Pathways of Intracellular Protein Turnover in Cultured Human Fibroblasts

Sarah Olenick

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LOYOLA UNIVERSITY OF CHICAGO

PATHWAYS OF INTRACELLULAR PROTEIN TURNOVER
IN CULTURED HUMAN FIBROBLASTS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MOLECULAR AND CELLULAR BIOCHEMISTRY

BY
SARAH OLENICK

DIRECTOR: ALLEN FRANKFATER, PH.D
CHICAGO, ILLINOIS
JANUARY, 1995
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Dr. Pamela Derstine for computerized graph plotting.

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ABBREVIATIONS

AV    Autophagic vacuoles
BSA   Bovine serum albumin
CBZ   Benzyloxycarbonyl
5(6)-CF 5(6)-Carboxyfluorescein
CHO   Chinese hamster ovary
CPM   Counts per minute
Cyto B Cytochalasin B
DMEM  Dulbecco's modified Eagle's medium
DOPE  Dioleyl phosphatidylethanolamine
DPPC  Dipalmitoyl phosphatidylcholine
DSPC  Distearyl phosphatidylcholine
DTT   L-Dithiothreitol
E_a   Activation energy
EDTA  Ethylenediamine tetraacetic acid
EGTA  Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER    Endoplasmic reticulum
FCS   Fetal calf serum
FD    Fluorescein isothiocyanate dextran
FITC  Fluorescein isothiocyanate
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<tr>
<td>FR</td>
<td>Ratio of fluorescence at Ex495 and Ex450</td>
</tr>
<tr>
<td>GCV</td>
<td>Giant complex vesicles</td>
</tr>
<tr>
<td>HC</td>
<td>Hydrocarbon</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LL</td>
<td>Lysolecithin</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell(s)</td>
</tr>
<tr>
<td>SA</td>
<td>Stearylamine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium lauryl sulfate</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCA-PTA</td>
<td>20% Trichloroacetic acid + 1% phosphotungstic acid</td>
</tr>
<tr>
<td>TES</td>
<td>N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid</td>
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<tr>
<td>Tris</td>
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OBJECTIVES

The purpose of this study is to determine whether the transfer of cellular proteins from the cytosol into lysosomes is an obligatory step in their degradation. To achieve the objectives of this study:

1. I will prepare large unilamellar phosphatidylserine (PS-LUV) liposomes as possible vehicles for the injection of radiolabeled proteins into the extralysosomal-cytoplasmic compartment of cultured cells.

2. I will develop assays to monitor the extent of vesicle-cell fusion. If this technique is successful, it will be possible to study the intracellular traffic and metabolism of "newly" introduced cytosolic proteins against an unlabeled cellular background.

3. If PS-LUV prove unsuitable as vehicles for the microinjection of proteins, I will investigate the degradation of cellular proteins by labeling them with $[^3\text{H}]$-leucine and monitor the appearance of TCA-soluble and insoluble products in the medium. In order to determine whether the lysosome is an intermediate compartment in the degradation of cytosolic proteins, protein turnover will be studied under culture conditions which are believed to alter the extent of sequestration of cellular proteins into lysosomes. The effects of pharmacological agents known to alter intracellular vesicular transport, intracellular protein transport and lysosomal function will also be investigated.
4. I will characterize the nature of any TCA-insoluble products released into the culture medium to determine whether they arise from cell leakage, cell detachment, vesicular shedding, or regurgitation of cellular proteins following their prior sequestration in lysosomes.

5. I will utilize the kinetic data to formulate a model to explain the appearance of both TCA-soluble and TCA-insoluble products released into the culture medium, the multiphasic nature of cellular protein turnover, and the effects of temperature, serum and amino acid deprivation, and added pharmacologic agents.
Phosphatidylserine large unilamellar liposomes were investigated as a means of injecting labeled proteins into fibroblasts to identify and quantitate pathways of cytosolic protein turnover. Methods were developed to quantify liposome-cell fusion. The results do not support liposome-plasma membrane fusion as the primary event in PS-LUV-fibroblast interaction. Rather, PS-LUV tightly adhere to the plasma membrane of fibroblasts and are difficult to remove by washing, giving the impression that liposome-cell fusion has occurred.

The unsuitability of PS-LUV as a means of microinjecting labeled proteins prompted an investigation of the degradation of cellular proteins metabolically labeled with [3H-4,5]-L-leucine. The turnover of cellular proteins results in the release into the cultured medium of both TCA-soluble and -insoluble products in 2 phases: a fast phase (largely completed by 5 hours) and a slow phase (half life of approximately 4.8 days).

Electrophoretic analysis of TCA-insoluble radiolabeled material suggested that a significant amount could have arisen from cell leakage or death. Protein synthesis inhibitors had no effect on the appearance of TCA-insoluble products.
Protein turnover under serum-supplemented conditions was inhibited by lysosomotropic amines, protein synthesis inhibitors, and serum and amino acid deprivation. However, lysosomal proteinase inhibitors had no effect on cellular protein degradation. This process was temperature-sensitive between 37° and 26° C with an Ea = 7.8 kcal/mol. Under conditions of amino acid and protein deprivation, protein degradation was diminished. This reduced turnover was no longer inhibited by lysosomotropic amines and was temperature-insensitive between 26° and 37° C.

These results indicate that there are at least 2 pathways for cellular protein turnover, one temperature-sensitive and the other temperature-insensitive. The temperature-sensitive pathway is inhibited by lysosomotropic amines, serum and amino acid deprivation, and protein synthesis inhibitors. It is presumed to be lysosomal and is responsive to rates of protein synthesis. The temperature-insensitive pathway is not sensitive to lysosomotropic amines and is not inhibited by amino acid and protein deprivation. It is presumed to be non-lysosomal and independent of rates of protein synthesis.
CHAPTER I

INTRODUCTION

General Concepts of Protein Degradation in Cells

The concentration of a specific cellular protein depends on both its rate of synthesis and its rate of degradation. Although the mechanisms for protein synthesis are fairly well understood, those of degradation have not been well-characterized. Both processes may be modified experimentally in cell culture (Goldberg & St. John, 1976) by manipulating the levels of serum, media constituents, hormones and growth factors, and pharmacological agents (Doherty & Mayer, 1985; Gronostajski et al., 1985; Thoene et al., 1985; Okada & Dice, 1984; Ahlberg et al., 1985; Etlinger & Goldberg, 1977; Marshall & Olefsky, 1983; Dice, 1982; Knowles & Ballard, 1981; Poole et al., 1978). Protein synthesis within growing cells is more sensitive to changes in high energy intermediates (Gronostajski et al., 1985): a 90% reduction in ATP levels causes only a 50% decrease in proteolysis in growing fibroblasts, while a 15% reduction in ATP causes a 50% inhibition of protein synthesis.

Cell growth occurs when protein synthesis exceeds proteolysis (Goldberg & St. John, 1976). There is evidence that cellular protein levels are largely controlled by rates of degradation rather than those of synthesis, which is more "economical" with respect to energy expenditure. It has been noted that protein gain in liver during starved mice refeeding is dominated by a marked decrease in degradation, with less dramatic increases...
in synthesis (Mortimore et al., 1983). In addition, growing (non-confluent) 3T3-L1 cultures degrade proteins more slowly than confluent cultures (Doherty & Mayer, 1985). This coincides with the observation that cathepsin activity is higher in high-density and quiescent cultures than in rapidly-proliferating and sparse cultures (Kenagy & Bierman, 1983).

Age may affect the rate of proteolysis within a cell. Microinjected $^{125}$I-labeled RNAase A has a $t_{1/2}$ of 90 h in young human lung fibroblast cultures, while half-life ($t_{1/2}$) is >120 h in senescent cultures. Protein $t_{1/2}$ is correlated with cell passage ($r = 0.873$) in this model. The same trends are generally observed with $^3$H-labeled RNAase A (reductive methylation) (Dice, 1982) and intracellular proteins (metabolically-labeled with $^3$H-Leucine) (Ballard et al., 1981; Okada & Dice, unpublished results, cited in Dice, 1982). Also, serum deprivation enhances proteolysis of microinjected RNAase A 1.6-fold in the younger cultures, but has little effect in the senescent cultures (Dice, 1982). However, the differences between young and old cultures on the proteolysis of metabolically-labeled proteins are of less magnitude than those of microinjected proteins. This may indicate that cellular aging does not affect the catabolism of all proteins identically (Dice, 1982). Recently, Dice and his associates have shown that RNAase A possesses a recognition signal which directs its sequestration by lysosomes under conditions of serum deprivation (Dice et al., 1986).

Experimentally, it has been observed that cellular proteins fall within 2 generalized groups: long-lived proteins which have slow turnover rates, and short-lived
proteins with comparatively high turnover rates (Hershko & Ciechanover, 1982). Within each group, there is substantial diversity with regard to degradation rates. It has been postulated that proteins which are degraded rapidly are rate-limiting enzymes for metabolic pathways (Goldberg & Dice, 1974), or proteins which are synthesized for secretion or routing to other cellular compartments which are degraded prematurely (Berg & Neblock, 1985). It has been reported that large, acidic proteins are degraded more rapidly than small, basic proteins (Neff et al., 1981). However, others have denied any relationship between the half-lives of cellular proteins and subunit molecular weight, isoelectric point, or secondary structure (Rechsteiner et al., 1984). Proteolysis of a specific protein was not found to vary much between different cell lines (Rechsteiner et al., 1984; Zavortink et al., 1979).

Serum deprivation studies in rat fibroblasts show a strong inverse correlation between proteolysis and thymidine incorporation (Poole et al., 1978). Degradation of long-lived proteins (\(^{14}\)C-labeled for 50 h) more than doubles in the absence of serum, while the breakdown of short-lived proteins (\(^{3}\)H-labeled for 1 h) is affected very little. These observations also occur in the presence of serum when amino acids, glucose, or phosphate are omitted from the medium. Addition of a mixture of insulin, dexamethasone, and fibroblast growth factor in 0% serum increases \(^{3}\)H-labeled thymidine incorporation and decreases the release of TCA-soluble \(^{14}\)C-labeled amino acids to about 75% of the level seen with 10% serum-supplemented fibroblasts (Poole et al., 1978). Recently, Dice et al. (1986; 1987) have shown that proteins which are
degraded more rapidly on serum deprivation contain the pentapeptide sequences related to Lys-Phe-Glu-Arg-Gln, and that such proteins are degraded in the lysosome.

