments run concurrently with those measuring the degradation of [^{3}H]-labelled intracellular proteins show that the inhibition of LDL degradation by chloroquine is consistently over 90% whereas the inhibition of intracellular proteolysis is 25-30% (Figure 1.14). NH_4Cl also inhibited most of the LDL degradation mechanism, although not as effectively as chloroquine, and simultaneously reduced intracellular proteolysis by only 22-27%. There was no significant additive effect produced on ^{125}I-LDL degradation in the presence of both chloroquine and NH_4Cl.

In the case of accelerated proteolysis, ^{125}I-LDL was used in simultaneous experiments where all the cell layers were incubated in minimal medium for measurements of the degradation of ^{125}I-labelled LDL and [^{3}H]-labelled intracellular proteins in the presence or absence of inhibitors. The effect of

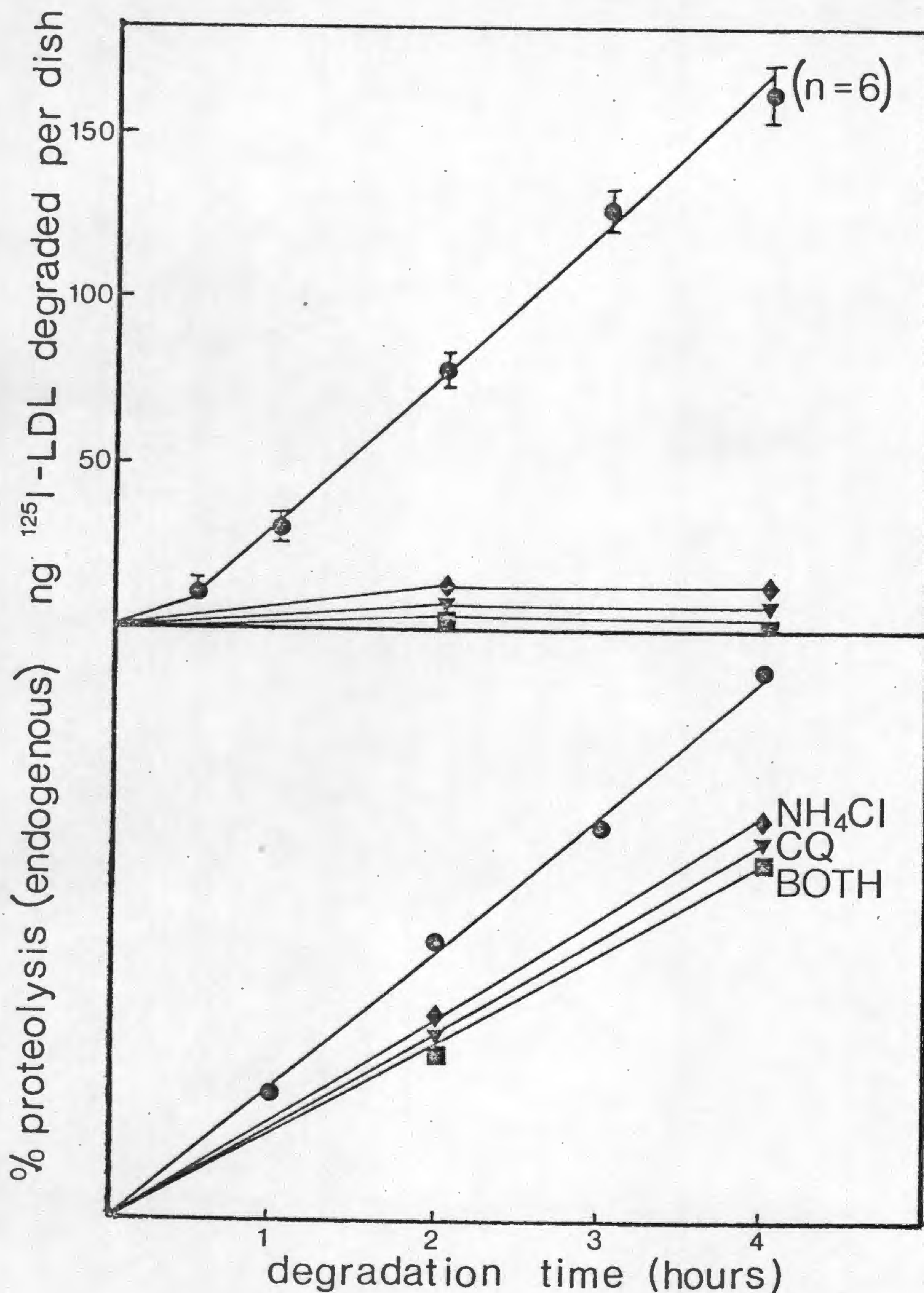


FIGURE 1.14. DEGRADATION OF ^{125}I-LDL COMPARED TO LONG-LIVED ENDOGENOUS PROTEINS UNDER BASAL CONDITIONS.

Endogenous proteolysis measurements were made as described under "Experimental Procedures, 3". Similar smooth muscle cell layers were preincubated in the presence of the indicated inhibitors for 2 hours, before the addition of 20 μg/ml ^{125}I-LDL in LPDS-containing medium. ^{125}I-LDL degradation was measured as described under "Experimental Procedures, 10". Unless otherwise indicated, each point is the mean of 3 cell dishes. ◆——◆ 10 mM NH_4Cl, ▼——▼ 70 μM chloroquine (CQ), ■——■ NH_4Cl + CQ.

CONDITIONS	ng ^{125}I-LDL BOUND PER DISH	ng ^{125}I-LDL INTERNALIZED PER DISH	ng ^{125}I-LDL DEGRADED PER DISH	% INHIBITION OF DEGRADATION
EXPT. (i)				
NO ADDITIONS	23,6	82,9	231,8	-
70 µM CHLOROQUINE	22,1	261,3	4,7	98%
10 mM NH_4Cl	23,9	285,1	16,3	93%
CQ + NH_4Cl	21,9	219,2	4,6	98%
EXPT. (ii)				
NO ADDITIONS	16,6	59,3	166,8	-
70 µM CHLOROQUINE	17,0	181,5	12,0	94%
10 mM NH_4Cl	15,8	170,8	21,9	87%
CQ + NH_4Cl	16,8	189,2	9,1	95%
EXPT. (iii)				
NO ADDITIONS	19,8	62,8	185,4	-
70 µM CHLOROQUINE	18,2	230,1	9,6	95%
10 mM NH_4Cl	19,5	251,8	10,9	94%
CQ + NH_4Cl	18,0	242,1	7,4	96%

TABLE 1.2. METABOLISM OF ^{125}I-LDL BY SMOOTH MUSCLE CELLS SHOWING EFFECTS OF NH_4Cl AND CHLOROQUINE. Smooth muscle cell layers were incubated for 2 hours in the presence of the inhibitors before the addition of 20 µg/ml ^{125}I-LDL in LPDS-containing medium. After 4 hours, the medium and cells were analyzed as described under "Experimental Procedures". Each value reported is the mean of duplicate cell dishes. Expt. (i) corresponded to the experiment from which the data shown in Figure 1.14 were obtained.

chloroquine and NH_4Cl on ^{125}I-LDL degradation was again an almost complete inhibition (82% and 88% for NH_4Cl and chloroquine respectively) (Figure 1.15).

To test the effectiveness of the lysosomotropic inhibitors on ^{125}I-LDL degradation under the conditions used for analysis of the degradation of short-lived proteins, the degradation of ^{125}I-LDL was determined. In order for the degradation of ^{125}I-LDL to be on the linear portion of the curve seen in Figure 1.14 , the cell layers were preloaded with ^{125}I-LDL prior to the measurement of degradation during the 1-hour period under test (see procedure under methods). Figure 1.16 illustrates the effectiveness of chloroquine and NH_4Cl in blocking ^{125}I-LDL degradation (over 90% inhibition in most cases) whilst their effect on the degradation of short-lived intracellular proteins remained minimal (10-12%).

In all the experiments described above, LDL binding, internalization and degradation were always measured to check that the effects of the inhibitors on degradation was in fact a true reflection of an inhibition of the degradative mechanism itself (see Tables 1.3 and 1.4). The inhibitors were found to have a minimal effect on LDL binding and uptake under each of the conditions employed.

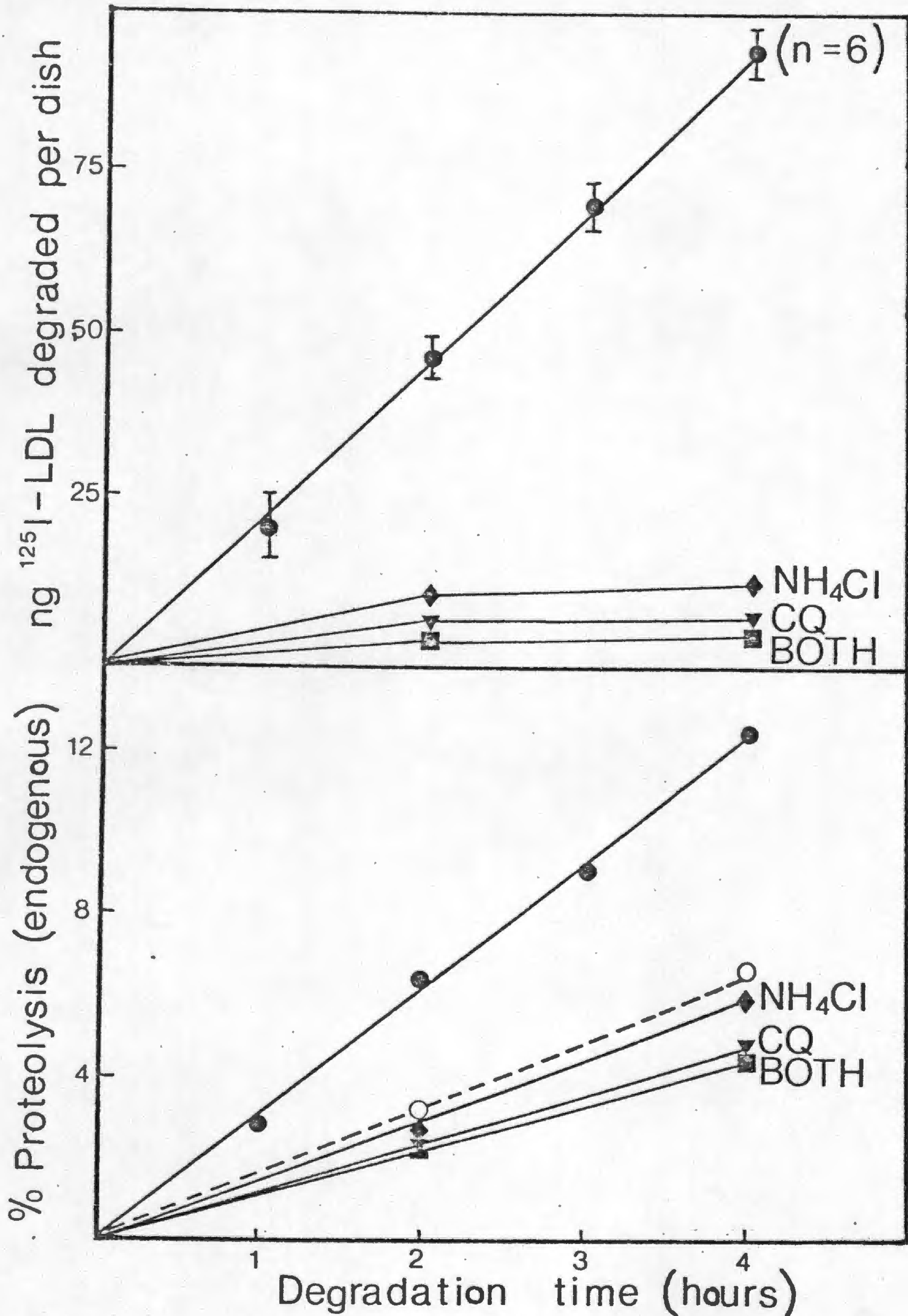


FIGURE 1.15. DEGRADATION OF ^{125}I-LDL AND ENDOGENOUS LONG-LIVED PROTEINS IN MINIMAL GROWTH MEDIUM (ACCELERATED PROTEOLYSIS).

Experimental procedure was the same as outlined in Figure 1.14. except that all incubations were carried out in minimal medium alone. Unless otherwise indicated, each point is the mean of 3 cell dishes. O---O indicates proteolysis rate in complete medium (basal proteolysis).

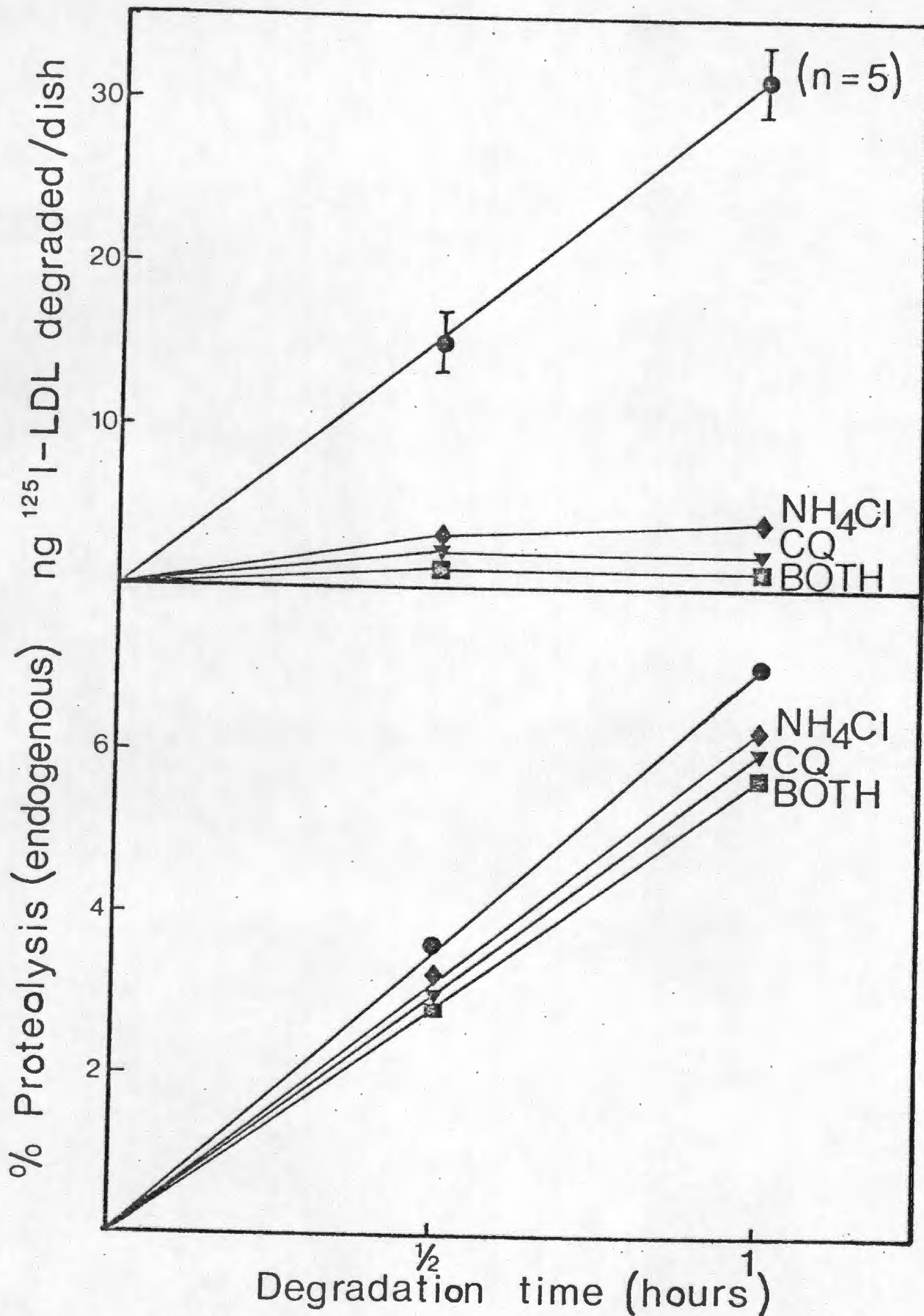


FIGURE 1.16. DEGRADATION OF ^{125}I-LDL COMPARED TO ENDOGENOUS SHORT-LIVED PROTEINS UNDER BASAL CONDITIONS. Endogenous proteolysis measurements were made as described "Experimental Procedures, 5". Similar smooth muscle cell layers were preincubated for 2 hours with 20 μg/ml ^{125}I-LDL and with the indicated inhibitors for 1 hour. Then fresh ^{125}I-LDL (20 μg/ml) and inhibitors were added, and ^{125}I-LDL degradation measured. Unless otherwise indicated, each point is the mean of 3 cell dishes.