**Cytosolic Degradative Systems**

The nature and locations of the proleolytic enzymes responsible for the degradation of cellular proteins is a matter of great controversy. Earlier studies have produced a general consensus that short-lived proteins are degraded to a larger extent by an ATP-dependent enzyme system within the cytosol (Etlinger & Goldberg, 1977; Knowles & Ballard, 1981; Rechsteiner et al., 1984; Bigelow et al., 1981). Proteolysis in the $10^5$ x g supernatant of reticulocytes is stimulated by 0.1-1 mM ATP, while ADP and $\alpha,\beta$-methylene-ATP cause slight increases. AMP, cAMP and $\beta,\gamma$-methylene-ATP have no significant effects on proteolysis (Etlinger, 1977). **Cytosolic proteolysis** is inhibited by chloromethyl ketones (TPCK and TLCK)\(^1\) and sulfhydryl blocking agents (N-ethylmaleimide and iodoacetamide), which suggests the participation of (a) sulfhydryl protease(s). Metalloenzyme activity is also suggested, since the heavy metal chelator o-phenanthroline also inhibits cytosolic proteolysis (Etlinger & Goldberg, 1977).

Recently, several high molecular weight nonlysosomal proteinases have been discovered in reticulocytes and other animal cells. These proteinases are composed of multiple subunits of $M_r$ 21,000 to 34,000 (Bond & Butler, 1987; McGuire et al., 1988) and appear to contain multiple catabolic sites (Bond & Butler, 1987). These enzymes

\(^1\)TPCK = L-1-tosylamido-2-phenylethyl-chloromethyl ketone
TLCK = N-$\alpha$-p-tosyl-L-lysine-chloromethyl ketone (Thoene et al., 1985).
have been termed multicatalytic proteinases. One group of multicatalytic proteinases, \( M_r 700,000 \), appears to be identical to a 20 S cylindrical or ring-like particle called a prosome (prosome) which has been found in many types of eukaryotic cells (Rivett, 1989). These enzymes appear to be cysteine proteinases because of their sensitivity to inhibition by thiol reagents, but there is evidence that some of these subunits may be serine endopeptidases (Rivett, 1989; Tanaka et al., 1988). Based on the sensitivity of this enzyme system to thiol reagents, it has also been called macropain (McGuire et al., 1989). The activity of the proteosome is latent, and it can be activated by treatment with polylysine (Tanaka et al., 1988) and detergents (Tanaka et al., 1989; McGuire et al., 1989). The requirement of proteosome proteolysis for ATP is a matter of some controversy. Driscoll and Goldberg (1989) have reported that the hydrolysis of peptide substrates by proteosomes is stimulated 12-fold by ATP, other nucleotide triphosphates, and by nonhydrolyzable ATP analogs. ATP also stimulated the hydrolysis of protein substrates such as casein. However, the caseinolytic activity of the proteosome is not stimulated by nonhydrolyzable ATP analogs, indicating that high energy phosphoanhydride bond hydrolysis is required for peptide bond cleavage with a protein substrate. In contrast, others have reported that ATP hydrolysis is not required for peptide bond cleavage (reviewed in Bond & Butler, 1987).

Reticulocytes contain a 26 S ATP/ubiquitin-dependent proteolytic system which is believed to function in the degradation of globin (Bond & Butler, 1987). A similar system has been shown to participate in the degradation of short-lived proteins in yeast
(Bachmair et al., 1986). These systems act by first conjugating a 9000-dalton peptide, ubiquitin, to protein substrates in an ATP-dependent fashion. Substrates which are multi-ubiquitinylated are then degraded by cysteine-like proteinases. Recently, McGuire et al. (1988) have reported that the proteosome aggregate (macropain) or an immunologically related protein can degrade ubiquitinylated protein substrates. Eytan et al. (1989) have demonstrated that the 26 S ATP/ubiquitin-dependent protease complex contains the 20 S "multicatalytic" protease as its catalytic core. The incorporation of the 20 S multicatalytic protease into the 26 S ubiquitin-dependent protease complex is an ATP requiring process (Eytan et al., 1989).

Bachmair et al. (1986), using recombinant DNA techniques, have demonstrated that the identity of the amino terminal residue in proteins is a major determinant of protein half-life in yeast. The amino-terminal amino acid residues Arg, Asn, Trp, Phe, Leu, Asp, and Lys destabilize cytosolic proteins by directing their rapid ubiquitinylation and degradation by the 26 S ubiquitin-dependent proteolytic system. Such proteins have half-lives of 2-3 minutes in yeast. In contrast, the presence of an amino terminal Ala, Cys, Gly, Met, Ser, Thr, or Val confers a marked stabilizing affect, and such proteins are typically long-lived in yeast. Bachmair et al. have termed this phenomenon the N-end rule for selective protein breakdown.

The role of the amino terminus in determining the rate of turnover of a protein has suggested additional mechanisms by which protein half-lives might by regulated. New amino termini might be exposed by limited endo- or exopeptidolytic cleavage of a
protein. Alternatively, the amino terminus of a protein may be altered in reactions catalyzed by aminoacyl-tRNA-protein transferases, which are known to exist in cells (Bachmair et al., 1986; Dice, 1987). Recently, it has been shown that ornithine decarboxylase, a protein which turns over rapidly in vivo, can undergo selective N-terminal arginylation (destabilizing) in vitro. Furthermore, an inhibitor of arginyl-tRNA: protein arginine transferase can strongly inhibit the turnover of ornithine decarboxylase in cultured hepatocytes in vivo (Kopitz et al., 1990).

The calpains represent another group of cytosolic proteinases. These are sulfhydryl-dependent proteases which are characterized by a requirement for millimolar to micromolar concentrations of calcium, depending on their type (Bond & Butler, 1987). The calpains appear to cleave specific categories of protein substrates and to initiate or regulate specific physiological responses. Calpains appear to be involved in the degradation of membrane and structural proteins in muscle and nerve cells (Wang & Beermann, 1988; Siman & Noszek, 1988; Inomata et al., 1989), the reorganization of the cytoskeleton in response to calcium (Nix, 1989), and the degradation of cellular proteins following injury-induced calcium influx (Kuwaki et al., 1989; Siman et al., 1989). Calpains also participate in myoblast fusion (Bond & Butler, 1987) and platelet activation (Tsujinaka et al., 1990). It has been suggested that proteins susceptible to proteolytic cleavage by calpains contain sequences rich in proline and acidic and hydroxylated amino acids known as PEST sequences (Siman & Christoph, 1989; Rogers et al., 1986).

There is some question as to the identity of the rate-limiting step in the putative
cytosolic proteolytic enzyme systems. Activation energies ($E_a$) (determined by Arrhenius plots of $\log k_D$ vs. $1/T$) for the turnover of a small number of proteins tested in vivo are $27 \pm 5$ kcal/mol. This rules out local protein unfolding and peptide bond hydrolysis for being the rate-limiting steps in cytosolic proteolysis, since these processes have $E_a$'s <15 kcal/mol. Ubiquitin-conjugated lysozyme was degraded 10-fold faster than non-conjugated lysozyme, but $E_a$'s were in the range $27 \pm 5$ kcal/mol, for both processes which eliminates ubiquitin conjugation as the rate-limiting reaction. It is postulated that the rate-limiting event(s) is (are) related to the recycling or assembly of the complicated ATP-dependent proteolytic system, loosely analogous to assembly of the components of the complement system (Rechsteiner et al., 1984).

**Lysosomal Degradative Systems**

Long-lived proteins ($t_{1/2} > 1$ day) are degraded--at least in part--within lysosomes (Doherty & Mayer, 1985; Ahlberg et al., 1985; Rechsteiner et al., 1984; Dean, 1975). Particulate proteins and proteins having low solubility in Triton X-100 are also principally degraded in lysosomes (Doherty & Mayer, 1985; Rechsteiner et al., 1984). The mechanism of entry of intracellular proteins into the lysosomes is ill-defined, but it appears that sequestration of the substrate into autophagic vacuoles (AV) or lysosomes may be the rate-limiting step in this pathway (Katunuma & Kominami, 1983; Bond & Aronson, 1983), since lysosomal proteases are present in excess (Katunuma & Kominami, 1983). AV are not by themselves sufficiently acidic for optimal proteolysis.
Hydrolytic enzymes are acquired by fusion with lysosomes or endoplasmic reticulum (ER) membranes which apparently form the limiting membranes of the AV. Incubation of these structures in an acidic (pH 5) environment markedly increases proteolytic activity of AV above that seen at neutral pH (Ahlberg et al., 1982).

Lysosomes appear to be acidified by an ATP-dependent proton pump; acidification appears to occur by electroneutral rather than electrogenic mechanisms. Acidification is optimal when cytosolic (external) pH is slightly alkaline (pH 7.5). Lysosomal hydrolases are not required, as lysosome "ghosts" (disrupted lysosomes containing <10% enzyme activity) are still capable of acidification (Schneider, 1981). The acidic environment dramatically reduces the binding between ligands and plasma membrane receptors, which facilitates receptor recycling (Gonzalez-Noriega et al., 1980).

Lysosomal enzymes are a subset of glycoproteins, and are synthesized, transported and processed in a directional manner. Translation and glycosylation occur in the rough endoplasmic reticulum (RER), the oligosaccharide side chains are modified in the Golgi apparatus, and the enzymes are packaged into lysosome-directed transport vesicles in the trans Golgi network (Goldberg & Kornfeld, 1983; Kornfeld, 1987). Most lysosomal enzymes contain the sugars N-acetylglucosamine, mannose, galactose and sialic acid (Hasilik, 1980). At least one of the mannose units is phosphorylated and serves as a lysosomal recognition marker (Neufeld, 1981; Pohlmann et al., 1982; Hasilik, 1980; Goldberg & Kornfeld, 1983; Kornfeld, 1987). Without the marker, lysosomal enzymes
are secreted in precursor form into the medium. This has been effectively demonstrated using I (inclusion)-cell disease fibroblasts (Neufeld, 1981). This cell line secretes enzymes which are not phosphorylated but are active, and this results in a deficiency of hydrolases within the lysosomes (Lightbody et al., 1971; Wiesmann et al., 1971; Wiesmann & Herschowitz, 1974). These secreted enzymes cannot be endocytosed by normal cell lines (Hickman & Neufeld, 1972). However, normal phosphorylated lysosomal enzymes added to culture medium can be endocytosed by the I-cell fibroblasts and are subsequently delivered to lysosomes (Neufeld, 1981).

Several thiol proteases have been isolated from the lysosomes. Cathepsin B [EC.3.4.22.1] is the best characterized and has a $M_r$ between 24-28,000 (Katunuma & Kominami, 1983). Studies using peptide [Katunuma et al., 1981 (1); Katunuma et al., 1981 (2); Towatari & Katunuma, 1983] and protein substrates (Nakai et al., 1978; Hannapel et al., 1982; Katunuma et al., 1982) indicate that cathepsin B acts primarily as a dipeptidyl carboxypeptidase which sequentially releases dipeptides from the C-terminus of the protein substrate. However, there is evidence that cathepsin B can also function as an endopeptidase (Bond & Butler, 1987), depending on pH (Polgar & Csoma, 1987). Cathepsin H [EC.3.4.22.16] ($M_r$ 21-24,000) appears to be both an endopeptidase and an aminopeptidase, although this enzyme has not been as well-studied. Cathepsin L [EC.3.4.22.15] ($M_r$ 21-24,000) appears to be an endopeptidase that cleaves peptide bonds which have neutral amino acids--Phe, Val, Leu, Trp, Tyr--in the $P_2$ position (Kargel et al., 1980; Katunuma et al., 1982; Katunuma et al., 1981). All three are unstable at pH's
greater than 6.0-7.0. Other thiol proteases include cathepsins S, T and N. Cathepsin S has a molecular weight of 25,000 and a substrate specificity similar to those of cathepsins L and N, but prefers small neutral residues in the P2 and P3 positions (Bromme et al., 1989). Cathepsin T ($M_r$ 33,500-35,000) was discovered on the basis of its ability to catalyze the limited proteolytic modification of tyrosine aminotransferase, converting it from the Type I form to the Type III form (Gohda & Pitot, 1980; Gohda & Pitot, 1981). Cathepsin N, isolated from human placenta ($M_r$ 34,600) and bovine spleen ($M_r$ 18-20,000), appears to have a specificity for collagen (Katunuma & Kominami, 1983).

Cathepsin D [EC.3.4.23.5] is the only known aspartate protease located within the lysosomes (Faust et al., 1985). It is an endopeptidase which primarily cleaves bonds at the carboxyl ends of aromatic amino acids (Barrett, 1977; Whitaker & Seyer, 1984). It shows homology with other aspartyl proteases, which suggests similar three-dimensional structures (Tang, 1979).

The cDNAs for cathepsins B, D, H, L and S have been cloned and sequenced (San Segundo et al., 1985; Faust et al., 1985; Qian et al., 1990; Ishido et al., 1987; Chan, unpublished results). In all cases, the enzymes are synthesized as single polypeptide chains which undergo successive cleavages to remove an amino terminal prepeptide signal sequence and a propeptide. Removal of the propeptide results in enzyme activation. Further maturation can involve C-terminal cleavages (Erickson & Blobel, 1983), and intra-polypeptide cleavages--possibly occurring within the lysosomes--to give light and heavy chains. For cathepsin D, there is a simple endoproteolytic cleavage
between amino acids 97 and 98 (Faust et al., 1985); an internal dipeptide between amino acids 47 and 48 (final product) is removed in cathepsin B (San Segundo et al., 1985). An internal dipeptide, Ser-177 Asn-178, is removed in cathepsin L (Ishidoh et al., 1987), and an endoproteolytic cleavage between Asn-177 and Gly-178 in cathepsin H (Ritonja et al., 1988). Both single- and double-chain forms of cathepsin D and cathepsin B are active (Huang et al., 1979; Takahashi et al., 1986).

**Compartmentalization and Regulation of Cellular Proteolysis**

There are many estimates of the contribution of lysosomal proteolysis in endogenous protein breakdown. These range from a low of 10 to 30% (Bigelow et al., 1981) to a high of 85 to 100% (Ahlberg et al., 1985; Hutson & Mortimore, 1982). Apparently, the tissue type and culture conditions largely determine the extent of lysosomal proteolysis. Fibroblasts (Gronostajski et al., 1985), macrophages (Poole et al., 1978) and rat yolk sacs (Knowles & Ballard, 1981) do not appear to utilize lysosomal proteolysis as extensively for endogenous proteins as the liver (Hutson & Mortimore, 1982; Ahlberg et al., 1985; Dean, 1975) and HeLa cells (Rechsteiner et al., 1984). However, many conclusions of these studies are hampered by short radioactive labeling and chase periods (<4 hours) (Poole et al., 1978; Knowles & Ballard, 1981). Thus, only a small fraction of the cellular protein pool is labeled and only a small amount of the labeled proteins are degraded during the period of observation. The amines and cathepsin inhibitors, which are used to "abolish" lysosomal protease activity, inhibit proteolysis
only partly, even when used in combination, possibly because of the presence of many different (redundant) proteases in lysosomes (Ahlberg et al., 1985). In addition, intracellular thiol protease inhibitors present in the cytosol (Kominami et al., 1982) inhibit lysosomal cathepsins when lysosomes are ruptured during tissue homogenization (Katunuma & Kominami, 1983). This may result in an overestimation of the degree of inhibition of lysosomal enzymes by added inhibitors and underestimation of the contribution of lysosomal proteolysis to intracellular protein turnover.

Proteolysis can be enhanced by serum deprivation. The preferred substrates have been reported to be long-lived (small, basic) proteins (Poole et al., 1978; Neff et al., 1981; Backer et al., 1983). Seventy percent of the deprivation-induced proteolytic activity is associated with the lysosomes in liver, while only 5% is associated with the cytosol (Mortimore & Ward, 1981). The selectivity for substrate is considerable; serum-deprived fibroblasts break down microinjected RNAase A in preference to microinjected RNAase S protein (Backer et al., 1983). Thus enhanced degradation of RNAase A in serum deprived conditions occurs largely in the lysosomal compartment (Backer et al., 1986). A pentapeptide sequence KFERQ (or in opposite orientation QREFK) has been shown to be responsible for the increased transfer of serum deprivation-responsive proteins to lysosomes on serum withdrawal (Backer et al., 1986; Dice, 1987; Dice, 1990). The covalent attachment of this peptide to heterologous proteins also causes their degradation rates to increase in response to serum withdrawal. Using antibodies to this peptide sequence, 30% of cytosolic proteins were shown to be immunoreactive in fibroblasts.
The degradation of these immunoreactive proteins was serum responsive, whereas the nonimmunoreactive proteins were degraded at the same rate in the absence of serum (Chiang & Dice, 1988). A member of the heat shock protein (hsp 70) family was found to bind to the KFERQ peptide region in RNAase A and to facilitate the direct transfer of the protein into lysosomes in vitro, possibly by a process related to the direct transfer of protein from the cytosol into mitochondria (Chiang et al., 1989). This protein, which has a molecular mass of 73 kDa, has been referred to as prp 73. Prp 73 increases in response to serum deprivation (Chiang et al., 1989) and has been shown to bind to proteins which undergo enhanced degradation on serum withdrawal (Dice, 1990).

Other mechanisms for targeting cytosolic proteins to lysosomes may also exist. A recent report suggests that the ubiquitin-dependent pathway and the lysosomal degradative pathway for protein turnover may converge. Thus, ubiquitin-protein conjugates are found to be specifically enriched in the lysosomal compartment of 3T3-L1 fibroblasts. Treatment with the lysosomal cysteine proteinase inhibitor E-64 causes an increase in the lysosomal compartment and an increase in the cellular content of ubiquitin-protein conjugates (Laszlo et al., 1990). In addition, Ciechanover (1990) has reported that the heat shock induced increases in cellular protein degradation which occurs in lysosomes is blocked in mutant Chinese hamster cells, which have a defect in ubiquitin conjugation to cellular proteins. This result suggests that the stress-induced lysosomal degradation of intracellular proteins is also mediated by the ubiquitin system. Interestingly, these cells are normal with respect to the basal turnover of long-lived
proteins indicating that the majority of long-lived proteins in Chinese hamster cells are degraded by a ubiquitin-independent system (Ciechanover, 1990).

In rat liver, the size of the lysosomal substrate pool is directly proportional to the measured rate of protein degradation. The largest accumulation of cellular substrates within lysosomes occurs in liver during glucagon-induced starvation, while the lysosomal compartment is smallest in liver from well-fed rats (basal conditions) (Mortimore & Ward, 1981). Furthermore, partial hepatectomy, which largely abolishes basal turnover in liver, causes a further reduction in the size of the lysosomal substrate pool (Scornik & Botbol, 1976; Swick & Ip, 1974). In rat liver homogenates, 80% of the short-lived (15 min label) and 90% of the long-lived (16 h label) proteins were degraded in isolated lysosomes under basal conditions (Ahlberg et al., 1985). These results have been interpreted to indicate that the lysosomes are the major site of protein degradation in rat liver (Mortimore & Ward, 1981).

Degradation of Microinjected and Endocytosed Proteins

For each specific protein tested in human diploid fibroblasts, loss of endogenous or microinjected label from cells exhibits first-order kinetics (Dice, 1982; Neff et al., 1981; Rechsteiner et al., 1984). Also, loss of label from cells is independent of the form of label (i.e. $^{125}$I, $^{3}$H) (Backer et al., 1983). Loss of microinjected label from the neoplastic HeLa cell line also followed a first-order time course for bovine serum albumin (Zavortink et al., 1979). However, these conclusions may be biased by the
manner in which the microinjection studies were conducted. In addition, not all microinjected proteins exhibit first-order degradation kinetics after injection into HeLa cells (Rechsteiner et al., 1984).

In contrast, it has been shown that release of endogenous label from isolated rat liver lysosomes occurs as the result of a multiphasic process (Mortimore & Ward, 1981). Specifically, there is an initial rapid phase (0-30 minutes) followed by a slower, somewhat linear phase (30-90 min); after 90 minutes, the release slows considerably, and an almost constant rate is observed after 120-150 minutes. In addition, it has been observed that the degradation of endocytosed proteins, which occurs in the lysosomes, is a multiphasic process, where the progress curves are described as the sum of individual first-order decay curves (Thyagarajan & Frankfater, 1985).