	CONDITIONS	ng ^{125}I-LDL BOUND PER DISH	ng LDL INTERNALIZED PER DISH	ng LDL DEGRADED PER DISH	% INHIBITION OF DEGRADATION
EXPT. (i)	-LPDS	9,1 $\pm$ 0,3	32,8 $\pm$ 1,1	72,9 $\pm$ 2,0	-
(n=3)	-LPDS + CQ	9,8 $\pm$ 0,6	83,4 $\pm$ 0,9	15,6 $\pm$ 0,2	79%
	-LPDS + NH_4Cl	8,9 $\pm$ 0,2	67,9 $\pm$ 1,3	15,8 $\pm$ 0,6	78%
	-LPDS + CQ + NH_4Cl	9,5 $\pm$ 0,9	81,1 $\pm$ 1,8	14,5 $\pm$ 0,6	81%
EXPT. (ii)	-LPDS	19,9 $\pm$ 0,8	110,3 $\pm$ 4,7	181,1 $\pm$ 2,9	-
(n=3)	-LPDS + CQ	22,0 $\pm$ 0,9	259,2 $\pm$ 2,8	31,2 $\pm$ 0,9	84%
	-LPDS + NH_4Cl	18,1 $\pm$ 1,1	220,9 $\pm$ 4,4	49,4 $\pm$ 1,8	73%
	-LPDS + CQ + NH_4Cl	20,7 $\pm$ 0,8	269,3 $\pm$ 1,8	29,8 $\pm$ 2,1	84%

TABLE 1.3. METABOLISM OF ^{125}I-LDL BY SMOOTH MUSCLE CELLS INCUBATED IN A MINIMAL MEDIUM SHOWING EFFECTS OF NH_4Cl AND CHLOROQUINE.

Experimental procedure as outlined under Table 1,1. except that all incubations were carried out in LPDS-free growth medium.

	CONDITIONS	ng ^{125}I-LDL BOUND PER DISH	ng LDL INTERNALIZED PER DISH	ng LDL DEGRADED PER DISH	% INHIBITION OF DEGRADATION
EXPT. (i)	NO ADDITIONS	27,6 ± 1,2	198,2 ± 3,1	29,4 ± 1,0	-
(n=3)	+ CQ	26,1 ± 0,9	263,7 ± 7,2	3,2 ± 0,3	91%
	+ NH_4Cl	22,9 ± 0,8	243,1 ± 6,4	5,2 ± 0,5	83%
	+ C+ Q NH_4Cl	20,8 ± 1,4	240,1 ± 9,3	0,7 ± 0,1	96%
EXPT. (ii)	NO ADDITIONS	10,9 ± 1,0	108,1 ± 6,2	30,6 ± 1,2	-
(n=3)	+ CQ	10,2 ± 0,8	129,0 ± 3,9	6,1 ± 0,8	82%
	+ NH_4Cl	10,8 ± 0,7	120,4 ± 8,3	9,2 ± 0,9	70%
	+ CQ + NH_4Cl	10,1 ± 1,1	128,9 ± 7,2	7,0 ± 1,1	79%

TABLE 1.4. METABOLISM OF ^{125}I-LDL BY SMOOTH MUSCLE CELLS DURING A 1 HOUR INCUBATION PERIOD SHOWING EFFECTS OF NH_4Cl AND CHLOROQUINE.

Smooth muscle cells were pre-incubated for 2 hours in the presence of 20 μg/ml ^{125}I-LDL in LPDS-medium and for 1 hour in the presence of the indicated inhibitors before fresh ^{125}I-LDL was added for a further period of one hour. Measurements of binding, internalization and degradation were then carried out as described in the legend accompanying Table 1.1.

1.2.5. Effect of other Proteinase Inhibitors on Intracellular Proteolysis

Of the three inhibitors described above, chloroquine and NH_4Cl are known to have a lysosomotropic mode of action and leupeptin did not show any significant inhibitory action in excess of that seen with chloroquine or NH_4Cl alone. To search for inhibitors which are specific for extralysosomal pathways of proteolysis, a number of other proteinase inhibitors have been investigated.

(a) Chymostatin

Chymostatin is a peptide-aldehyde produced by actinomycetes which is known to inhibit chymotrypsin-like enzymes as well as papain and cathepsin B. Chymostatin (100 μg/ml) decreased the rate of degradation of long-lived proteins by 25% and the degradation rate of short-lived proteins by 15%. (Figure 1.17). Because chymostatin was solubilized in dimethyl sulphoxide (DMSO), the effect of this solvent alone was always tested and generally caused a slight increase in the protein degradation rate. Under conditions of accelerated proteolysis of long-lived proteins, the effect of chymostatin was reduced, showing only 13% inhibition. The effect of combining chymostatin and chloroquine in the incubation medium reduced the proteolysis rates further, with these two inhibitors showing complete additivity in most cases (Figure 1.17). Chymostatin is the only inhibitor used in this study that consistently showed complete additivity with chloroquine, and thus appears to be most diagnostic for extralysosomal proteolysis. Further, it is noteworthy that chymostatin inhibited the degradation of short-lived proteins more effectively than leupeptin, chloroquine or NH_4Cl, and this degradative process appears to be largely extralysosomal.

Chymostatin (100 μg/ml) did not inhibit the degradation of ^{125}I-LDL by these cells by amounts greater than did the presence of DMSO alone in the incubation medium (Table 1.5), which was therefore consistent with an extralysosomal site of action.

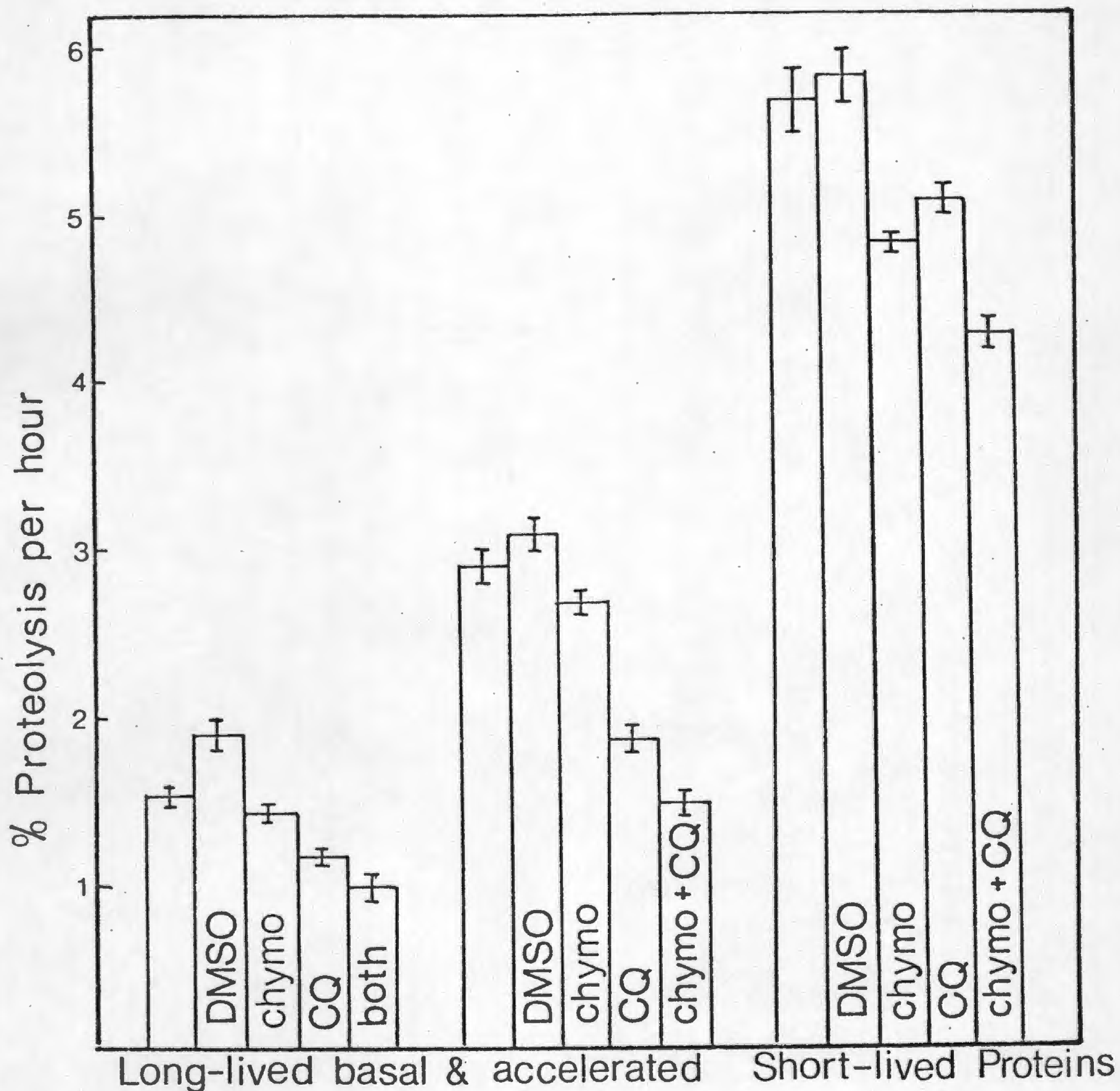


FIGURE 1.17. EFFECT OF CHYMOSTATIN ON INTRACELLULAR PROTEIN DEGRADATION.

Smooth muscle cells were labelled and proteolysis measurements made under the indicated conditions as described under "Experimental Procedures". Chymostatin (100 µg/ml) was dissolved in DMSO which was present at a concentration of 2,5% in the medium. Chymostatin and chloroquine (70 µM) were preincubated with the cells during the prechase wash periods. Each value represents the mean ± S.E. for 4 experiments.

CONDITIONS	ng ^{125}I-LDL BOUND PER DISH	ng ^{125}I-LDL INTERNALIZED PER DISH	ng ^{125}I-LDL DEGRADED PER DISH	% INHIBITION OF DEGRADATION
NO ADDITIONS	11,5 ± 1,1	66,7 ± 5,0	37,7 ± 3,1	-
+ 70 µM CHLOROQUINE	11,2 ± 2,0	99,4 ± 3,8	2,3 ± 0,8	95%
+ 2,5% DMSO	9,9 ± 0,9	38,9 ± 2,1	24,9 ± 2,8	34%
+ 100 µg/ml CHYMOSTATIN	10,4 ± 1,0	43,2 ± 2,8	25,1 ± 3,0	0% ✗?
+ 100 µg/ml PEPSTATIN	9,2 ± 0,8	45,9 ± 2,6	21,8 ± 1,7	12%

TABLE 1.5. EFFECT OF CHYMOSTATIN AND PEPSTATIN ON ^{125}I-LDL METABOLISM.

Smooth muscle cell layers were pre-incubated with inhibitors for 2 hours, before 20 µg/ml ^{125}I-LDL in LPDS-medium was added for an incubation period of 4 hours. Measurements of binding, internalization and degradation were then made as described under "Experimental Procedures". All values are Mean ± S.E. of 5 cell dishes.

(b) Pepstatin

Pepstatin is another proteinase inhibitor produced by actinomycetes cultures that inhibits carboxyl proteinases of which intralysosomal cathepsin D is the only known intracellular example. Pepstatin (100 μg/ml), dissolved in DMSO to facilitate its entry into the cells, produced a small but significant inhibition of the basal intracellular degradation of long-lived proteins (8%) but a much greater inhibition (22%) of the accelerated proteolysis of this group of proteins (Table 1.6). When the effect of this inhibitor was simultaneously tested on the degradation of ^{125}I-LDL by these cell layers, it was found to inhibit 10% in excess of degradation found in the presence of DMSO alone. The binding and internalization processes were not affected (Table 1.5). These results indicate that pepstatin may not be permeating the smooth muscle cells to the site of proteolysis under the experimental condition used. The inhibition values reported would thus be underestimates of the potential inhibition of pepstatin, as is particularly apparent in the case of LDL degradation (only 12% inhibition). The problem of low permeability of pepstatin has been previously reported (Libby and Goldberg, 1978; Ward *et al*., 1979) and in some cases, the inhibitor has been entrapped within phagocytosable liposomes to facilitate its permeation (Bohley *et al*., 1977a; Dean, 1975d). However, these results do indicate an increased role of lysosomal cathepsin D under accelerated proteolytic conditions, which is consistent with the results obtained using other lysosomotropic inhibitors.

(c) Chloromethyl Ketones

N-α-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK) inhibits the action of chymotrypsin-like proteinases and N-α-Tosyl-L-lysyl chloromethyl ketone (TLCK) inhibits the action of trypsin-like proteinases. Both inhibitors act as substrate analogues and inactivate the respective enzymes by irreversible covalent modification.

CONDITIONS	% PROTEOLYSIS PER HOUR	% INHIBITION
LONG-LIVED PROTEINS:		
(a) BASAL DEGRADATION	1,52 ± 0,08	-
+ 2,5% DMSO:	1,80 ± 0,07	-
+ 100 μg/ml PEPSTATIN	1,63 ± 0,04	10%
(b) ACCELERATED DEGRADATION	2,90 ± 0,10	-
+ 2,5% DMSO	3,11 ± 0,09	-
+ 100 μg/ml PEPSTATIN	2,36 ± 0,04	24%

TABLE 1.6. EFFECT OF PEPSTATIN ON DEGRADATION OF LONG-LIVED PROTEINS.

Smooth muscle cell proteins were labelled and proteolysis measured after 4 hours as described under Experimental Procedures". Pepstatin was dissolved in DMSO and preincubated with the cells for 2 hours. Each value is the Mean ± S.E. of 5 cell dishes.

(Shaw, Mares-Guia and Cohen, 1965). TPCK and TLCK are highly toxic to cells and there is only a narrow margin between an effective and a toxic dose. Under the experimental conditions used in this study, a dose of 100 μg/ml of each inhibitor was found to be effective and non-toxic. The viability of cell layers after treatment with inhibitor was evaluated by their ability to survive a further 24 hour incubation period after removal of inhibitor, and in each of the experiments reported here cell viability was judged to be 100%. TPCK and TLCK both exerted marked effects on the degradation of long-lived and short-lived proteins as shown in Figure 1.18.

TPCK inhibition was most marked under accelerated conditions (38%) and was found to be completely additive to the effect of chloroquine under all conditions. TLCK inhibited protein degradation to an even greater extent with inhibition most marked under accelerated conditions (89%), and additivity to chloroquine was also evident. Although it appeared that the chloromethyl ketones may be useful in identifying extralysosomal proteolysis, Table 1.7. shows that they have severe effects on ^{125}I-LDL degradation at a dose of 100 μg/ml, without affecting LDL binding or internalization. Thus, it seems that TPCK and TLCK are having some non-specific effects on smooth muscle cell metabolism, which does not allow definitive statements concerning pathways of protein degradation to be made from the results obtained.