The relative proportions of endocytosed proteins and microinjected proteins which are degraded vary for individual proteins; 20% to >50% of the label is released as trichloroacetic acid (TCA)-insoluble products (Zavortink et al., 1979; Thyagarajan & Frankfater, 1985; Buktenica et al., 1987). The remainder of the label (50-80%) appears in a TCA-soluble form, primarily labeled amino acids (Dice, 1982; Zavortink et al., 1979; Thyagarajan & Frankfater, 1985; Buktenica et al., 1987).

Endocytosed and microinjected proteins are regurgitated in parallel with degradation (Rechsteiner et al., 1984; Buktenica et al., 1987; Thyagarajan & Frankfater, 1985; Isenman & Dice, 1989). With endocytosed proteins, the observed rate constants ($k_{obs}$) for the multiphasic appearance of TCA/PTA precipitable products are identical
with those of TCA-/PTA soluble products, which implies either a common rate-determining step for the release of both products or a common compartment as the source of both products (Buktenica et al., 1987; Thyagarajan & Frankfater, 1985). The regurgitated material appears in the medium as the intact protein and peptide fragments (Buktenica et al., 1987). Both degradative and regurgitative processes are inhibited by inhibitors of degradation, thus preserving the similarity in rate constants observed for the appearance of TCA/PTA-soluble and -insoluble products Buktenica et al., 1987). This indicates that the TCA/PTA-soluble products and regurgitated proteins are derived from a common intracellular degradative compartment, presumably the lysosomes (Buktenica et al., 1987; Thyagarajan & Frankfater, 1985).

Vehicles for Cellular Microinjection

The study of proteolysis for endogenous (metabolically-labeled) proteins is difficult, as all cellular proteins are labeled. Therefore, it is not always possible to observe the fate of individual proteins. Consequently, several techniques have been developed to attempt the delivery of labeled proteins into the cytosol of unlabeled cells. Microinjection via loaded erythrocytes (RBC ghosts) results in a random distribution of injected proteins within the cytoplasm as shown by autoradiography (Neff et al., 1981; Zavortink et al., 1979). Fluorescent-labeled proteins injected through erythrocytes or by scrape-loading tend to have a perinuclear distribution similar to that of lysosomes (Zavortink et al., 1979; Stacey & Allfrey, 1977; McNeil et al., 1984). However, it has
been shown that injected proteins may not have the same initial distribution within the cytosol as endogenous cytosolic proteins, with the former associated with sedimentable structures (Doherty & Mayer, 1985). Proteins microinjected through capillaries show a uniform cytoplasmic distribution immediately upon injection. Subsequently, many of these proteins appear to become localized within "vacuoles", although it has not been proven that these structures are truly membrane-bound. Eventually, the injected proteins assume a perinuclear distribution (Stacey & Allfrey, 1977). The time-course for vacuolization is dependent upon the protein injected (3 hours to 3 days), and there is a tendency for larger, more acidic proteins to be "vacuolized" more rapidly. While some believe microinjected proteins are broken down chiefly through lysosomal processes (Doherty & Mayer, 1985), others maintain that cytoplasmic systems provide a significant contribution to their degradation (Bigelow et al., 1981). Results from capillary microinjection have shown that vacuolized proteins eventually have the same distribution as lysosome-specific acid phosphatase; however, not all proteins are vacuolized. This suggests that not all proteins are processed identically and that there is more than one degradation process (Stacey & Allfrey, 1977).

In this present study, I have explored the feasibility of using liposomes for microinjecting proteins into cells. The potential advantages of this method are the ease of preparation of liposomes, the low cost of raw materials, no disruption of the cell monolayer, and the higher probability of a random distribution of injected material.

The principle theory behind cell microinjection through a liposome vehicle is
vesicular phospholipid fusion with cellular membranes. Although liposome-cell fusion has never been absolutely proven to occur, numerous studies have provided circumstantial evidence to indicate that fusion does take place.

**Bivalent Cations and Membrane Phospholipid Interactions**

Bivalent cations are reported to modulate membrane fusion with acidic, unilamellar liposomes. At physiological monovalent salt concentrations, only acidic phospholipids (PS, PA) bind strongly to bivalent metal ions (Papahadjopoulos, 1968). PS appears to be the principle phospholipid involved in Ca\(^{++}\)-mediated permeability changes within biological membranes. PS is usually 10-20% of the total cell membrane phospholipid. Membranes lacking PS (prokaryotes, mitochondria and chloroplasts) usually contain PG or other glycerol-containing phospholipids (Papahadjopoulos, 1971).

Membranes are generally stable structures. For membrane fusion to take place, there must be destabilization at some point (Papahadjopoulos et al., 1977). The parameters of fusion have been stated (Papahadjopoulos et al., 1976):

1. Lipids must be in the fluid phase.
2. There is a threshold concentration of Ca\(^{++}\) or Mg\(^{++}\) required for fusion, and this concentration varies for each phospholipid.
3. Induction of fusion by cations is not due to electrostatic charge neutralization.
4. Ca\(^{++}\)-induced fusion results in the crystallization of acyl chains and an increase in the phase transition temperature.
The ability of Ca\(^{++}\) to increase liposome membrane permeability (decrease membrane stability) appears to be related to differences in the Ca\(^{++}\) concentration between the inside and outside of the liposome. Thus, addition of the calcium ionophore A23187 reduces Ca\(^{++}\) concentration differences across the liposome membrane and also reduces the effect of Ca\(^{++}\) on membrane permeability and fusion. When Ca\(^{++}\) is added to a phospholipid vesicle suspension, ions bind only to the outer monolayer. Some charge neutralization occurs, and the vesicles begin to aggregate. As the Ca\(^{++}\) concentration is increased above >1 mM, more condensation of the phospholipids occurs to the point of crystallization of the outer monolayer. However, the inner layer remains fluid. Structural defects may occur, exposing the hydrocarbon chains to water. This creates a very unstable state, which is suggested to cause increased permeability and susceptibility toward fusion. As the bilayer crystallizes, phospholipid molecules cannot accommodate the high curvature of the original vesicular structure. Ruptures occur, and flattened bilayer disks are formed in which hydrocarbon (HC) edges are exposed to water. These hydrocarbon-water interactions are unfavorable, so the HC structures tend to fuse, producing large membranous sheets (Papahadjopoulos et al., 1977).

**Liposome Visualization**

Nearly all visualization studies of liposomes have been performed using electron microscopy, which is necessarily a static process. Therefore, these studies are limited to structural characteristics rather than dynamic properties. One group (Rand et al., 1985;
Kachar et al., 1986) recently observed the structural changes in giant complex phospholipid vesicles (GCV)--prepared from either pure PS, dioleyl phosphatidylethanolamine (DOPE)/PS or PC/PS lipid mixtures--in a Ca\textsuperscript{++}-containing buffer using video-enhanced differential interference light microscopy and rapid mixing freeze fracture electron microscopy. Addition of 60 mM CaCl\textsubscript{2} to the phospholipid suspensions caused adhesion of the vesicles. The zone of adhesion between PS-LUV and between DOPE/PS GCV vesicles appeared as a flat diaphragm made up of two tightly apposed bilayers. The vesicles often deformed as the contact area enlarged, with the interior angles between the diaphragm and the vesicle bilayer at the margins of the contact zone becoming progressively smaller. This produced such a high level of membrane curvature and stress in the membrane adjacent to the contact region that the vesicles either remained adherent but lost volume as a consequence of membrane rupture or leakage, or the interface between the vesicles ruptured, resulting in vesicle fusion. Fusion was often observed to occur when at least one of the vesicles was multilamellar or when there was a high proportion of smaller vesicles present. Fusion and rupture were equally likely events with PS-LUV aggregates. Eventually, an end-state was reached, which consisted of multilamellar bilayer stacks, as revealed by freeze fracture electron microscopy. The formation of choclate cylinders as described by Papahadjopoulos (1970) was not reported in this study (Rand et al., 1985).

Somewhat different results were obtained with lipid vesicles of differing composition. With DOPE/PS-GCV, vesicle adhesion and diaphragm formation occured
and was then apparently followed by either vesicle fusion or vesicle deflation. Although adherant vesicles frequently collapsed, there was no evidence of vesicle rupture. It was suggested that vesicle collapse could result from leakage of vesicle contents across highly stressed membrane regions at the margins of the contact zones (diaphragms). Eventually, the system decayed with the extrusion of the vesicle contents. The individual lipids then separate to form bulk lipid phases with appearances similar to that of pure DOPE (hexagonal phase lipid) and pure PS-Ca (lamellar phase lipid). In contrast, when Ca++ was added to PC/PS vesicles, large aggregates were formed. These PC/PS vesicle aggregates were stable (no fusion, no loss of volume) for at least several hours. X-ray diffraction of these aggregates revealed an amorphous structure with no evidence for a regular multilamellar phase as seen with pure PS-Ca.

It has been proposed (Kachar et al., 1986) that mechanical bilayer tension is required—in addition to enhanced contact—for bilayer fusion or rupture. Bilayer tensions greater than 3 dynes/cm caused by high surface curvature and surface area increases greater than 3% caused by osmotic stress lead to bilayer rupture (Evans & Parsegian, 1983; Evans & Kwok, 1982). In contrast, stable aggregation (as observed for PC/PS vesicles) may be attained if low-adhesion energies result in tensions below the critical level. When bilayer tension is high, stress may be relieved by rupture of the contact area (fusion, by rupture of the bilayer adjacent to the contact zone seen with PS-LUV), or by volume loss without rupture (seen with DOPE/PS-GCV) (Kachar et al., 1986). PS-LUV have been observed to rupture rapidly prior to formation of very large aggregates (Rand
et al., 1985). DOPE/PS vesicles form large, stable aggregates before rupture tensions are attained (Kachar et al., 1986). Calcium, which has a high affinity for pure PS vesicles, produces adhesions with high surface tensions (Parsegian & Rand, 1983). Ca\(^{2+}\)-PS-Ca complexes appear to form very early (Rand et al., 1985). This results in dehydration of PS bilayers, permitting their close apposition (Rand et al., 1985). In addition, calcium might increase bilayer-bilayer adhesion by forming a bridge (PS-Ca\(^{2+}\)-PS) between the polar head groups of adjacent PS-containing bilayers.