1.2.6. Effect of Metabolic Inhibitors

In order to investigate the energy-dependence of the lysosomal and non-lysosomal pathways of protein degradation, a series of metabolic inhibitors in the presence or absence of chloroquine were employed. The degradation of long-lived proteins in the presence of three metabolic inhibitors, sodium fluoride, sodium azide and potassium cyanide, was significantly reduced by 45%, 30% and 12% respectively. All these inhibition values were found to be partially additive to the effect of chloroquine (Table 1.8), indicating that the requirement for energy is not

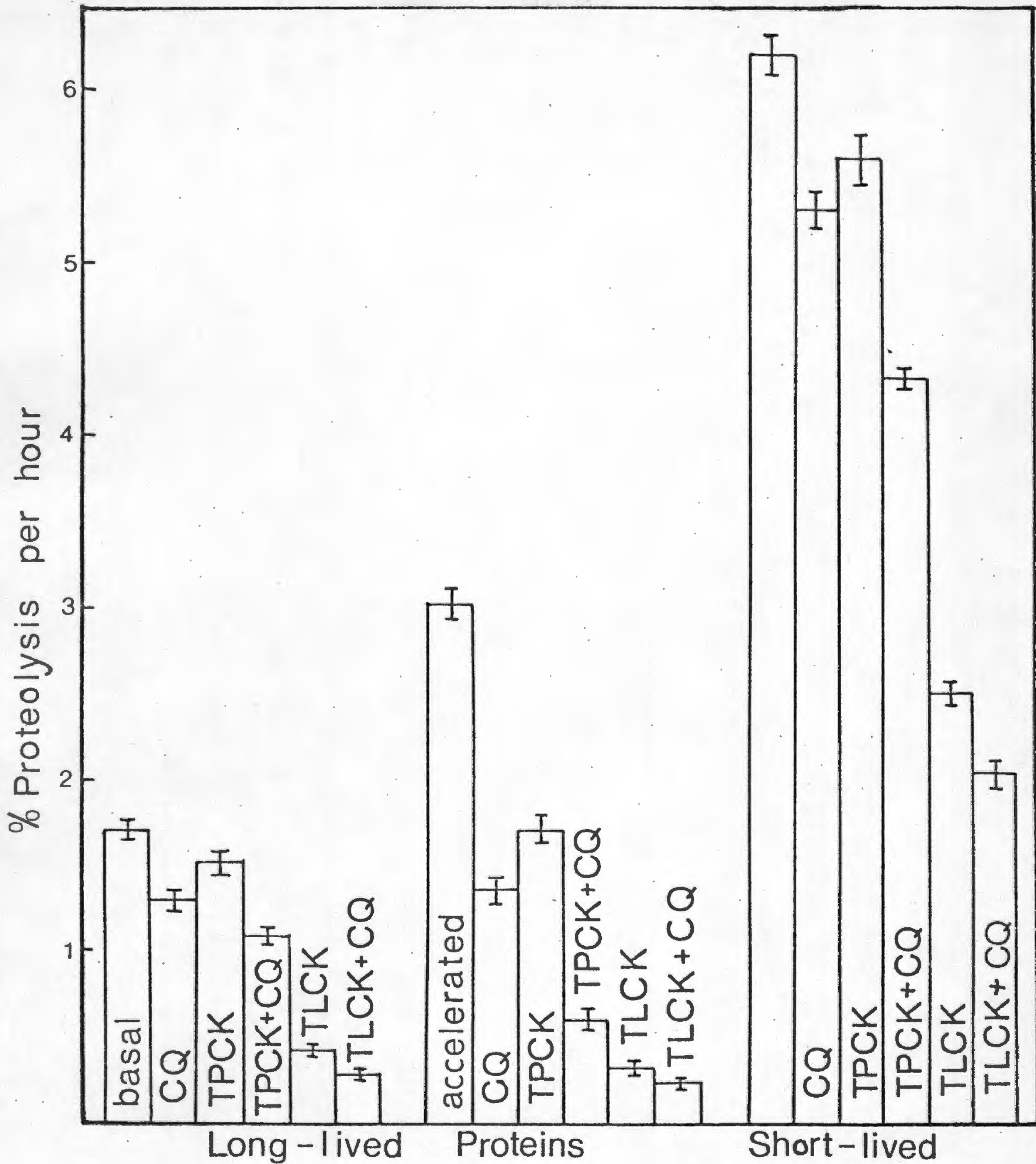


FIGURE 1.18. EFFECT OF TPCK AND TLCK ON INTRACELLULAR PROTEIN DEGRADATION

Smooth muscle cells were labelled and proteolysis measured under the indicated conditions. TPCK and TLCK (100 µg/ml) and chloroquine (70 µM) were preincubated with the cells during the prechase wash periods in each experiment. Each value represents the mean ± S.E. for three experiments.

CONDITIONS	ng ^{125}I-LDL BOUND PER DISH	ng ^{125}I-LDL INTERNALIZED PER DISH	ng ^{125}I-LDL DEGRADED PER DISH	% INHIBITION OF DEGRADATION
NO ADDITIONS	20,5 ± 1,8	211,6 ± 8,4	78,1 ± 2,3	-
+ 70 μM CHLOROQUINE	18,2 ± 0,9	301,2 ± 15,2	3,6 ± 0,3	96%
+ 50 μg/ml TPCK	15,9 ± 1,6	209,0 ± 11,3	61,0 ± 1,9	11%
+ 100 μg/ml TPCK	16,4 ± 1,9	211,9 ± 9,5	23,4 ± 0,9	70%
+ 50 μg/ml TLCK	15,2 ± 0,8	200,7 ± 6,9	3,9 ± 0,5	95%
+ 100 μg/ml TLCK	15,9 ± 0,7	198,7 ± 3,9	1,9 ± 0,2	98%

TABLE 1.7. EFFECT OF CHLOROMETHYL KETONES ON ^{125}I-LDL METABOLISM.

Smooth muscle cells were preincubated with the inhibitors for 2 hours before 20 μg/ml ^{125}I-LDL in LPDS-medium was added for an incubation period of 4 hours. Each value is the Mean ± S.E. for 6 cell dishes.

A: "LONG-LIVED" PROTEINS

CONDITIONS	% PROTEOLYSIS PER HOUR	% INHIBITION
NO ADDITIONS	1,51 ± 0,04 (n=4)	-
+ 70 μM CHLOROQUINE	1,16 ± 0,03 (n=6)	23%
+ 10 mM NaF	0,83 ± 0,03 (n=6)	45%
+ 20 mM NaN_3	1,06 ± 0,06 (n=4)	30%
+ 1 mM KCN	1,33 ± 0,04 (n=4)	12%
+ NaF + CQ	0,60 ± 0,05 (n=6)	60%
+ NaN_3 + CQ	0,87 ± 0,05 (n=4)	42%
+ KCN + CQ	1,09 ± 0,05 (n=4)	28%

B: "SHORT-LIVED" PROTEINS

CONDITIONS	% PROTEOLYSIS PER HOUR	% INHIBITION
NO ADDITIONS	7,04 ± 0,09 (n=6)	-
+ 70 μM CHLOROQUINE	6,12 ± 0,10 (n=6)	13%
+ 10 mM NaF	4,65 ± 0,06 (n=4)	34%
+ NaF + CQ	3,80 ± 0,08 (n=4)	46%

TABLE 1.8. EFFECT OF METABOLIC INHIBITORS ON INTRACELLULAR PROTEOLYSIS RATES.

A: "Long-lived" proteins were prepared as described in the legend accompanying Figure 1.9. Inhibitors were pre-incubated with the cells during the 2-hour wash period prior to the 4-hour degradation incubation (in the presence of 1.1 mM phenylalanine) after which proteolysis measurements were made.

B: "Short-lived" proteins were prepared as described in the legend accompanying Figure 1.11. The inhibitors were preincubated with the cells during the 1-hour wash period prior to the 1-hour degradation incubation (in the presence of 1.1 mM phenylalanine) after which proteolysis measurements were made.

exclusively intralysosomal or extralysosomal but rather that both sites for protein degradation require some energy. The effect of these inhibitors on the degradation of short-lived proteins was less marked, for example, sodium fluoride only inhibited degradation by 30%. In this case, the effect of NaF was found to be almost completely additive to the inhibition obtained with chloroquine (Table 1.8), indicating that the greater part of the energy requirement is for extralysosomal mechanisms.

1.2.7. Effect of Lowered Oxygen Tension on Protein Degradation

The result of incubating the smooth muscle cell layers in an atmosphere containing only 5% oxygen instead of the normal 20% O_2 during the four-hour degradation period, reduced the basal rate of degradation of intracellular long-lived proteins by a small (8-10%) but significant amount (Table 1.9). However, when the cells were incubated under N_2 to inhibit energy-requiring cellular processes, i.e. anoxic conditions, the degradation rate of long-lived proteins was inhibited by 35-40%. The inhibition was significantly greater than that of chloroquine (28%), and showed a partially additive effect with chloroquine (Table 1.9), again indicating that both extra- and intra-lysosomal mechanisms require energy, with the bulk of the requirement apparently for extralysosomal degradation.

1.2.8. Effect of Decreased Temperature

In an attempt to obtain independent evidence that long and short-lived proteins may be hydrolyzed by distinct mechanisms, the temperature dependence of these processes was compared. Smooth muscle cell cultures were treated as described above to label predominantly long-lived or short-lived proteins. At the end of the labelling period, cultures were transferred to fresh incubation medium containing 10 mM HEPES buffer, and maintained at temperatures of 37°C, 28°C, 20°C or 15°C. The degradation of labelled proteins was compared and Figure 1.19. shows that at lower temperatures, the release of radioactivity from both types of protein was significantly reduced below

CONDITIONS	% PROTEOLYSIS PER HOUR	% INHIBITION
20% O_2	1,54 ± 0,06 (n=9)	-
5% O_2	1,40 ± 0,05 (n=5)	10%
20% O_2 + 70 µM CHLOROQUINE	1,11 ± 0,07 (n=5)	28%
0% O_2 (ANOXIC)	0,95 ± 0,04 (n=6)	38%
0% O_2 + 70 µM CHLOROQUINE	0,68 ± 0,07 (n=6)	56%

TABLE 1.9. EFFECT OF O_2 TENSION ON INTRACELLULAR PROTEIN DEGRADATION

Smooth muscle cells were labelled for 16 hours with 1 µCi/ml [^{3}H]-phenylalanine, washed for 2 hours at 37°C during which time they were preincubated under conditions indicated above in growth medium containing 10 mM HEPES buffer. Proteolysis measurements were made after a following 4-hour incubation under indicated conditions in the presence of 1.1 mM non-radioactive phenylalanine.

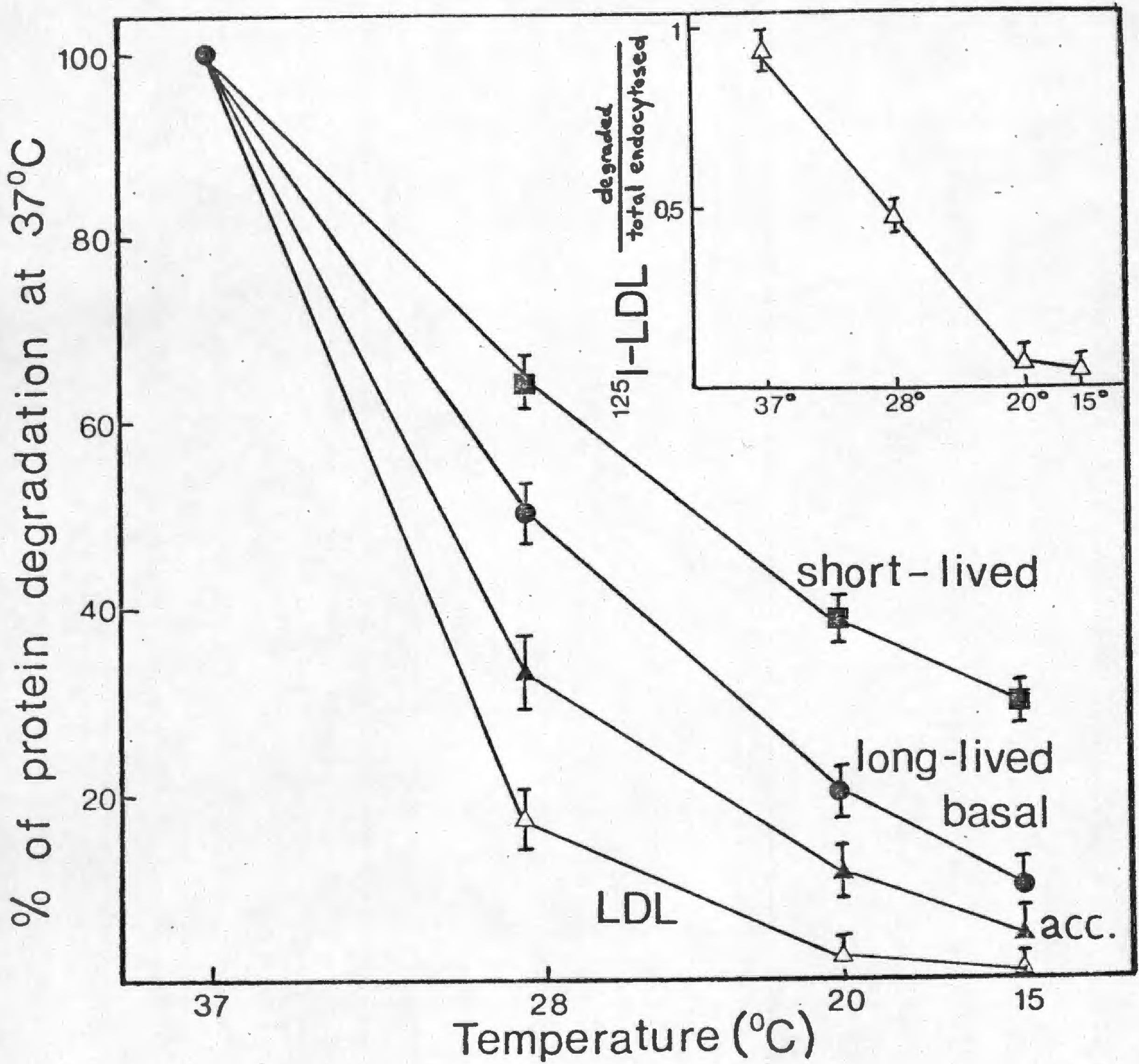


FIGURE 1.19. INHIBITION OF PROTEIN DEGRADATION AT DECREASED TEMPERATURE

Smooth muscle cells were labelled and protein degradation was measured for each indicated class of proteins as described under "Experimental Procedures 3,4,5". Each point is the mean ± S.E. for 4 cell dishes.

rates at 37°C. These differences in the observed temperature dependence was not due to a failure of the radioactive amino acids to exit from intracellular pools into the medium at lower temperatures since the intracellular acid-soluble pools remained constant during the degradation period.

In the case of short-lived proteins, a fairly linear relationship was found between the rate of degradation and temperature (Figure 1.19). The response to temperature decrease was markedly greater for long-lived proteins, particularly under accelerated conditions. In addition, a more marked deviation from linearity was evident, particularly under accelerated conditions.

The effect of temperature on LDL degradation was also analyzed. In order to control for variations in the amount of LDL taken up by the cells at the different temperatures, degradation was also expressed as a function of the total amount of LDL taken up by the cells during the incubation period (i.e. "internalized" LDL + "degraded" LDL) (Figure 1.19). Degradation showed a marked dependence on temperature with a sharp increase in rate above approximately 20°C. It has been postulated that at a critical temperature, the fusion of endocytotic vesicles with lysosomes may be prevented, and that the sharp inflection seen in the degradation curve may reflect the inhibition of such a fusion process.

The results reported here confirm the finding of a non-linear temperature response for the degradation of an extracellular protein, and suggest a fundamental difference in the degradative mechanisms operating for long-lived and short-lived proteins. The results also indicate the possibility that processes involving membrane fusion may be operating in the case of long-lived proteins, especially under accelerated conditions.

1.2.9. Effect of Ascorbate on Protein Degradation

Since it has been reported that LDL apoprotein degradation is affected by ascorbic acid (Coetzee, G.A., Stein, O. and Stein, Y. (1979)

Atherosclerosis 32, 277-287) the effect of ascorbate on endogenous protein degradation was tested. The effect of incubating smooth muscle cell layers in the presence of 50 μg/ml ascorbate for a period of 24 hours did not significantly alter the rate of intracellular protein degradation, although there was an increased incorporation of label during this period (Table 1.10A). However, the effect of ascorbate on the degradation of ^{125}I-LDL by these cells was very marked, increasing by 2 to 3-fold in each case (Table 1.10B). Incubation of ascorbate in combination with inhibitors of protein degradation did not affect the inhibitory activity of ammonium chloride or chloroquine but generally had some effect on the activity of leupeptin. In the case of intracellular proteolysis, the effect of leupeptin appeared to be reduced or eliminated in the presence of ascorbate. Conversely, the effect of leupeptin on the degradation of ^{125}I-LDL was significantly greater in the presence of ascorbate.

1.2.10. The Effect of Extracellular Calcium and Ionophore A23187 on Protein Degradation

In view of the possible importance of Ca^{++} in the regulation of muscle protein degradation and the existence of Ca^{++}-activated proteinase in muscle tissues (see Introduction 3.5), it seemed important to investigate whether manipulation of intracellular Ca^{++} levels in cultured muscle cells could affect the rate of protein degradation. The addition of excess Ca^{++} to the incubation medium (up to 10 mM $CaCl_2$) resulted in an increased rate of intracellular proteolysis which was maximal at 5 mM excess $CaCl_2$ (Figure 1.20). The rate of intracellular proteolysis could be further increased by the inclusion of ionophore A23187 (1 μg/ml) in the presence of 5 mM excess $CaCl_2$. The preparation of A23187 used was found to be extremely toxic to the smooth muscle cell layers if used at concentrations exceeding 1 μg/ml. The effect of the ionophore solvent alone, absolute ethanol (final conc. 0,5%), was also measured in each experiment. The effect of ionophore A23187 was insignificant if used alone, and depended on the presence of extracellular Ca^{++}. The increase in the proteolysis rate

A.