In summary, model studies employing PS-liposomes indicate that Ca\(^{++}\) mediates a tight aggregation of vesicles with resulting stress to the bilayer. This stress is relieved by either vesicle fusion or vesicle rupture, with both being equally likely. Incorporation of PC into PS liposomes reduces the interaction energy between adherent bilayers and causes vesicle fusion and/or rupture to become less likely.

**Liposome-Cell Membrane Interactions**

After reviewing the theoretical concepts of lipid-lipid interactions and fusion, the practical experimental aspects of lipid-cell membrane interactions must be examined. It must again be stressed that liposome-cell fusion has not been conclusively proven to occur in any experiment, although there is considerable indirect evidence that it does. It is known that mammalian cells can incorporate large numbers of lipid vesicles without cytotoxicity. Fusion with plasma membranes, endocytosis, surface adsorption and molecular exchange may all be methods of transport between vesicles and cells.
Charge neutralization by Ca$^{++}$ (and to some extent Mg$^{++}$) of acidic phospholipids allows vesicle aggregation and fusion (Papahadjopoulos et al., 1977 & 1976). Presumably, the same principles apply to vesicle-cell membrane fusion.

The route of vesicular incorporation is highly dependent on both the electrical charge and the phase of the phospholipids within the vesicles. $^{14}$C-sucrose uptake by 3T3 cells was studied using 3 types of vesicles:

1. Solid-charged (PS/DSPC/DPPC)
2. Fluid-neutral (PC)

Treatment of cells with the endocytosis inhibitor cytochalasin B (Cyto B) resulted in an 80 to 90% inhibition of sucrose uptake compared to controls for solid-charged and fluid-neutral vesicles. In contrast, the uptake of sucrose from charged-fluid vesicles was inhibited only 35% by Cyto B. Much more $^{14}$C-sucrose was incorporated into Cyto B-treated cells from charged fluid than from charged solid vesicles (Poste & Papahadjopoulos, 1976).

Inhibitors of glycolysis and respiration, when used in combination, effectively inhibit endocytosis but not fusion. Uptake of $^{14}$C-sucrose from fluid-neutral and solid-charged vesicles was completely inhibited in cells treated with these inhibitors, while charged-fluid vesicle uptake was decreased only 30-40%. The temperature dependence of uptake of fluid-charged vesicles shows a marked change in slope at 16-18$^\circ$ C, while
fluid-neutral and solid-charged vesicles do not demonstrate such a slope change. Membrane fusion processes have distinct transition temperatures at 18° C, but endocytosis has a simple linear dependence (Poste & Papahadjopoulos, 1976).

These results suggest that fluid-neutral and solid-charged vesicles are incorporated into 3T3 cells primarily by endocytosis, while charged-fluid vesicles may be incorporated at least 60% by fusion with the plasma membrane. They also suggest that vesicles may be targeted; different lipids can be used to introduce substances into different cellular regions. Solid-charged and fluid-neutral vesicles which enter by endocytosis would deliver materials to the lysosomes, while charged-fluid fused vesicles would release their contents into the cytoplasm. Fusion of charged-fluid vesicles may also change the composition of the plasma membrane.

Other studies have also suggested that fusion, rather than molecular exchange, occurs between charged-fluid lipid vesicles and cells (Papahadjopoulos et al., 1974). L929 and 3T3 cells after incubation with PS/PC/cholesterol liposomes containing 14C-cholesterol and 3H-dipalmitoylphosphatidyl choline (DPPC) show nearly identical ratios of 3H: 14C (1: 1.8) as that of the original liposomes (1: 1.9). If molecular exchange was the mode of transfer of label from liposomes to cells, cholesterol uptake was expected to have been much greater than PC uptake. Electron micrographs of RBC ghosts incubated with multi-lamellar vesicles (PS/PC) revealed "patches" devoid of intramembrane particles (integral membrane proteins) not seen in untreated controls. The dimensions of the patches were similar to those of the vesicles. In addition, the surface density of
intramembranous particles on vesicle-treated cells not showing patches was lower than in untreated cells, which suggests an increase in cell surface area (Papahadjopoulos et al., 1974).

An enzyme repletion study was conducted with invertase-deficient macrophages and fibroblasts (Gregoriadis & Buckland, 1973). [Invertase converts sucrose to glucose + fructose.] These cells were incubated with sucrose, which resulted in the appearance of phase-lucent vacuoles in the perinuclear region. The vacuoles had not disappeared by 5 hours (macrophages) or 24 hours (fibroblasts) after the cells were exposed to a sucrose-free medium.

Incubation of the sucrose-loaded cells with liposomes (egg lecithin, cholesterol, phosphatidic acid, 7: 2: 1) containing invertase for 3 hours (macrophages) or 24 hours (fibroblasts) resulted in the disappearance of the vacuoles in most of the cells. These observations were supported by radioimmunoasays in which the cells were incubated with U-14C-sucrose. After incubation with invertase-containing liposomes, much more radioactivity was released from the cells into the medium (glucose and fructose diffused out) than from cells incubated with denatured invertase-containing liposomes or controls (no invertase).

The authors (Gregoriadis & Buckland, 1973) suggest that invertase entrapped in liposomes is taken up by "liposome uptake", and finds its way to a sucrose-containing secondary lysosome. The liposomal membrane is then disrupted, perhaps by lysosomal lipases, and the enzyme is released. These results are consistent with those of Poste, et al.
(1976) which suggest that PC enriched liposomes are targeted to lysosomes.

Several studies involving viral infectivity have been done with PS liposomes, which indicate that the viral particles are entrapped within the vesicle interior and are introduced into the cell by a non-endocytotic mechanism. PS vesicles capture naked nucleotides at 5-10% efficiency (Wilson et al., 1979; Mayhew et al., 1977). It appears that a minimal nucleotide concentration of 1 ng/nmol lipid is required for maximal capture (Wilson et al., 1979). The maximum efficiency of polio virus RNA infection is 1 RNA molecule/vesicle. This implies that the RNA is highly infectious once it enters the cell, and that the rate-limiting step is the vesicle-cell interaction. It also implies that the encapsulated RNA is not significantly degraded by lysosomal nucleases. Therefore, endocytosis is probably not the mode of uptake of the encapsulated RNA (Wilson et al., 1979). The encapsulated RNA is also resistant to added ribonuclease (Taber et al., 1978). In fact, RNAase treatment of the vesicles increases infectivity 10-15 fold (Wilson et al., 1979). However, this effect may be due to the high positive charge of RNAase, because the denatured enzyme had a similar effect. The positively-charged RNAase may neutralize the negative charge on the vesicles, which may enhance vesicle-cell interactions (Wilson et al., 1979).

The synthetic interferon inducer polyribosyl inosine phosphate/polyribosyl cytosine phosphate (rI_n.rC_n) has been encapsulated by liposomes. Uptake of rI_n.rC_n by cells was 2.5-9 fold higher when the polynucleotides were in vesicles than when free in solution. In free solution, the rC_n strand was taken up more slowly than rI_n, even though
they were from the same double helix. However, $rI_n.rC_n$ uptake from vesicles showed
that both strands entered the cell unaltered. Furthermore, only vesicle-entrapped $rI_n.rC_n$
produced significant increases in antiviral activity in human fibroblasts compared with
free $rI_n.rC_n$. Vesicle-entrapped single-stranded $rI_n$ and $rC_n$ and mixtures of
nonencapsulated $rI_n.rC_n +$ pre-formed vesicles had no effect (Mayhew et al., 1977).

Poliovirus has been encapsulated in PS liposomes. The encapsulated viruses were
antibody resistant; antibody decreased free virus infectivity $10^5$ fold (Taber et al., 1978),
while the "vesicular" virus infectivity was reduced less than 10 fold (Taber et al., 1978;
Wilson et al., 1977). Chinese hamster ovary (CHO) cells, which do not have the primate-
specific membrane receptor for poliovirus, can be infected by the encapsulated viruses.
Free virus, free virus + vesicles and vesicles alone have no effect (Taber et al., 1978).

These studies give, at best, indirect proof for the validity of the fusion mechanism.
Furthermore, they involve potent molecules which produce strong responses (e.g.
cytotoxicity, infections, resistance) when only a relatively small number are delivered.
Thus, such studies do not provide quantitative information about the efficiency of
liposome-cell fusion as a means for injecting substances into cells in bulk. One purpose
of this study was to evaluate liposomes as a vehicle for the bulk injection of proteins into
the cytosol of cultured cells. This required the development of an assay to quantify
liposome-cell fusion.
CHAPTER II
MATERIALS AND METHODS

PART I. LIPOSOME-MEDIATED MICROINJECTION OF PROTEINS INTO FIBROBLASTS

Cell Culture

Normal adult human diploid fibroblasts, GM3440, were obtained from N.I.G.M.S. Human Genetic Mutant Cell Repository located in the Institute for Medical Research (Camden, NJ). Fibroblasts were grown in medium consisting of 85% Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories), 13% heat-inactivated (56-65° C, 30 min) fetal bovine serum (Gibco), and 2% of a penicillin-streptomycin solution (200 units penicillin, 200 µg streptomycin per 1 ml complete medium) (Gibco). Cells were grown either in 10 ml complete medium on 75 cm² tissue culture flasks (Falcon or Lux) or in 5 ml medium on 60 x 15 mm tissue culture dishes (Falcon or Corning) at 37° C in a 95% air/5% CO₂ atmosphere (McGarrity, 1979).