CONDITIONS	% PROTEOLYSIS PER HOUR	% INHIBITION
NO ADDITIONS	1,65 ± 0,07 (n=4)	-
+ 50 μg/ml ASCORBATE	1,54 ± 0,10 (n=4)	7%
+ 100 μg/ml LEUPEPTIN	1,48 ± 0,03 (n=4)	10%
+ 70 μM CHLOROQUINE	1,20 ± 0,05 (n=4)	27%
+ 10 mM NH_4Cl	1,27 ± 0,06 (n=4)	23%
ASCORBATE + LEUPEPTIN	1,55 ± 0,04 (n=4)	0%
ASCORBATE + NH_4Cl	1,11 ± 0,03 (n=4)	28%
ASCORBATE + CQ	1,20 ± 0,05 (n=4)	22%

B.

CONDITIONS (n=4)	ng ^{125}I-LDL BOUND PER mg PROTEIN	ng ^{125}I-LDL INTERNALIZED/mg PROTEIN	ng ^{125}I-LDL DEGRADED PER mg PROTEIN	% INHIBITION DEGRADATION
NO ADDITIONS	53,4 ± 3,1	175,6 ± 9,2	244,2 ± 12,8	-
+ 50 μg/ml ASCORBATE	58,9 ± 4,5	244,0 ± 15,4	866,0 ± 13,0	-
+ 100 μg/ml LEUPEPTIN	53,4 ± 2,9	295,6 ± 12,2	110,7 ± 7,2	55%
+ ASCORBATE + LEUPEPTIN	62,2 ± 3,7	685,9 ± 18,8	272,7 ± 10,3	69%

TABLE 1.10. EFFECT OF ASCORBATE ON PROTEIN DEGRADATION

A. Smooth muscle cells were labelled and degradation of "long-lived" proteins was measured after 4 hours. Ascorbate and leupeptin were preincubated with the cells for 18 hours, whilst NH_4Cl and chloroquine were preincubated for 2 hours.

B. Smooth muscle cells were incubated with 20 μg/ml ^{125}I-LDL in LPDS-medium for 4 hours before binding, internalization and degradation were measured. Ascorbate and leupeptin were preincubated with the cells for 18 hours in normal growth medium.

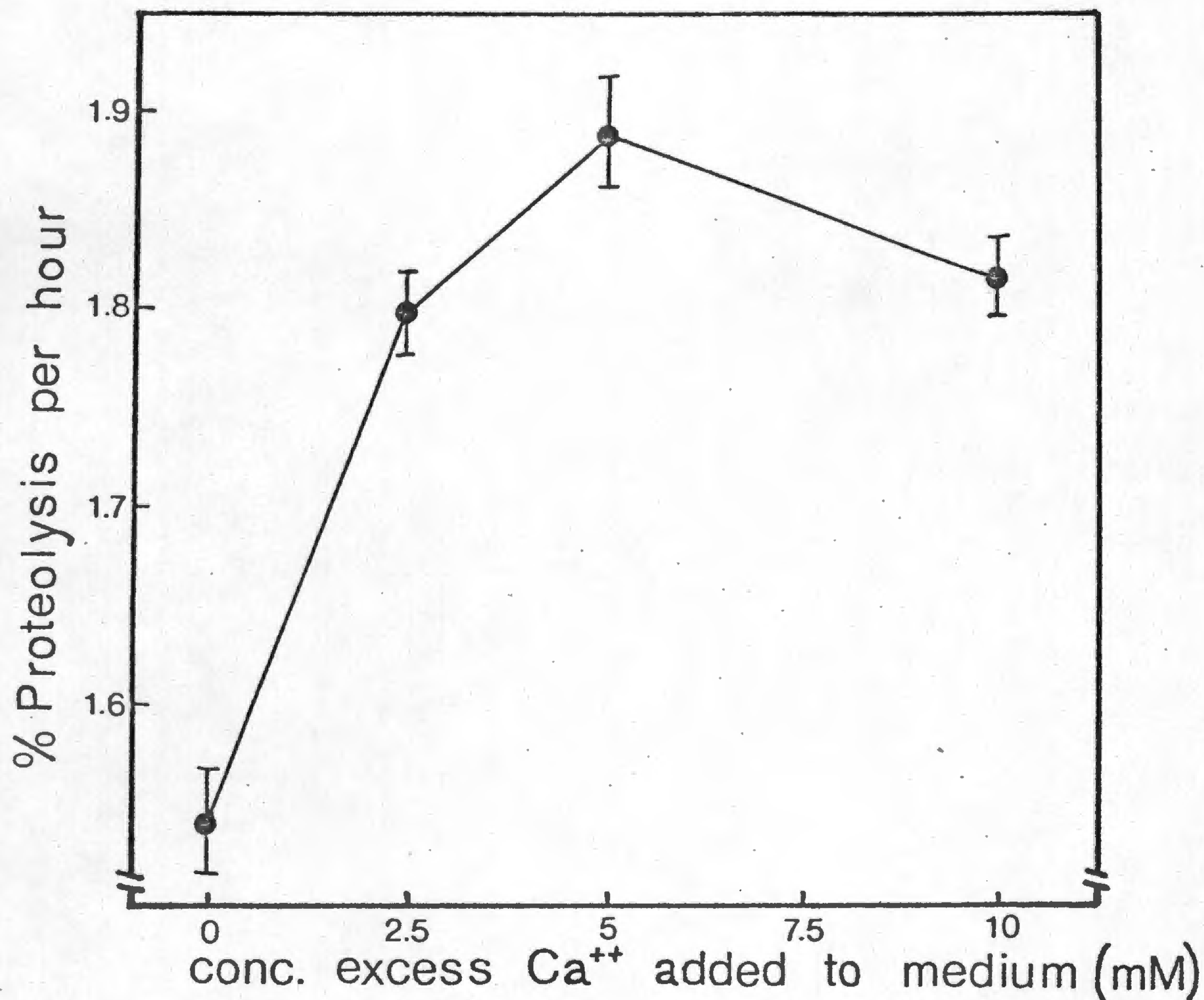


FIGURE 1.20. EFFECT OF EXTRACELLULAR Ca^{++} CONCENTRATION ON THE DEGRADATION OF LONG-LIVED PROTEINS.

Smooth muscle cells were labelled with 1 μCi/ml ^{3}H-phenylalnine for 16 hours, and degradation was measured over 4 hours in the presence of different concentrations of $CaCl_2$. Each point is the mean ± S.E. of 3 cell dishes.

appeared to be completely inhibited by the presence of leupeptin (100 µg/ml) in the incubation medium (Table 1.11). It is noteworthy that this inhibition by leupeptin (23%) was larger than any previously obtained value (see Figure 1.10).

1.3. PROTEIN DEGRADATION IN SKELETAL MUSCLE CELLS

1.3.1. Characterization of L8 Cell Line

1.3.1.1. Growth of cells on different substrates

L8 cells from rat skeletal muscle were seeded (2×10^5 cells/ 60 mm petri dish) in dishes coated with collagen by gluteraldehyde fixation, in dishes coated with 0,5% gelatin or in uncoated dishes. The process of growth and myogenesis was monitored by cell counts, visually under phase-contrast microscopy and by analyzing cellular protein using SDS-polyacrylamide gel electrophoresis. The cells seeded in uncoated dishes were found to attach and proliferate more quickly than those seeded in the coated dishes. The cells also remained viable in the differentiated state (generally attained by 7-8 days) for a further 5-6 days on uncoated dishes, and this period was sufficient for all manipulations to be performed.

1.3.1.2. Myogenesis in culture

The growth pattern of L8 cells on uncoated dishes in culture is shown in the series of photographs of Figure 1.21. Figure 1.22A shows that after 10 days of growth, approximately 90% of nuclei are present within myotubes. The growth curve of these cells, shown in Figure 1.22B, illustrates the continual increase in the amount of protein in the culture dish and shows the arrest of DNA synthesis shortly after the onset of myoblast fusion. Beating of differentiated myotubes was visible from about the third day after the onset of fusion and myogenesis was accompanied by a marked accumulation of myosin and actin (data not shown).

CONDITIONS	% PROTEOLYSIS PER HOUR	COMMENT
LONG-LIVED PROTEINS:		
BASAL DEGRADATION	1,63 ± 0,05	-
+ 0,5% ETHANOL	1,60 ± 0,06	NO SIGNIFICANT EFFECT
+ 1 μg/ml A23187	1,61 ± 0,08	NO CHANGE
+ 5 mM $CaCl_2$	2,00 ± 0,03	22% INCREASE
+ A23187 + 5 mM $CaCl_2$	2,07 ± 0,04	27% INCREASE
+ A23187 + 5 mM $CaCl_2$ + 100 μg/ml LEUPEPTIN	1,61 ± 0,04	NO CHANGE

TABLE 1.11. EFFECT OF IONOPHORE A23187 AND Ca^{++} CONCENTRATION ON PROTEOLYSIS OF LONG-LIVED PROTEINS

Smooth muscle cells were preincubated with A23187 and leupeptin for 2 hours. Proteolysis measurements were made after a further 4-hour incubation. Each value is the mean ± S.E. of three cell dishes.

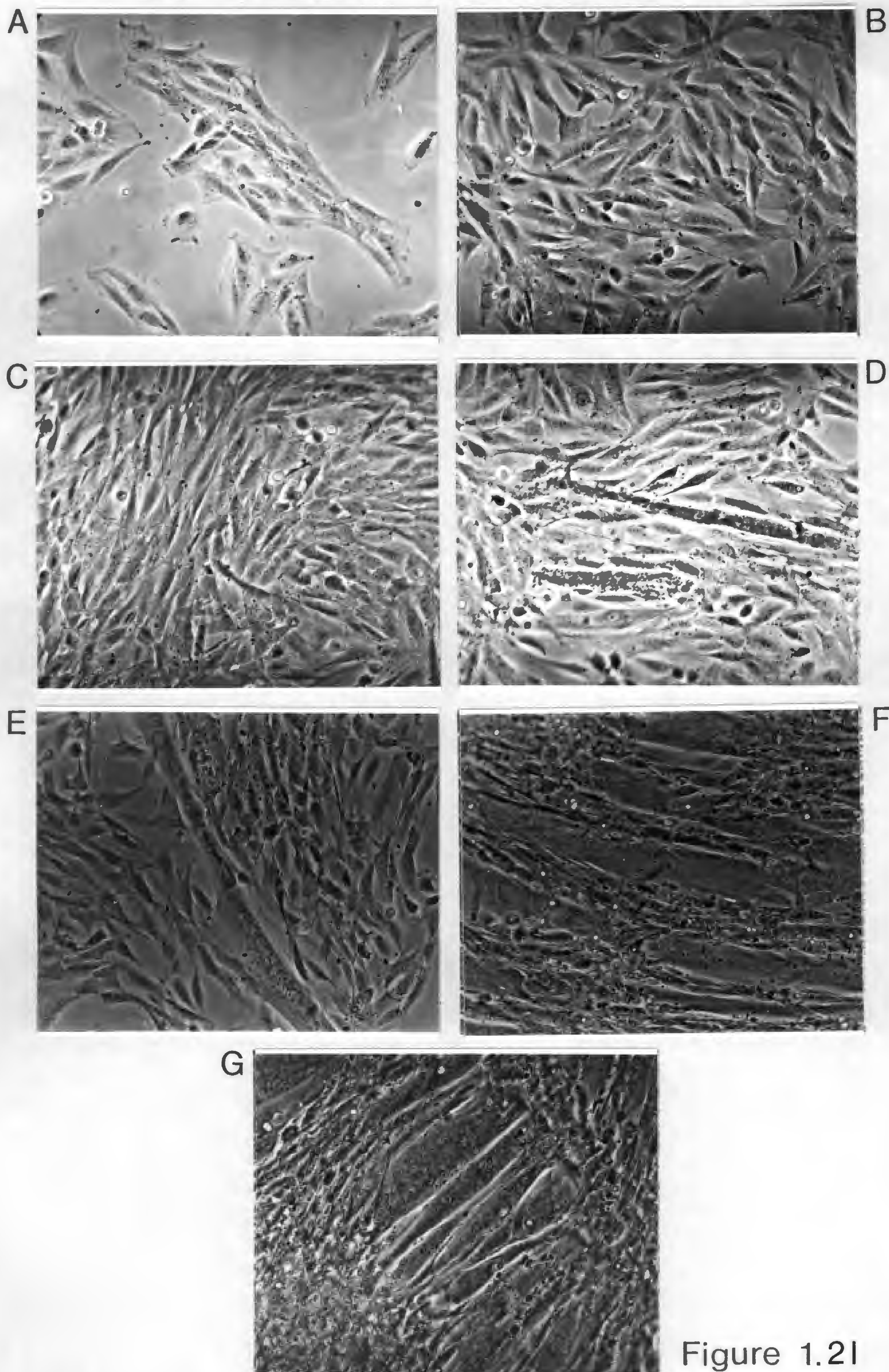


Figure 1.21

FIGURE 1.21. PHASE CONTRAST MICROGRAPHS OF L8 SKELETAL MUSCLE CELLS IN CULTURE

(Magnification 600x)

A: 24 hours in culture (Day 1). Cells seeded at 10^5 myoblasts per 35 mm culture dish.

B: Day 2 - rapid myoblast proliferation to confluence.

C: Day 3 - confluent monolayers, cells lining up for fusion.

D: Day 4 } Progressive fusion to form large multinucleate

E: Day 5 } syncitia (myotubes).

F: Day 6 - 70-80% fusion, synthesis of muscle-specific proteins begins.

G: Day 7 - up to 90% fusion; spontaneous contraction of the myotubues becomes visible.

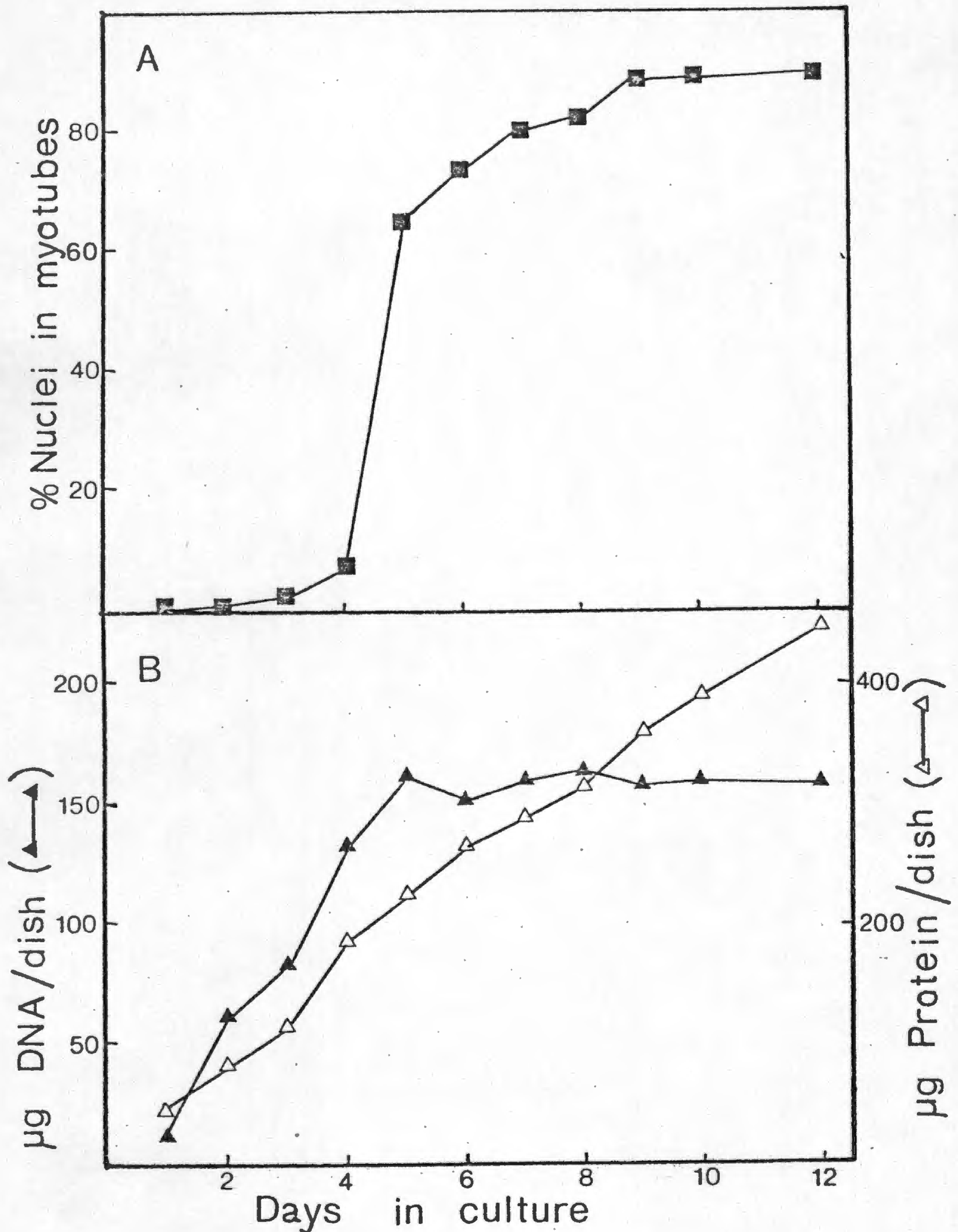


FIGURE 1.22. GROWTH CURVE OF L8 CELLS IN CULTURE.

A: Cell layers were fixed and stained as described under "Experimental Procedures 1.2". Each point was determined by counting 10000 nuclei in at least 15 fields of view.

B: L8 myoblasts were plated at $2x10^5$ cells/60 mm dish and harvested daily for estimation of DNA and protein as described under "Experimental Procedures, 2".

1.3.2. Basal Proteolysis of Long-Lived Proteins

Under nutritionally optimal conditions, the rate of degradation of intracellular proteins prelabelled with [^{3}H]-pnenylalanine for 16 hours, was found to be 1,2 - 1,3% per hour. This rate proceeded linearly for the 12-hour period tested (Figure 1.23). Leupeptin (100 µg/ml) inhibited this degradation rate by 5%, and this inhibition value was not increased on increasing the preincubation period with the inhibitor (Figure 1.24). NH_4Cl (10 mM) was found to reduce the degradation rate by 22% and chloroquine (70 µM) had a larger inhibition value of 28%. No significant additive effects could be obtained by combining these inhibitors (Figure 1.24).

1.3.3. Accelerated Proteolysis of Long-Lived Proteins

Incubation of differentiated cell layers labelled for 16 hours with [^{3}H]-phenylalanine in a minimal medium, i.e. horse serum and chick embryo extract removed, did not accelerate the basal degradation to the same extent with smooth muscle cells (approx. 100% increase), but the protein degradation rate was nevertheless accelerated by about 50% (Figure 1.23). The effects of the inhibitors were markedly increased under accelerated conditions, with leupeptin inhibition increasing from 5% to 25%, and NH_4Cl and chloroquine resulting in inhibitions of 36% and 45%, respectively. No significant additivity was evident when these inhibitors were used in combination (Figure 1.24).

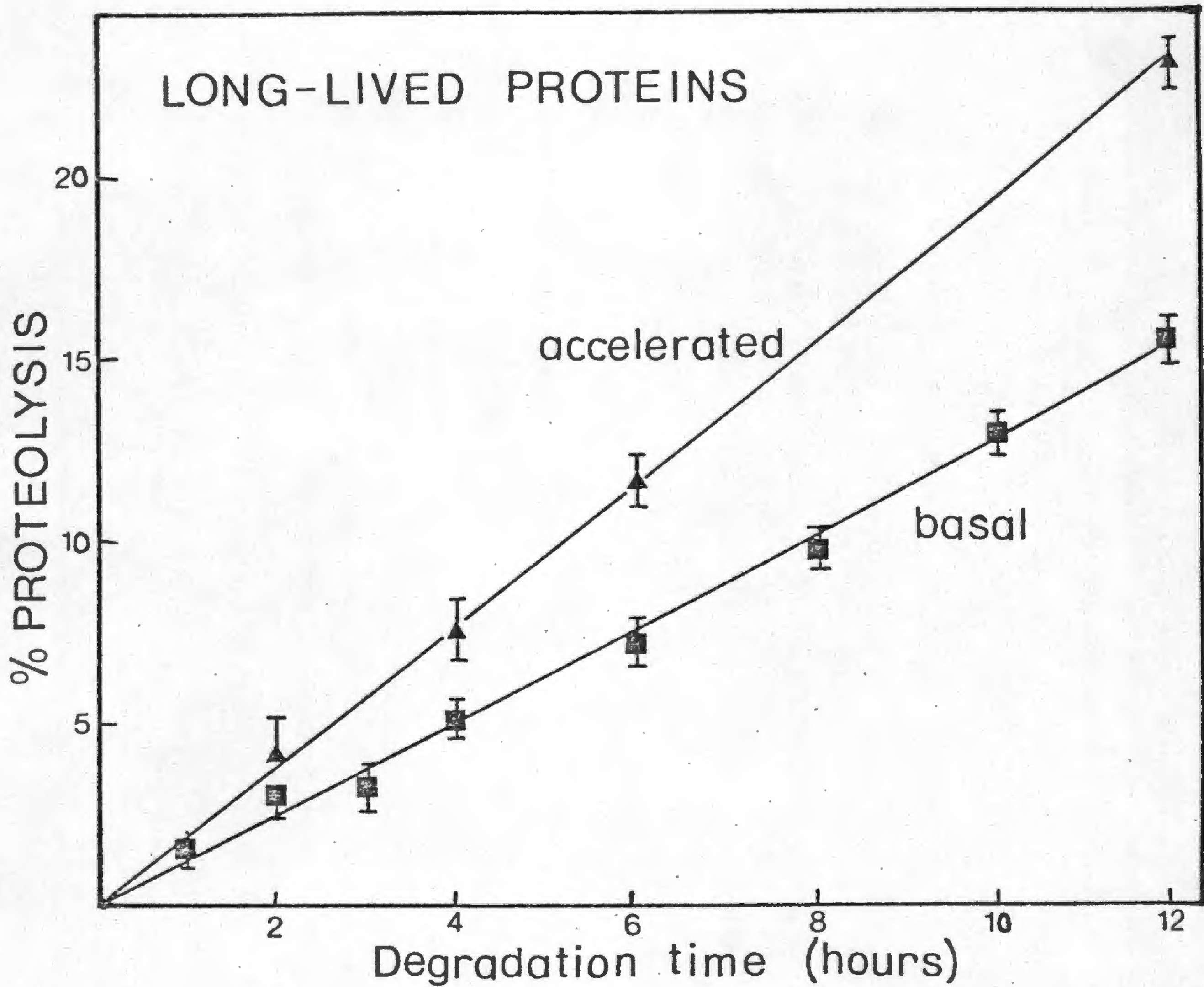


FIGURE 1.23. BASAL AND ACCELERATED DEGRADATION OF LONG-LIVED SKELETAL MUSCLE PROTEINS.

L8 skeletal muscle cells were labelled for 16 hours with 1 µCi/ml ^{3}H-phenylalanine, and proteolysis measurements were made under basal and accelerated conditions as described under "Experimental Procedures 3,4". Each point is the mean ± S.E. for 4 cell dishes.
■——■ Incubations in complete growth medium (basal proteolysis)
▲——▲ Incubations in minimal medium (accelerated proteolysis).

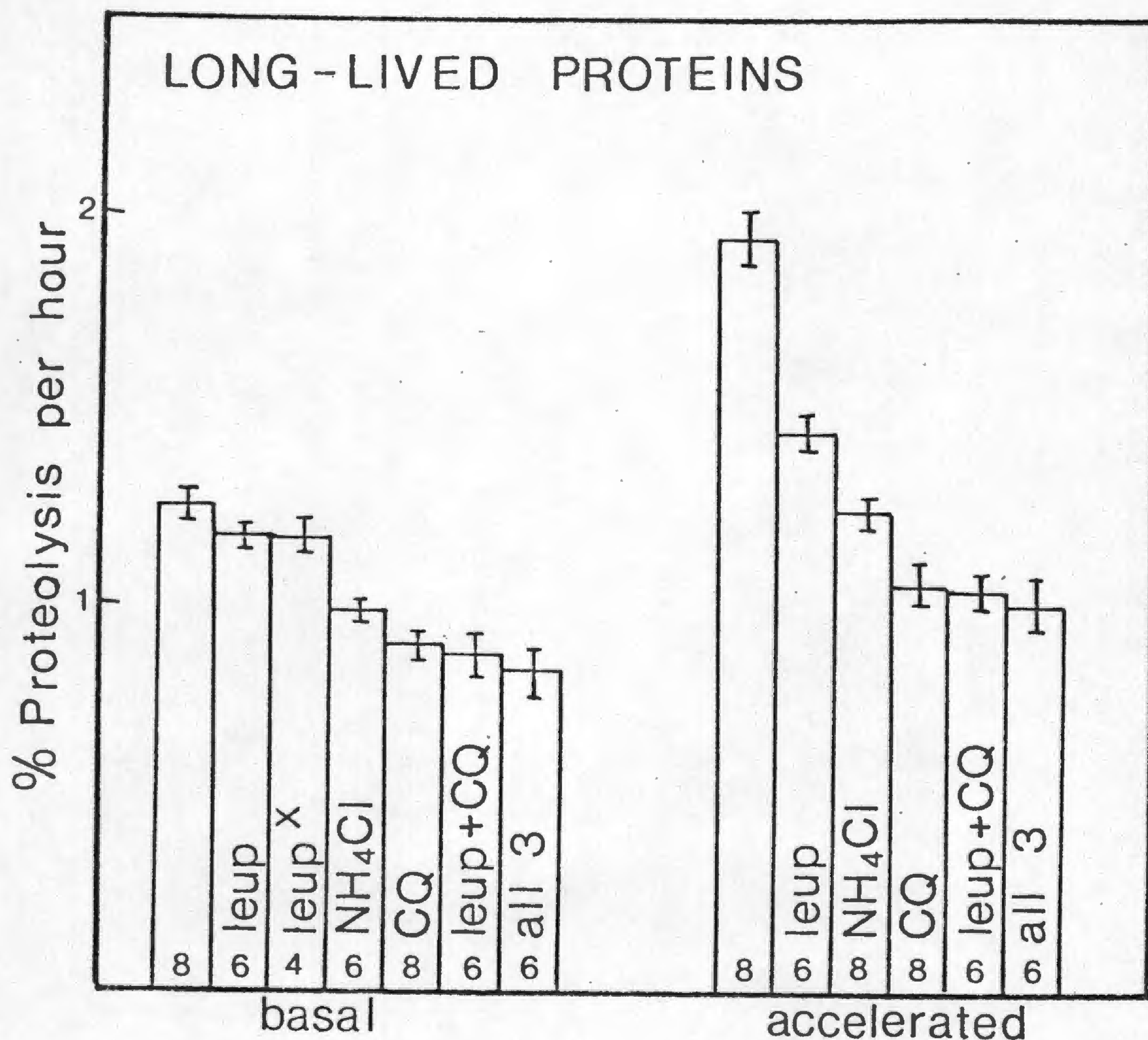


FIGURE 1.24. INHIBITION OF PROTEOLYSIS IN L8 SKELETAL MUSCLE CELLS.

All measurements were made after 4 hours of degradation and the inhibitors were preincubated with the cells for 2 hours as described under "Experimental Procedures, 6". Numbers indicate the amount of cell dishes analyzed in at least 3 different experiments.

x indicates an 18-hour preincubation of leupeptin with the cells.

DISCUSSION

The basal intracellular protein turnover rate of 1,5% per hour found in the cultured muscle cells used in this study, is consistent with other values reported for cells in culture, generally 1-2% per hour under normal growth conditions (Amenta et al., 1977, 1978; Epstein et al., 1975; Warburton and Poole, 1977; Castor, 1977; Bradley 1977; Robinson et al., 1976; Tanaka and Ichihara, 1978). The results of this study also show that nutritional deprivation is a significant stimulus to the degradation rate of intracellular muscle cell proteins in accordance with previous reports (Mayorek et al., 1979; Jenkins et al., 1979; Ward et al., 1979; Vandenburgh and Kaufman, 1980), and that both smooth and skeletal muscle cells respond similarly to nutritional step down conditions.

Most cell types have been found to contain proteins of very heterogeneous half-lives. Skeletal muscle, for example, appears to have a small group of proteins with rapid turnovers such as rate-limiting enzymes involved in metabolic control, and a larger group of structural proteins such as myosin and actin which turn over much more slowly (Shemin and Rittenberg, 1944; Berlin and Schimke, 1965). Muscle cells labelled by the techniques described here, will differentially incorporate label into rapidly turning over proteins and slowly turning over proteins (Knowles and Ballard, 1976; Vandenburgh and Kaufman, 1980; Neff et al., 1979), and it was found that short-lived proteins were generally much less affected than long-lived proteins by the various experimental manipulations described in this study. The co-ordinated investigation of the degradation of normal long-lived and short-lived proteins, and of abnormal proteins in a single cell type has pointed towards the possible functioning of separate catabolic pathways for these classes of proteins in cultured muscle cells.

The results presented in this study provide several lines of evidence for the functioning of non-lysosomal as well as lysosomal pathways for protein degradation, with the non-lysosomal pathway playing an important role in basal protein

turnover, particularly with respect to the short-lived class of proteins. This finding is in agreement with a number of previous reports (Knowles and Ballard, 1976; Amenta et al., 1978c; Warburton and Poole, 1977), but in this study, the proposal is more firmly substantiated because the basal rate of degradation of long-lived and short-lived proteins was only affected to a limited extent (maximum of 30% and 15% respectively) by lysosomotropic agents such as chloroquine and ammonium chloride which fully arrested the breakdown of endocytosed ^{125}I-labelled LDL (see Figure 1.14 and 1.16). The use of a lipoprotein, internalized by the cell through endocytosis, to evaluate the effectiveness of lysosomotropic inhibitors has been invaluable in the interpretation of the results in this study, because it has, for the first time, given a clear indication of their extent of action on the intralysosomal degradation pathway. The fact that chloroquine, at the concentration routinely used (70 μM), blocked lysosomal degradation by over 95% without significantly affecting the receptor-binding and internalization of LDL, makes it a most useful diagnostic agent for lysosomal proteolysis (see Tables 1.2, 1.3, 1.4).

In a recent study of protein degradation in rat yolk sacs (Livesey et al., 1980), lysosomotropic weak bases were found to substantially lower the rate of pinocytosis of ^{125}I-labelled albumin. These results illustrate the necessity of measuring the rates of uptake of albumin in order to assess the effects of the weak bases on intralysosomal proteolysis since uptake is known to be rate-limiting in the overall process of ingestion and breakdown of this exogenous protein. The use of endocytosed LDL with cultured smooth muscle cells preclude any complex corrections of this nature since the uptake process for LDL generally remained unaltered. This allowed direct comparisons of the effectiveness of several lysosomotropic agents on endogenous and exogenous proteolysis. Therefore, because chloroquine only showed a limited inhibition of endogenous protein degradation, it clarifies the role of the lysosome in basal proteolysis, restricting it to a maximum of

30%, and thus confirms that extralysosomal degradative mechanisms are responsible for approximately 70% of the average basal turnover of endogenous muscle proteins.

However, the accelerated rate of endogenous proteolysis (3% per hour) found under nutritional step-down conditions was reduced to the basal level by NH_4Cl and chloroquine, in accordance with findings reported by Amenta and co-workers (1977, 1978b,c). A nutritional step-down, therefore, seems specifically to activate the lysosomal pathway of degradation, suggesting that one of the functions of this pathway may be to serve as an emergency system for the nutritional utilization of available protein reserves during starvation. These findings are, however, inconsistent with those reported by Jenkins et al. (1979) and Ward et al. (1979). The former workers have related the increased muscle proteolysis in the nutrient-deprived state to an increase in non-lysosomal proteolytic activity and the latter group have proposed that the proteolytic pathways operating under normal and deprived conditions in perfused rat liver and heart are proportionately the same, with no selective activation of either pathway in starvation. The findings based on the use of leupeptin and pepstatin may have the severe limitation of the inaccessibility of these inhibitors to the intracellular sites of proteolysis.

The results of this study are consistent with the view that the majority of short-lived proteins are degraded by non-lysosomal pathways (Ballard, 1977; Knowles and Ballard, 1976). The degradation of short-lived muscle proteins appeared to be almost exclusively extralysosomal because of the very small effects of chloroquine and NH_4Cl (12-15% inhibition) at concentration which almost fully arrested LDL degradation (see Figure 1.16). The rapid degradation of these proteins was not accelerated by nutritional step-down conditions, indicating that this class of proteins is probably not a readily available protein reserve during periods of dietary stress. The increased lysosomal activity did nevertheless result in a greater proportion of short-lived proteins being degraded intralysosomally.