When cells reached confluency, they were detached with 0.5% Trypsin (ICN) in phosphate buffered saline (Flow) and passed 1:3 (75 cm²) or 1:6 (60 x 15 mm). Time to confluency generally ranged between 5-14 days, depending on cell passage. Unless otherwise stated, all experiments were done with confluent monolayers of cells having passage numbers between 7 and 17.
**Preparation of Large Unilamellar Liposomes**

Large unilamellar liposomes (LUV) were prepared by formation of Ca$^{2+}$-cochleate structures (Papahadjopoulos, 1970) or multibilayer stacks (1985) from small unilamellar vesicles (SUV), followed by chelating the Ca$^{2+}$ with EDTA (or EGTA) (Wilson et al., 1977; Mayhew et al., 1977; Papahadjopoulos et al., 1975). Ten μmol of bovine brain phosphatidylserine (Sigma, P-L Biochemicals) was transferred to a screw-cap test tube which had been flushed with N$_2$, and the CHCl$_3$ solvent was evaporated under N$_2$. One ml of buffered saline (100 mM NaCl, 2 mM histidine, 0.1 mM EDTA, 2 mM TES, pH 7.4) was added under N$_2$. The test tube was capped, vortexed 10 minutes to suspend the lipid, then sonicated 30 min with a Branson bath-type sonicator with a cooling coil. The resulting suspension, which contained small unilamellar liposomes, was left at room temperature in a nitrogen atmosphere for 1 h before further processing.

Seventy μl of 0.1 M CaCl$_2$ was added to the liposome suspension and the resulting material incubated for 1 h at 37$^\circ$ C. [At this point, the Ca$^{2+}$ cochleate of phosphatidylserine could be stored overnight at 4$^\circ$ C]. The suspension was centrifuged at 10,000 x g for 10 min at 20$^\circ$ C and the supernatant discarded. [At this point, the pellet may be stored at -20$^\circ$ C under a N$_2$ atmosphere for several months]. The pellet was mixed with 0.1 ml of saline buffer containing the material to be entrapped, and 3 glass beads were added. The suspension was vortexed 10 seconds, then sonicated 10 sec to maximize dispersion. Eighty μl 0.1 M EDTA and 50 μl 0.1 M NaOH were added (or 150 μl 0.1 M EGTA, pH 7.4), and the mixture was shaken for 10 min at 37$^\circ$ C. After
equilibration for 30 min at 37°, 0.5 ml buffer was added. This suspension was either
dialyzed against 1 of buffer for 30 h. Alternatively liposomes were collected by
centrifugation at 40,000 xg for 25 minutes at 20° and the pellet resuspended in 1 ml buffer
three times to remove nonencapsulated materials. ²

**Phosphorus Determination**

Phospholipid concentration was measured by determining phospholipid phosphorus (Bartlett, 1959; Bottcher et al., 1961). Five µl aliquots of the final liposome suspension were pipetted into acid-washed screw-cap test tubes, to which were added 0.2 ml 70% perchloric acid (HClO₄) (Mallinckrodt). The tubes were then placed in a heating block (Tecam Dri-Block) at 168° C for 40 min. The tubes were cooled to about 50° and 0.2 ml 5% ammonium molybdate (Fisher) and 0.2 ml Fiske-Subbarow reagent were added. The tubes were vortexed, loosely capped, and then incubated in a boiling water bath for 7 min. The tubes were cooled and distilled water was added to the 5.0 ml mark. Absorbance was measured at 830 nm. A series of dilutions of a standard phosphate solution were made from a 10⁻⁴ M stock NaH₂PO₄ (Sigma) solution to obtain phosphorus contents ranging from 0-0.20µmoles.

The Fiske-Subbarow reagent was made by adding 0.5 g of purified 1-amino-2-naphthol-4-sulfonic acid to 200 ml of a freshly prepared 15% NaHSO₃ (Mallinckrodt)

²All glassware for liposome preparation was soaked in HNO₃:H₂SO₄ (1:10) for 1 h and rinsed thoroughly with deionized water. Stirring bars were soaked in a solution of EDTA/EGTA and rinsed with deionized water. All aqueous solutions were degassed before use.
solution, followed by 1.0 g Na₂SO₃ (anhydrous) (Allied Chemical). The suspension was heated to dissolve the solutes, and the solution was stored in a dark bottle at 4°.

To purify 1-amino-2-naphthol-4-sulfonic acid, 1.5 g of the crude material (Eastman Chemicals) was added to 100 ml water containing 15 g NaHSO₃ and 1 g Na₂SO₃ and heated to 90°. The mixture was stirred until all but an amorphous impurity was dissolved. The hot solution was suction filtered, and the filtrate cooled thoroughly. One ml of 12 N HCl was added to precipitate the product, and the mixture was suction filtered. The precipitate was washed with 30 ml cold water followed by cold ethanol until the washings were colorless (Fiske & Subbarow, 1925).

**Electron Microscopy**

Liposomes were characterized by electron microscopy using a pH-neutral negative stain. The liposomes were prepared as before, except that the saline buffer contained 1% phosphotungstate (Baker) (Egerdie & Singer, 1982). Samples were placed on prepared copper mesh grids. After 1 min, the grids were drained, then allowed to air dry. Photographs were taken with Kodak electron microscope film-4489.

The negatives were developed in the following manner:

- D-19 developer: 4 min
- H₂O: 1.5 min
- Kodak fixer: 8 min
- H₂O: 1 min
Kodak hyperclear  2 min
H₂O  5 min

The prints were made the following way:
Dektol  1 min
Stop bath (Kodak indicator)  15-30 sec
Kodak fixer  2-4 min
H₂O  5 min

Measurement of Entrapped Aqueous Space

¹⁴C-labeled sucrose was added to the saline buffer during the entrapment step in order to determine the efficiency of the trapping step and the average size of liposomes (calculated from surface area to volume ratios). Two μCi [U⁻¹⁴C] sucrose (10 μl sterilized aqueous solution containing 3% ethanol, 200 μCi/ml) (Amersham) was added with 0.1 ml of saline buffer prior to the addition of EDTA. The untrapped sucrose was removed by dialysis before the liposomes were isolated in the final pellet. The liposome mixture was placed in dialysis tubing which had been pretreated with EDTA/EGTA and rinsed thoroughly with deionized water. The bag was dialyzed against 1000 ml of NaCl buffer (pH 7.4) overnight. The retentate was again dialyzed against 1000 ml fresh buffer for 5 h. Samples taken from the dialysates and retentates were dissolved in 10 ml Aquasol and radioactivity was determined by liquid scintillation (Beckman LG 7500).
Calculation of Average Liposome Size

Average liposome size was determined by 3 methods: (1) Direct measurement by electron microscopy; (2) molecular sieve chromatography; and (3) calculation of the average liposome diameter, D, from the entrapped volume. The relationship between diameter, volume and surface area is given by the following equation:

\[ D = 6 \times \frac{\text{Vol}}{\text{Area}} \]

\[ D = \frac{6 \times (\mu\text{l trapped}) \times 10^{18} \text{nm}^3/\mu\text{l}}{(\mu\text{mol PS}) \times 1.89 \times 10^{17} \text{nm}^2/\mu\text{mol}} \]

Volume is the \( \mu\text{l} \) of entrapped aqueous space per \( \mu\text{mole} \) of phospholipid recovered, and is calculated from the amount of \(^{14}\text{C}\) sucrose and phospholipid recovered from the liposomes. Area is the surface area of a bilayer which can be formed from 1 \( \mu\text{mole} \) of phospholipid, assuming a packing density found in hydrated bilayers formed from egg lecithin (62.7 Å/polar head group) (Levine & Wilkens, 1971).

Further experiments for characterizing liposome size were completed by others in this laboratory (Thyagarajan, 1985). PS-LUV liposomes were prepared as previously described in the presence of either \(^{14}\text{C}\) sucrose (2-4 \( \mu\text{Ci/ml} \)), FD (40 mg/ml) or \(^{125}\text{I}\)-labeled phosphorylase b 100 \( \mu\text{Ci/ml} \) in the NaCl buffer. These were separated from unentrapped solutes by gel filtration on a 1.5 x 50 cm column of Sepharose CL4B presaturated with PS and eluted with the same buffer. This column was previously calibrated using microspheres of defined sizes as standards.
Preparation of $\text{[}^{14}\text{C}]$-Cholesterol-Labeled Liposomes

Liposomes were sometimes prepared with $^{14}$C-cholesterol. The procedure was identical to that described above, except that 5 µCi $[4-^{14}\text{C}]$ cholesterol (5 µl toluene solution, 100 µCi/ml, specific activity = 58.4 mCi/mmol) (Amersham) was added to the phosphatidyl serine solution before solvent evaporation. The liposomes were dialyzed as for the sucrose-labeled liposomes.

Entrapment of Fluorescent Probes in Liposomes

Fluorescein isothiocyanate-dextran (average MW = 10,500) (Sigma) was entrapped into LUV to be used as a pH-sensitive fluorescent probe in order to detect liposome-cell fusion (Okuma & Poole, 1978). Liposomes were prepared as before, except that 10.4 mg fluorescein isothiocyanate-dextran (FD) was added with 0.1 ml buffer prior to the addition of EDTA. The liposomes were washed 3 times by centrifugation.

In some experiments, 6-carboxyfluorescein (Sigma) (6-CF) was entrapped within LUV. To purify the compound, 750 mg 6-CF was stirred in 50 ml H$_2$O and solubilized with a few drops of NaOH. The solution was filtered and adjusted to pH 7.4 with acid. Molarity was determined to be 0.388 M by absorbance measurements. Liposomes were prepared as before, except that 80 µl of a purified 0.388 M 6-CF stock aqueous solution and 20 µl of 5 x saline buffer, were added prior to the addition of EGTA. The liposomes were washed 3 times by centrifugation.
Fluorescence was detected with a Perkin-Elmer MPF-44B fluorescence spectrophotometer operated in the Ratio mode, and corrected fluorescence spectra were obtained. Instrument settings were as follows:

- Emission slit width = 20 nm
- Excitation slit width = 10 nm
- Emission = 519 nm
- Excitation = 400-500 nm in the scanning mode, or 450 & 495 nm in the fixed wavelength mode

Neutral density plate 4

A standard curve of A495/A450 (excitation wavelengths) vs. pH was made by adding 100 µl of a 300 µg/ml FD solution to 3 ml of buffers ranging in pH from 4.0-8.5. To determine the pH dependency of liposome-entrapped FD, 100 µl of the final liposome preparation was added to 3 ml of buffer at pH 4.75, 6.8, and 8.5. Fluorescence of liposome-entrapped 6-CF was determined by adding 10 µl LUV (or 20 µl phosphatidylcholine SUV) to 3.0 ml NaCl-Tris buffer in the presence and absence of 0.1 ml of 10% Triton X-100 [Excitation = 492 nm; Emission = 520 nm].