Chymostatin has been found to inhibit lysosomal proteinases cathepsin B, G and L (Libby and Goldberg, 1980), the Ca^{++}-activated proteinase in skeletal muscle (Toyo-Oka _et al_., 1978), chymotrypsin and is also a potent inhibitor of alkaline proteolytic activity in rat muscle homogenates (Libby and Goldberg, 1980) although this activity probably originates from mast cells rather than myocytes (Woodbury and Neurath, 1978). Since chymostatin did not produce toxic effects in cultured muscle cells at concentrations that inhibited protein degradation, it could be used as a probe to study intracellular proteolytic pathways. It was unique among the inhibitors tested in causing an exclusive and pronounced effect on non-lysosomal proteolysis, deduced from its complete additive effect with chloroquine (see Figure 1.17). The action of chymostatin was also unique in that it produced a smaller inhibition of accelerated compared to basal proteolysis, pointing to an extralysosomal site of action in the former. Chymostatin also had no effect on the degradation of endocytosed LDL (see Table 1.5) and should therefore prove to be a powerful tool in the investigation of extralysosomal pathways of protein degradation.

Use of the chloromethyl ketones (TPCK and TLCK) indicated the possible extralysosomal involvement of trypsin-like (TLCK-sensitive) and chymotrypsin-like (TPCK-sensitive) enzymes, but their usefulness was limited by cell toxicity and other non-specific effects. The results were probably most meaningful in the case of short-lived proteins where the short exposure times limited their toxic effects (see Figure 1.18).

The studies using metabolic inhibitors show, in accordance with other findings, that energy-dependence is not restricted to either intra- or extra-lysosomal proteolysis (Seglen, _et al_., 1979). In this case, most of the energy appeared to be required extralysosomally with a small portion required intra-lysosomally, shown by incomplete additivity of the effects of the inhibitors with chloroquine (Table 1.8). Lowering the O_2 tension of the environment also illustrated an energy-requirement for protein degradation, with most of the requirement

still evident under conditions which block lysosomal activity (Table 1.9).

The differences observed between the temperature-dependence of the degradation of various class of proteins (see Figure 1.19) also suggest distinct proteolytic pathways or at least distinct rate-limiting steps. The non-linear response of degradation to increased temperature in the case of long-lived proteins, particularly under starvation conditions, is suggestive of a membrane-involved process such as membrane fusion. Possibly this process corresponds to a fusion of autophagic vacuoles with lysosomes, a process expected to occur more extensively under starvation conditions and not to involve short-lived proteins. These experiments therefore clearly support the conclusions based on the inhibitor studies.

The results presented here suggest that an increase in the intracellular concentration of Ca^{++} can increase the rate of muscle protein degradation as has been previously reported (Sugden, 1980). Inclusion of ionophore A23187, however, did not further increase the proteolytic rate, but this may be due to the very low levels used to preclude cell toxicity. The increase may be due to activation of the Ca^{++}-activated proteinase in muscle which is also known to be inhibited by leupeptin.

CHAPTER 2: CHARACTERIZATION OF INTRACELLULAR PROTEIN DEGRADATION IN CULTURED MUSCLE CELLS

2.1. CORRELATION BETWEEN SUBUNIT SIZE AND RELATIVE DEGRADATION RATE OF INTRACELLULAR PROTEINS

(a) Smooth Muscle Cells

Double-labelled intracellular proteins were run on gradient SDS-polyacrylamide gels, giving protein profiles as illustrated in Figure 2.1. Figure 2.2. shows that a clear correlation exists between protein subunit size and half-life for intracellular proteins from cultured smooth muscle cells incubated under normal nutritional conditions, since the larger molecular weight polypeptides tended to have higher ^{3}H : ^{14}C ratios. The dotted line in Figure 2.2. indicates the variation in ratio obtained in a control experiment using proteins from cells labelled simultaneously with both isotopes ($[^{3}H]$-phenylalanine and $[^{14}C]$-phenylalanine) for 16 hours before being harvested. No systematic variations in the ^{3}H : ^{14}C ratios are evident. Figure 2.1. also shows that the relationship between protein size and degradation rate is largely obliterated when smooth muscle cells are incubated under starved conditions. Similar trends in double label ratios were found in four repeat experiments.

Figures 2.3. and 2.4. show results obtained when different double-labelling protocols, chosen to analyze preferentially, proteins having relatively shorter and longer half-lives respectively, were used. The relationship between relative degradation rate and polypeptide size was always evident, and incubation under starved conditions always showed a reduced correlation. This analysis was also carried out on the soluble intracellular proteins from smooth muscle cells. The results obtained are shown in Figure 2.5., and illustrate the very clear relationship between half-life and molecular size of proteins in the soluble fraction under normal conditions. Further, a

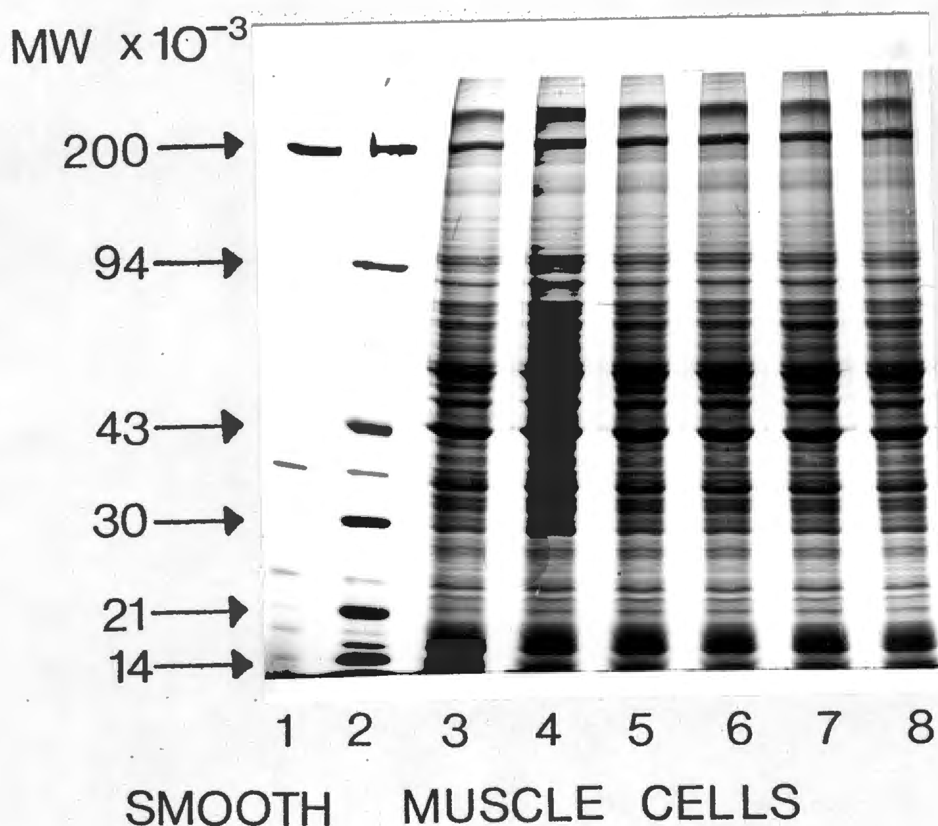


FIGURE 2.1. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF DOUBLE-LABELLED SMOOTH MUSCLE CELL PROTEINS.

Smooth muscle cells were double-labelled as described under "Experimental Procedures 12.2" and run on SDS polyacrylamide gradient slab gels as described under "Experimental Procedures, 13".

Lane 1,2: Molecular weight standards as described on page 40.

Lane 3,4: Cells labelled under control conditions (•---• Fig.2.2)

Lane 5,6: Cells labelled under basal proteolytic conditions (●—● in Fig. 2.2)

Lane 7,8: Cells labelled under accelerated proteolytic conditions (○—○ in Fig. 2.2).

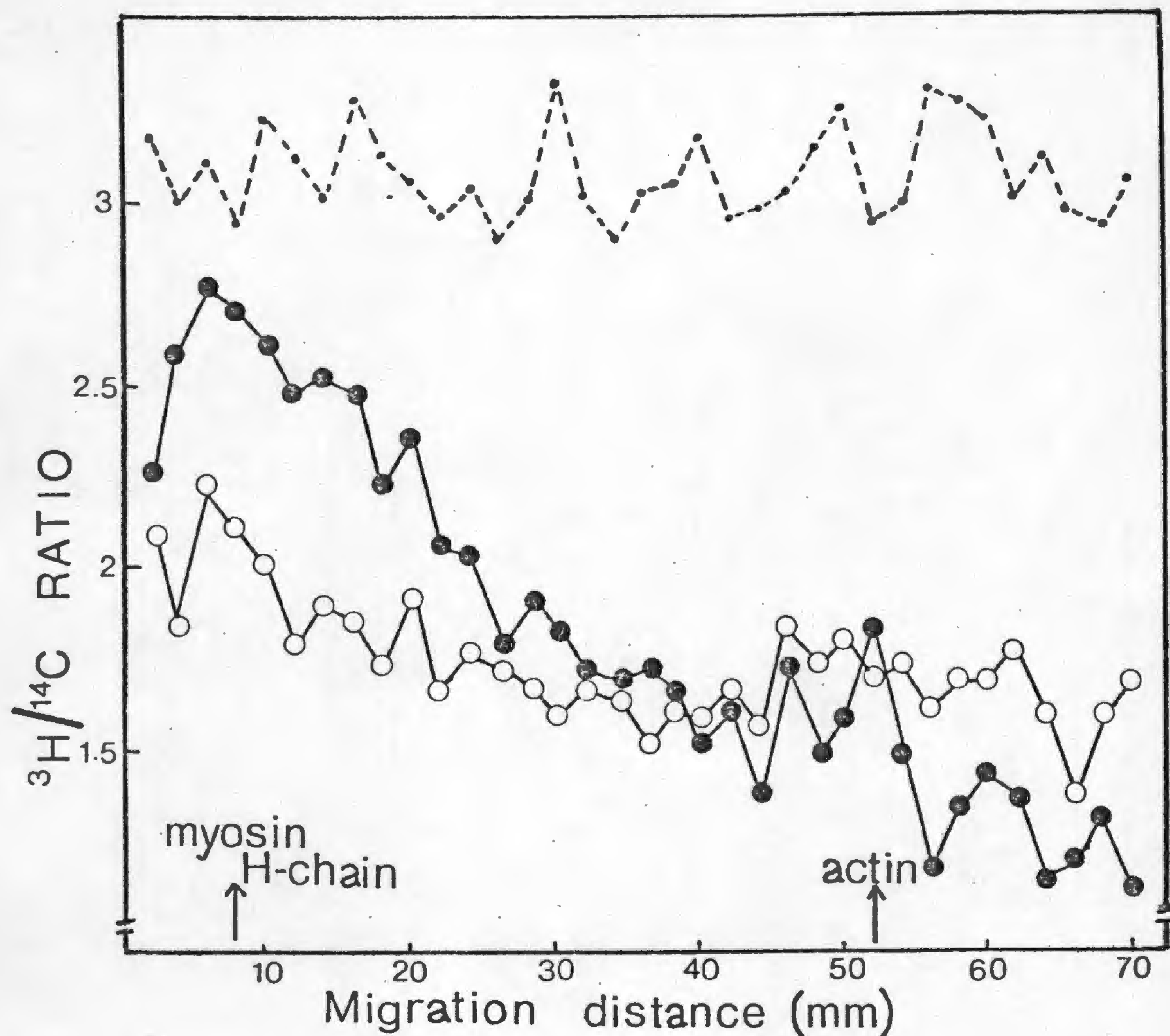


FIGURE 2.2. RELATIVE DEGRADATION RATES OF SMOOTH MUSCLE CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Smooth muscle cells were labelled for 16 hours with 3 μCi/ml ^{14}C-phenylalanine, chased for 24 hours under normal (●—●) or starved conditions (○—○) before 10 μCi/ml ^{3}H-phenylalanine was added to fresh cells for 2 hours. Cells were harvested and proteins run on gradient slab gels as described under "Experimental Procedures, 13", which were then sliced and counted. Direction of migration was from left to right, so the slowly migrating, large molecularweight proteins are on the left. Arrows indicate the position of marker proteins run simultaneously in the gel. Ratios were not plotted for fractions containing less than 80 dpm of ^{14}C or 320 dpm for ^{3}H.
•-----• indicates results of a control experiment using proteins from cells given 3 μCi/ml ^{14}C-phenylalanine and 10 μCi/ml ^{3}H-phenylalanine for 16 hours before harvesting.

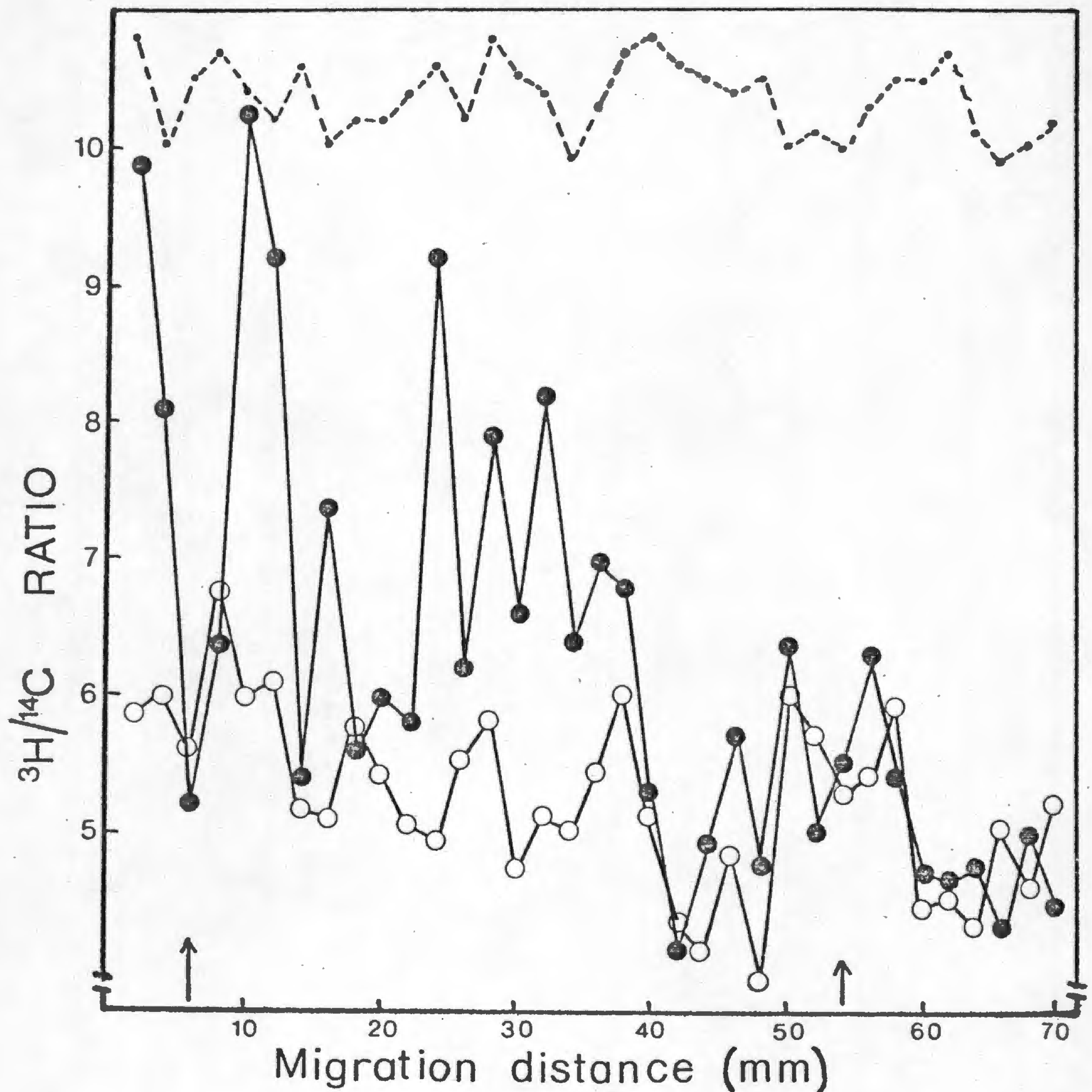


FIGURE 2.3. RELATIVE DEGRADATION RATES OF SMOOTH MUSCLE CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Experimental details as given in Figure 2.2, except that labelling with 3 μCi/ml ^{14}C-phenylalanine was reduced to 8 hours, followed by a 12 hour chase before labelling with 10 μCi/ml ^{3}H-phenylalanine for a further 2 hours.

●—● Complete medium during chase

○—○ Starved during chase

•----• Control experiment as described in Figure 2.2.

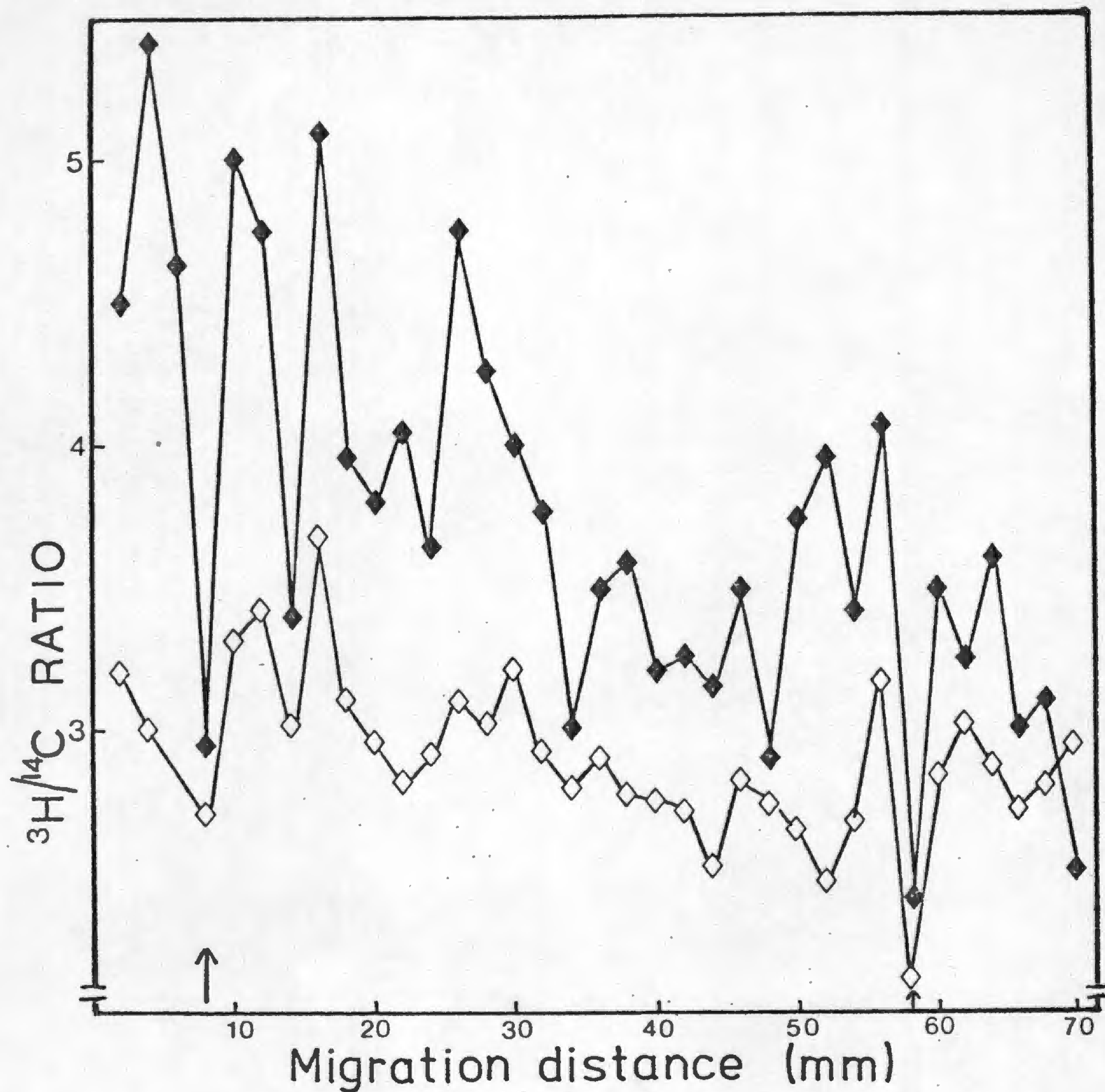


FIGURE 2.4. RELATIVE DEGRADATION RATES OF SMOOTH MUSCLE CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Experimental details as given in Figure 2.2 except that the cells were exposed to 3 µCi/ml ^{14}C-phenylalanine for 30 hours, followed by a 24 hour chase before labelling with 10 µCi/ml ^{3}H-phenylalanine for a further 2 hours.

◆—◆ Complete medium during chase

◇—◇ Starved during chase.

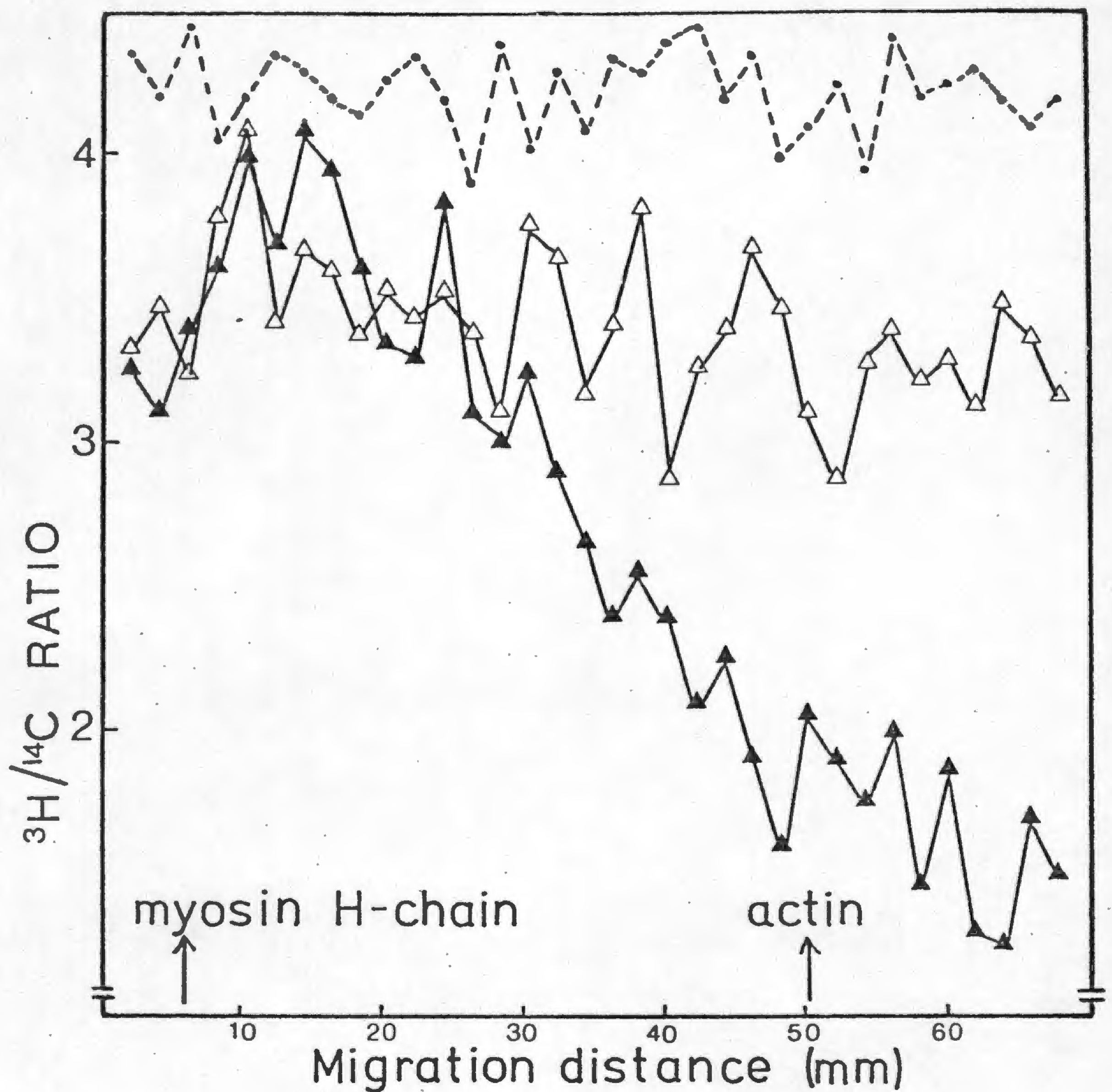


FIGURE 2.5. RELATIVE DEGRADATION RATES OF SOLUBLE SMOOTH MUSCLE CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Cells were labelled as described in Figure 2.2, then soluble intracellular proteins were prepared as described under "Experimental Procedures, 12.3" before application to the gel.

▲——▲ Complete medium during chase

△——△ Starved during chase

•----• Control experiment as in Figure 2.2.

distinct situation exists under starved conditions in which no size-"half-life" correlation exists.

(b) L8 Skeletal Muscle Cells

Figures 2.6, 2.7, and 2.8 show that there is a clear correlation between polypeptide size of intracellular skeletal muscle cell proteins and their relative degradation rates for three different double-labelling protocols used. However, when these cells were incubated under starved conditions, this relationship was largely maintained and not markedly reduced as in the case of the smooth muscle cells. The dotted line in Figure 2.6 indicates the variation in ratio obtained in a control experiment using double-labelled proteins from cells labelled simultaneously with the ^{3}H- and ^{14}C-isotopes for 16 hours before being harvested. No systematic variations in the $^{3}H : ^{14}C$ ratios were evident. Similar trends in double label ratios were found in three repeat experiments.

2.2. CORRELATION BETWEEN GLYCOPROTEIN CONTENT AND RELATIVE DEGRADATION RATES OF SOLUBLE INTRACELLULAR PROTEINS

Concanavalin A (Con A) binds molecules which contain α-D-mannopyrranosyl, α-D-glucopyranosyl and sterically related residues, and thus ConA-agarose is a generally applicable group-specific adsorbent for the separation and purification of glycoproteins, polysaccharides and glycolipids. The glycoprotein can be eluted from Con A by a solution containing α-methyl-D-mannoside.

(a) Smooth Muscle Cells

Figure 2.9A illustrates results obtained by measuring $^{3}H : ^{14}C$ ratios for non-glycoprotein and glycoprotein fractions obtained by affinity chromatography with Con A-agarose. It can be seen that the significant relationship between carbohydrate content and protein half-life which is obtained under normal conditions, is considerably

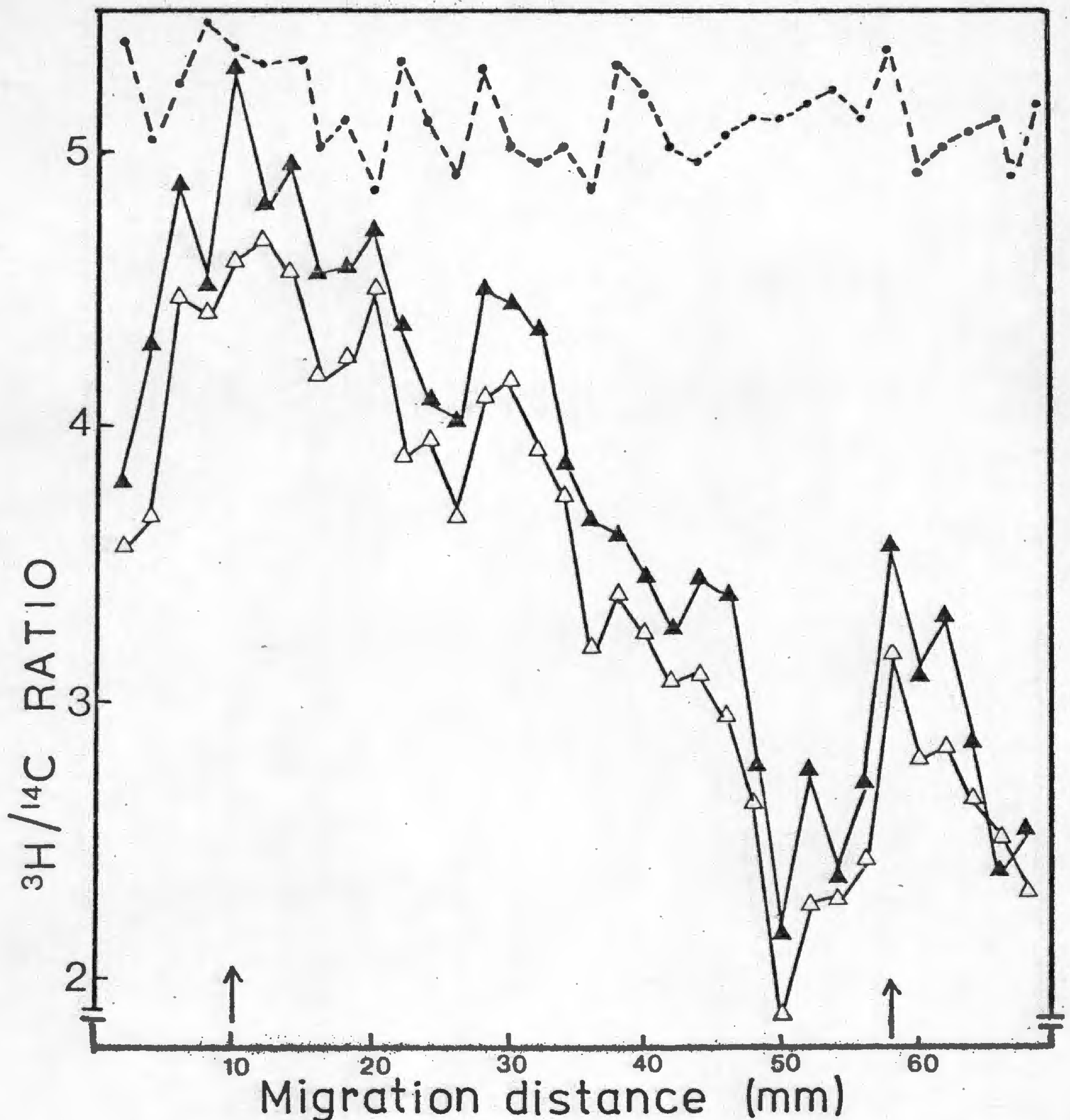


FIGURE 2.6. RELATIVE DEGRADATION RATES OF SKELETAL MUSCLE CELL (L8) PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

L8 cells were labelled and proteins prepared as described in Figure 2.2. Double-labelled proteins were sujected to gradient gel analysis and counted as described under "Experimental Procedures 13, 14". Ratios were not plotted for fractions containing less than 80 dpm for ^{14}C or 320 dpm for ^{3}H.

▲—▲ Complete medium during chase

△—△ Starved during chase

•----• indicates a control experiment as described in Figure 2.2.