Liposome-Cell Fusion

Confluent monolayers of GM 3440 fibroblasts grown in 75 cm² flasks were used for all experiments. The growth media was removed from the cell cultures, and the cells were washed with 5 ml of phosphate buffered saline (PBS) (Flow). Two ml fresh PBS +
1 ml liposomes were added to the "experimental" flask. "Control" flasks contained 3 ml PBS and either 1 µl ¹⁴C-sucrose or 1 µl ¹⁴C-cholesterol for the radioactive experiments. Controls for the fluorescent experiments consisted of cells which had been incubated with empty liposomes. The cells were incubated 2.5 h at 37°, then washed 3 times with 5 ml PBS. The cells were either detached with 2 ml 0.5% trypsin or disrupted by adding 1 ml of 0.1% Triton X-100 and sonicating 10 min in a Bendix bath-type sonicator. One ml of cell suspension was dissolved in 10 ml Aquasol to count radioactivity.

**Preparation of Fluorescein-Labeled Bovine Serum Albumin**

Forty mg bovine serum albumin (BSA) (Sigma) was dissolved in 4 ml 0.05 M Tris, 0.1 M NaCl (pH 9.2) buffer. Eight mg fluorescein isothiocyanate (FITC) (Sigma) was dissolved in 10 ml dimethyl formamide to give a 2 mM solution. Forty µl of the FITC solution was added to the BSA solution and allowed to react for 45 min.

The reaction mixture was loaded onto a 1.5 x 25 cm Sephadex G-25 column equilibrated with Ca⁺⁺-free/Mg⁺⁺-free PBS (pH 7.4) and eluted with the same buffer (flow rate = 33.0 ml/h). One ml fractions were collected and analyzed for fluorescence:

2.9 ml PBS + 0.1 ml fraction

Excitation  = 492 nm

Emission  = 520 nm

Signal gain = 3

Fluorescent fractions which eluted with the void volume of the column were pooled and
stored at -20° C.

Preparation of Small Unilamellar Vesicles (SUV)

All lipids were purchased from Sigma. Five mg egg yolk L-α-phosphatidylcholine (PC) + 1.05 mg lysolecithin + 0.27 mg stearylamine (molar ration of lipids 70: 20: 10, respectively) were placed in a 50 ml round-bottom flask and dissolved in 9 ml CHCl₃ and 1 ml methanol. The solvents were evaporated to dryness on a rotary evaporator (2 h). The entrapment buffer was added: 1 ml Ca⁺⁺-free/Mg⁺⁺-free PBS + 10 mg FD or 0.7 ml FITC-BSA stock solution + 0.8 ml PBS. The flask was flushed with N₂ for 3 min, then sonicated 45 min with a Branson bath-type sonicator. The suspension was allowed to equilibrate 1 h at room temperature. The liposomes were washed by centrifuging 3 times at 40,000 x g (18,000 rpm) at 20° C for 25 min. The final pellet was suspended in 1 ml PBS (Ca⁺⁺-free/Mg⁺⁺-free).

Percent entrapment was determined by fluorescence measurements. The fluorescence of a blank solution containing 2.9 ml (2.9) ml PBS + 0.1 ml 0.1% Triton X-100 was set to 0. The stock FITC-BSA solution was analyzed by adding 5 µl to 2.9 ml PBS + 0.1 ml 0.1% Triton X-100. Liposome fluorescence was measured by adding 50 µl of the suspension to 2.8 ml PBS + 0.1 ml 0.1% Triton X-100.

Excitation  = 492 nm

Emission  = 520 nm
Preparation of Cells for Microscopic Examination

Cells were prepared for microscopic examination by (1) suspension of trypsinized cells in PBS and (2) formaldehyde fixation of cells grown on coverslips. For the first method, 5 ml PBS was added to trypsinized cells (as previously described). The cells were pelleted by centrifugation and resuspended in 1 ml PBS. One drop of the suspension was placed on a microscope slide and observed under a fluorescence microscope.

For the second method, glass coverslips were coated with 1 ml 10% FCS in PBS and incubated at least 1 h at room temperature in a tissue culture hood. The FCS solution was removed and the coverslips were air-dried and sterilized under a U.V. light for 24 h.

Medium was removed from the fibroblasts, and the cells were washed with 5 ml PBS. Five ml medium were added, and the cells were detached by scraping. The cells were pelleted by centrifugation and resuspended in complete medium. One ml of the suspension and 1 ml of medium were added to each treated coverslip. The fibroblasts were allowed to incubate at 37° at least 1 day to permit attachment and spreading.

After incubation, the medium was removed, and the coverslips were washed with 2 ml sterile PBS. The cells were then incubated with SUV prepared from PC, lysolecithin and stearylamine containing either FD or FITC-BSA. Two methods of incubation were used:

1. The coverslips were incubated 1 h at 37° C with 1.0 ml PBS (containing no Ca++ or Mg++), 0.9 ml of the liposome preparation, and 0.1 ml Ca++ (final Ca++
concentration = 40 mM). [The control coverslips were incubated in 2.0 ml PBS which was made 40 mM in Ca\textsuperscript{++}.] The coverslips were washed 3 times with 2 ml PBS (Whittaker) and fixed with 2 ml Immunofix II for 4 min at room temperature. The coverslips were washed at least 3 times with PBS, then glued to alcohol-washed slides with UVInert.

2. The coverslips were incubated 2 h at 37\textdegree with 0.5 ml liposome suspension and 1 ml PBS (Whittaker). After rinsing with PBS, one coverslip was immediately fixed with Immunofix II (as described in 1). The second coverslip was incubated for 2 h at 37\textdegree in complete growth medium, rinsed with PBS, and fixed with Immunofix II.

An endocytosis experiment was also performed with the cells seeded on coverslips. The coverslips were incubated with 2 ml complete growth medium containing 1 mg FD/ml medium for 18 h at 37\textdegree. The medium was removed, and the cells were washed several times to remove the fluorescent compound. The cells were then fixed with Immunofix II.

Immunofix II was prepared by heating 0.25 g paraformaldehyde (Sigma) in 2.5 ml H\textsubscript{2}O until it began to steam (about 60\textdegree C). Care was taken to avoid boiling. Two to four drops of 0.1 N NaOH was added to clarify the solution. Cacodylate buffer (12.5 ml of 0.2 M, pH 7.4) and 10 ml H\textsubscript{2}O were then added (final volume 25 ml).
Photography

Photographs of the trypsinized cells were made as observed under U.V. lighting:

ASA = 400

Recip. = 3

Exposure time = automatic

Photographs were made of the cells as observed under phase-contrast and U.V. lighting. Black and white Tri-X and Ektachrome 400 color films were used.

ASA = 800

Timing = Automatic mode

The slides and prints were made professionally (Loyola Photography Department).

Measurement of Intracellular pH

Confluent monolayers of fibroblasts were pretreated with empty PS-LUV for 2 h in PBS containing Ca++ and Mg++. The monolayer was then washed 4 times and the cells harvested by trypsin treatment. Washed cells were then resuspended in ice cold 50 mM MES buffer, pH 6.2, containing the non-fluorescent derivative (5)6-carboxyfluorescein (CF) diacetate (35 µM). After a 15 minute incubation at 40°C, the cells were washed, resuspended in ice cold PBS, and aliquots removed for fluorescence measurements in buffers of various pH. The aliquots of the medium were also analyzed for fluorescence in the various buffers (Thyagarajan et al., 1985).

Excitation = 450 nm, 495 nm

Emission = 519 nm
PART II. DEGRADATION OF ENDOGENOUS CELLULAR PROTEINS

Measurements of Cell Leakage by the Lactate Dehydrogenase Method

Media was removed from confluent monolayers of fibroblasts grown on 60 x 15 mm dishes. The cells were washed 3 times with 2 ml of complete growth medium warmed to 37°C. Media samples were collected in two ways:

1. Five ml fresh medium at 37°C was added, and cells were incubated at 37°C. At various time points, 1 ml of the medium was withdrawn for assay and replaced with 1 ml fresh medium. The samples were then assayed immediately for lactate dehydrogenase activity with a Perkin-Elmer 320 spectrophotometer.

2. One ml fresh medium at 37°C was added, and cells were incubated at 37°C. At intervals of 1 h, the entire medium (1 ml) was removed and replaced with 1 ml of fresh medium (37°C). All samples were assayed immediately as before.

After the last time point, the cells were washed 3 times with 2 ml warmed media, and 2 ml of fresh media were added. The cells were scraped into the media, and 20 µl 20% Triton X-100 were added. The cells were disrupted in ice by a Cole-Parmer ultrasonic probe-type homogenizer (4710 series) and 0.1 ml was removed for measurement of lactate dehydrogenase.

Lactate dehydrogenase activity was measured by the method outlined by Kornberg (Kornberg, 1955). The assay mixture contained 2.7 ml 0.03 M phosphate buffer (pH 7.4), 0.1 ml 0.002 M NADH (pH 8) (Sigma), 0.1 ml 0.01 M sodium pyruvate (pH 7) (Eastman) and 0.1 ml of the sample. Change in absorbance was measured at
340 nm for at least 2 min. Activity was recorded as µmol lactate formed/ml of medium or cell lysate/min.

SDS-Polyacrylamide Gel Electrophoresis

Acrylamide and N,N'-methylene bisacrylamide were purchased from Eastman. Trizma base, glycine, Coomassie Brilliant Blue, bromphenol blue, and sodium lauryl sulfate (SDS) were purchased from Sigma. Mercaptoethanol and TEMED were purchased from Bio-Rad. Acetic acid and methanol were purchased from American Scientific Products.

A stock acrylamide solution consisted of 30% acrylamide + 0.8% bisacrylamide. This solution was stored for no longer than 1 month at 4°. The lower gel buffer consisted of 1.5 M Tris (pH 8.8) and 0.4% SDS. The upper gel buffer consisted of 0.5 M Tris (pH 6.3) and 0.4% SDS. Running buffer was made from a 1:10 dilution of 0.25 M Tris, 1.92 M glycine and 1% SDS (pH 8.3). The reducing sample buffer (2 x) was prepared with 10 ml glycerol, 23 ml of a 10% SDS solution, 5 ml of β-mercaptoethanol, 2 ml of a 0.1% bromphenol blue solution, 8.3 ml of the upper gel buffer and 1.7 ml of deionized water.