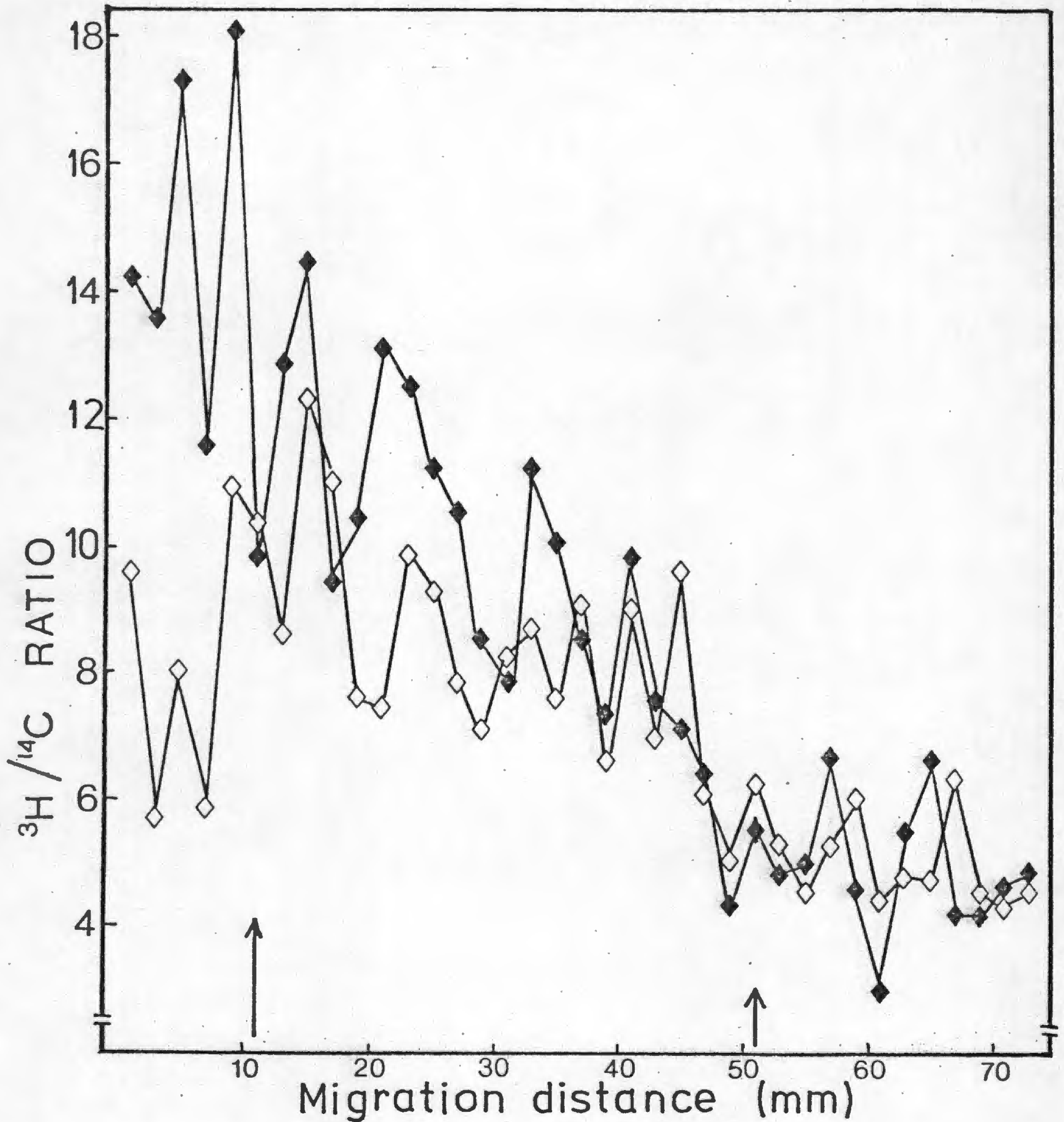


FIGURE 2.7. RELATIVE DEGRADATION RATES OF L8 CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Cells were labelled for 8 hours with 3 μCi/ml ^{14}C-phenylalanine, chased for 12 hours before fresh cells were labelled with 10 μCi/ml ^{3}H-phenylalanine for 2 hours. Labelled proteins were then subjected to gradient gel analysis and counted.

◆—◆ Complete medium during chase.

◇—◇ Starved during chase.

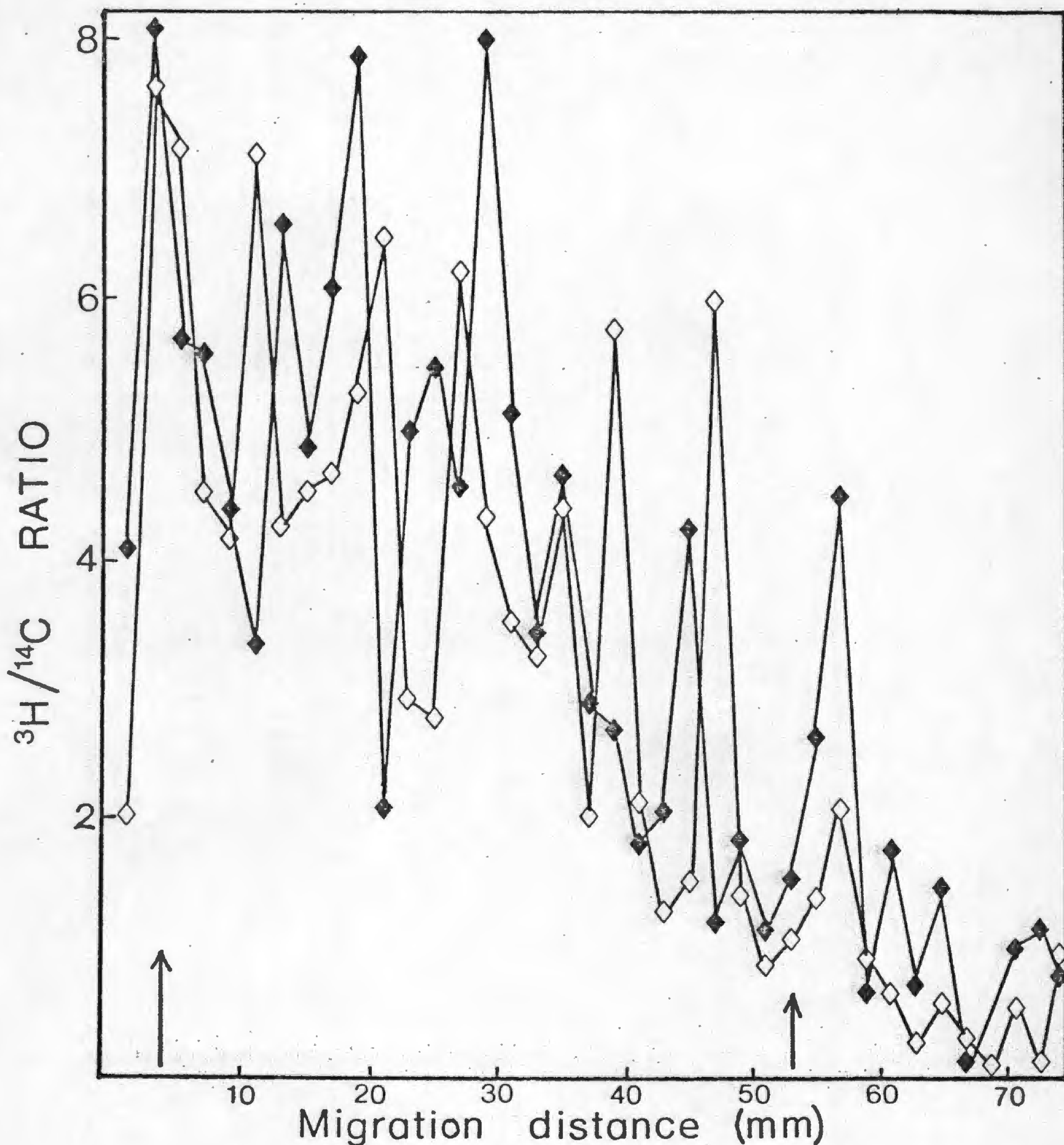


FIGURE 2.8. RELATIVE DEGRADATION RATES OF L8 CELL PROTEINS AS SEPARATED BY SDS-POLYACRYLAMIDE ELECTROPHORESIS.

Cells were labelled for 30 hours with 3 µCi/ml ^{14}C-phenylalanine, chased for 24 hours before fresh cells were labelled with 10 µCi/ml ^{3}H-phenylalanine for 2 hours. Labelled proteins were then subjected to gradient gel analysis and counted.

◆—◆ Complete medium during chase

◇—◇ Starved during chase.

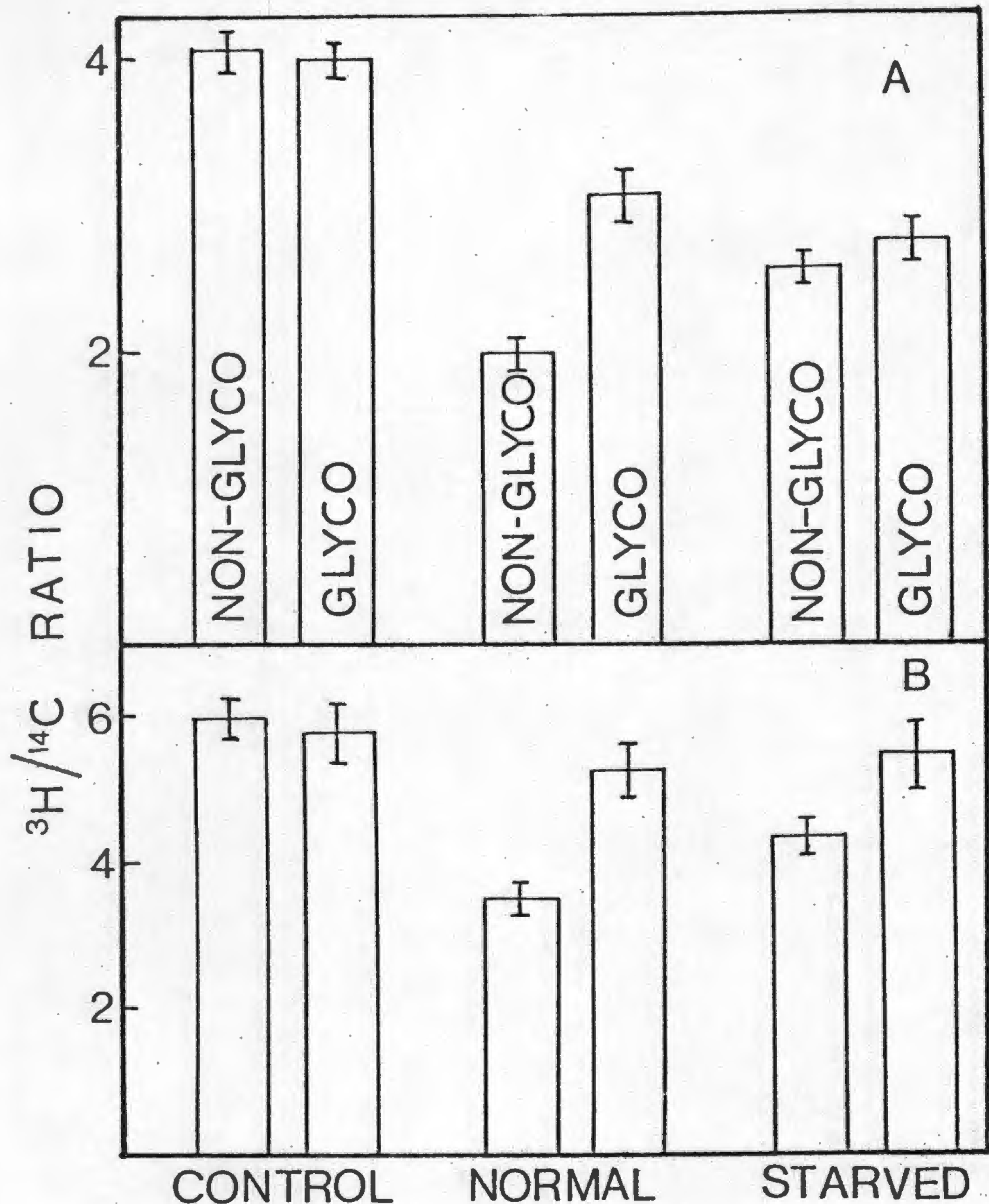


FIGURE 2.9. RELATIONSHIP BETWEEN CARBOHYDRATE CONTENT AND RELATIVE DEGRADATION RATES OF MUSCLE CELL PROTEINS.

Smooth muscle (A) and L8 skeletal muscle (B) cells were labelled as described in Figure 2.2. and soluble double-labelled proteins were prepared as described under "Experimental Procedures, 12.3". Proteins were applied to to ConA-agarose columns to separate nonglycoproteins (NON-GLYCO) from glycoproteins (GLYCO). Values represent mean ± S.E. of three experiments using the same double-labelled proteins. 5-6% of total protein bound to ConA-agarose in each case.

reduced under starved conditions. A control experiment, in which cell layers were exposed simultaneously to both isotopes for 16 hours before being harvested, was also used to prepare double-labelled proteins, which were analyzed in the same way and showed no significant difference in the isotope ratios obtained from the glycoprotein and non-glycoprotein fractions (see Figure 2.9).

(b) Skeletal Muscle Cells

Figure 2.9B shows that the correlation between carbohydrate content and relative degradation rate can also be shown under the normal incubation conditions for L8 skeletal muscle cells. However, this relationship does not appear to be as markedly reduced under starved incubation conditions, as was the case for smooth muscle cells.

DISCUSSION

The double-isotope method as used in this study has proved to be very valuable in providing information about the general features of protein degradation in muscle cells under normal and starved conditions, since catabolic rates of many proteins can be compared in a single experiment.

These experiments have demonstrated a correlation between subunit size and ^{3}H : ^{14}C ratio among the intracellular rpoteins of both smooth and skeletal muscle cells, in accordance with reports by other workers that larger proteins tend to be degraded more rapidly than smaller proteins (Dice et al., 1973). It has also been shown that the glycoproteins of both muscle cell types tend to be degraded faster than non-glycoproteins, a result which has previously been reported by Dice et al. (1978) in liver muscle and brain tissue. The new finding reported here is that in cultured cells, the accelerated protein degradation associated with starvation conditions, especially in smooth muscle cells, is fundamentally different from normal protein catabolism. This is consistent with the findings of other workers who have compared normal to accelerated protein catabolism during nutrient deprivation, and have found that breakdown of stable cell proteins is preferentially enhanced (Knowles and Ballard, 1976; Epstein et al., 1975; Poole and Wibo, 1973; Bradley, 1977). In this study, the rates of breakdown of small proteins and non-glycoproteins of smooth muscle cells (which appear to be two classes of stable intracellular proteins) approach that of large proteins or glycoproteins (which appear to be more labile intracellular proteins). The preferential degradation of more stable intracellular proteins in times of dietary stress would seem to be advantageous to the cells because these proteins tend not to be crucial metabolic regulatory enzymes (Schimke, 1970; Goldberg and St. John, 1976) and could perhaps be degraded with the minimum disruption to cellular function. The loss of correlation was not quite so clear in the case of skeletal muscle cells, where the general correlation between the two

physicochemical properties and degradation rates appeared to be largely maintained under nutrient deprivation. This result is therefore consistent with the findings that the increase in proteolytic rate observed under nutrient deprivation was not as marked in skeletal muscle cells as in smooth muscle cells.

In an attempt to preferentially label different classes of intracellular proteins, the labelling protocols for the two radioisotopes were altered so as to detect proteins having either shorter or longer half-lives than those being detected by the standard procedure (Zak et al., 1979; Russell et al., 1980). The trends in relative degradation rates were not greatly changed by incorporating label into "short-lived" proteins or "long-lived" proteins (half lives of 1-24 hours and 1-3 days respectively). The ability to discriminate between relative half lives within these ranges may have been slightly improved (see Figures 2.2, 2.3 and 2.4), but the overall trends were the same in each case.

These results support the theory that protein subunits rather than protein multimers are the basic units of degradation (Dice et al., 1973) and the idea that protein multimers may be in a state of continual association-dissociation in which their subunits rapidly exchange with free subunits. Thus, the protein composition of structures containing a number of protein subunits may be altered without necessitating resynthesis of the entire complex. Models involving exchange of protein constituents between free and associated states have previously been proposed in order to explain the characteristics of degradation of membrane proteins (Dehlinger and Schimke, 1971) and ribosomal proteins (Dice and Schimke, 1972).

The accelerated proteolysis seen in nutrient deprivation could be due to an alteration in the degradative mechanism(s) and/or due to structural changes in proteins making them more susceptible to hydrolytic cleavage (this has been discussed more fully in the "Introduction"). Bearing in mind the results

obtained using inhibitors (see Chapter 1, Results) these results would support an intracellular degradative machinery which is composed of at least two components as originally suggested by Schimke (1970) and clarified more recently by Knowles and Ballard (1976). Since the lysosomal system appears to play the more significant role in accelerated proteolysis, it can be argued that the lysosomal autophagic mechanism of protein degradation is non-selective since all types of proteins are degraded at similar rates. The increased lysosomal activity under nutrient deprivation tends to equalize the relative degradation rates of various protein classes in smooth muscle cells, because autophagy appears to account for the larger proportion of protein catabolism under these conditions.

It is important to emphasize that the results presented in this section of the thesis only apply as a general rule for proteins and do not provide information about individual proteins or polypeptides. However, the results are in accordance with those obtained using protease inhibitors (Chapter 1, Results), and have therefore led to a greater understanding of the degradation pathways operating in cultured muscle cells under normal and nutritionally-poor conditions.

SUMMARY

The observations in this study are compatible with the dual-pathway model proposed by Knowles and Ballard (1976) and strongly support the existence of multiple degradative pathways in muscle. The experiments indicate the existence of one pathway for the hydrolysis of short-lived proteins under all growth conditions, which appears to be extralysosomal in location. The role of the lysosome in degrading long-lived proteins has been clarified, and its involvement appears to be restricted to one-third of basal proteolysis, whereas it plays a more substantial role in starvation-induced proteolysis.

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