The 9% lower running gel was prepared with 9 ml of the lower gel buffer, 10.8 ml of the acrylamide solution and 16.2 ml of deionized H2O. After degassing, 75 µl of a 10% freshly prepared ammonium peroxydisulfate solution and 10 µ of TEMED were added to initiate polymerization. The gel was poured immediately into a Bio-Rad gel
apparatus, covered with distilled H₂O, and allowed to polymerize overnight.

The 4.5% stacking gel was made by combining 2.5 ml of the upper gel buffer, 1.5 ml acrylamide solution, and 6.0 ml of deionized H₂O. After degassing, 300 µl of 10% ammonium peroxydisulfate and 10 µl of TEMED were added to initiate polymerization. The gel was poured around an insert comb and allowed to polymerize. The comb was then removed in the presence of the running buffer.

The gel was placed in the upper tank of a Bio-Rad Protean 16CM electrophoresis apparatus and the upper gel buffer was added. Either 50 µl of the prepared samples or 20 µl of the molecular weight standards were added to the sample wells. The lower tank was filled with running buffer, the upper tank was placed within the lower tank, and buffer was added to cover the cathode. The gel was run overnight (20 h) at 8 mamps (constant current) (Laemmli, 1970).

When the dye front reached the bottom of the gel, the power source was disconnected, and the gel was removed from the plates and placed immediately into a staining solution containing 46% methanol, 8% acetic acid, and 0.1% Coomassie Blue. After staining overnight, the gel was placed in a destaining solution containing 20% methanol and 8% acetic acid. The gel was destained until the bands were clearly visible (2-3 days). The gel was then photographed on a light box with Polaroid type 667 film [F-stop =16, shutter speed = 60, U.V. filter in place] (Laemmli, 1970).
**Fluorography**

Fluorography of the gels was performed according to Chamberlain's procedure (Chamberlain, 1979). The destained gel was soaked 30 min in 1 M salicylate (pH 7.0) (Sigma), then rinsed with 30% methanol. The gel was then placed on filter paper and covered with plastic wrap. The gel was dried overnight on a Hoefer Scientific Instruments Slab Gel Dryer (Model SE 540), then placed in a Wolf X-ray Corporation cassette equipped with Du Pont intensifier screens. Du Pont X-ray film was placed on the gel in total darkness and exposed 1 month at -70°C. The film was developed in total darkness with Kodak GBX developer for 5 min and Kodak GBX fixer for 5 min. The developed film was washed with tap water.

**Protein Metabolism in Cultured Fibroblasts**

L-Leucine and trichloroacetic acid (TCA) were purchased from Sigma. \[^{3}\text{H}-4,5\] L-leucine was purchased from ICN and Amersham. Leucine-free/glutamine-free medium was purchased from Hazelton. Phosphotungstic acid (PTA) was purchased from Baker.

Media was removed from confluent fibroblasts grown on 60 x 15 mm dishes, and the cells were washed with 2 ml PBS. The cells were then labeled by incubating them in 2 ml complete growth medium containing 1 µCi \[^{3}\text{H}-4,5\] L-leucine (0.5 µCi/ml) for approximately 40 h. After the labeling period, the media was discarded, and the cells were washed 3 times with 2 ml PBS. The cells were then incubated 5 min at 37°C with 5 ml of experimental media containing 2 mM Leu (non radioactive, "cold") to remove
free \(^3\)H-Leu. This media was removed and discarded. To measure intracellular protein degradation, 1 ml of fresh medium was added, and the cells were incubated at 37\(^\circ\)C (Gronostajski et al., 1985; Neff et al., 1977). Periodically, the entire medium was removed, and 1 ml of fresh medium was added. Each medium sample so obtained was treated with an equal volume of ice cold 20\% TCA-1\% PTA solution (Buktenica, 1983). This suspension was centrifuged at 5000 rpm for 10 min at 4\(^\circ\). The supernatant was removed and dissolved in 10 ml Ecoscint. The pellet was dissolved in 1 ml 1 N NaOH and added to 10 ml Ecoscint (Bond & Aronson, 1983). At the conclusion of the experiment, the cells were disrupted with 2 ml of 0.6\% SDS-0.01 M EDTA (Hirt, 1967) and dissolved in 10 ml Ecoscint. Radioactivity was measured with a Beckman LS7500 liquid scintillation spectrometer.

These experiments were conducted in the presence of a variety of agents known to affect cellular protein metabolism. The essential amino acid leucine was added to the complete growth medium at concentrations of 0, 2 and 10 mM; the control level was set at 2 mM Leu. The protein synthesis inhibitor cycloheximide (Sigma) was used at concentrations of 10 and 50 µM (Thoene et al., 1985). The lysosomotrophic agents ammonium chloride and chloroquine (Sigma) were added in 10 mM and 25 µM concentrations, respectively (Doherty & Mayer, 1985; Hasilik & Neufeld, 1980; Gonzalez-Noriega et al., 1980). The effects of two thiol proteinase inhibitors--leupeptin and antipain (Protein Research Foundation)--were also examined. Leupeptin was used at concentrations of 20 µM and 200 µM, while the concentration of antipain was 40 µg/ml
(Okada & Dice, 1984; Mego, 1984; Umezawa & Aoyagi, 1977). Vinblastine (Sigma), a microtubule inhibitor, was used at 55 µM (Ahlberg, 1982). Cytochalasin B (Sigma), an actin inhibitor, was used at 6.6 µg/ml (Stacey & Allfrey, 1977). Experiments with control medium were also performed at 26° C (Dunn et al., 1980).

Experiments were also performed in the absence of serum with leucine-free/glutamine-free medium (Poole et al., 1978). At least 1 compound from each class of agents listed above was tested. Under these conditions, the concentrations were changed to enhance the effects or to prevent cell death. The concentration of antipain was raised to 100 µg/ml. The carboxyl protease inhibitor pepstatin A (Protein Research Foundation) was added as a saturated solution (Mego, 1984; Umezawa & Aoygi, 1977). The chloroquine concentration was reduced to 5 µM. The vinblastine concentration was reduced to 12 µM. The concentration of cytochalasin B was changed to 7.5 µg/ml.

For electrophoresis, cells were labeled as before, except that 55.6 µCi [35S]-methionine (27.8 µCi/ml) (Amersham) was used in place of 3H-Leu. Two incubation periods were used: 30 min and 42 h. The experimental medium consisted of complete growth medium containing 2 mM Met (Sigma). Samples were treated with equal volumes of 2 x electrophoresis sample buffer and heated in boiling water for at least 2 min. Samples were stored at -20° prior to electrophoresis (Hasilik & Neufeld, 1980; Sahagian & Gottesman, 1982).
Measurements of Cell Detachment and Vesicle Shedding

The cells were metabolically labeled and washed as before. Cells were incubated in either complete growth medium or in serum-free, Leu-free, Gln-free medium for 3 h at 37°. All 1 ml of the medium was then removed to Beckman 343778 centrifuge tubes and balanced to the nearest 0.01 g. The media was centrifuged at 18,000 rpm (14,116 x g) on a Beckman TL-100 ultracentrifuge for 15 min at 4°. The supernatant was removed to new tubes; the pellet was dissolved in 0.5 ml of the 0.6% SDS-0.01 M EDTA solution and added to 10 ml Ecoscint.

The supernatants were balanced as before, then centrifuged at 50,000 rpm (108,920 x g) for 1 h at 4°. The supernatant was removed and precipitated with 1 ml ice cold TCA-PTA. This suspension was centrifuged at 5000 rpm for 10 min at 4°, and the supernatant was dissolved in 10 ml Ecoscint. The pellet was dissolved in 1 ml 1 N NaOH and added to 10 ml Ecoscint. The high-speed pellet was dissolved in 0.5 ml SDS-EDTA and added to 10 ml Ecoscint.

Two ml fresh media were added to the cells, which were then incubated overnight at 37°. In the afternoon of the second day, the medium was removed, and the cells were washed 3 times with 2 ml PBS. The cells were next incubated overnight with 1 ml fresh medium. All 1 ml samples of medium were transferred to centrifuge tubes and centrifuged at 18,000 rpm and 50,000 rpm as before. The monolayer was disrupted with 2 ml SDS-EDTA and the extract was dissolved in 10 ml Ecoscint. Radioactivity was determined by liquid scintillation spectrometry.
**Cathepsin B Activity in Cultured Fibroblasts**

CBZ-L-Arg-L-Arg-4-methoxy-β-naphthylamide, ethylenediamine tetraacetic acid (EDTA) and L-dithiothreitol (DTT) were purchased from Sigma.

Media was removed from confluent fibroblasts grown on 60 x 15 mm dishes, and the cells were washed with 2 ml PBS. The cells were incubated for 66 h in 4 ml of each of the following media:

1. Complete growth medium containing 2 mM Leu.
2. Leu-free/Gln-free medium containing penicillin-streptomycin.
3. Complete growth medium containing 2 mM Leu and 100 µg/ml antipain.

The cells were washed 3 times with 2 ml PBS, then incubated at room temperature in 2 ml of hypotonic 50 mM acetate/1 mM EDTA buffer (pH 5.2) for 2 h on a shaking water bath. Samples (100 µl) of the resulting cellular extract were incubated with 100 µ activator (30 M DTT, 15 mM EDTA, pH 5.2) for 30 min at 37°C. The reactions were initiated by the addition of 200 µl 0.2 M citrate/phosphate buffer (pH 6.2) and 100 µl of the substrate (0.68 mM CBZ-L-Arg-L-Arg-4-methoxy-β-naphthylamide). At intervals of 5, 15, and 25 min, the reactions were terminated with 2 ml ice cold 1 N HCl. A product standard was prepared with of 100 µl of the acetate buffer, 100 µl of the activator, 200 µl of the citrate/phosphate buffer, 100 µl of 4-methoxy-β-naphthylamide (final concentration 100 µM), and 2 ml 1 N HCl.

Fluorescence was measured with a Perkin-Elmer MPF-44B fluorescence spectrometer at the following settings:
Excitation = 300 nm

Emission = 420 nm

Excitation slit width = 10

Emission slit width = 10

Signal gain = 1

The 100 nM standard was set to read 120 fluorescence units.

**Analysis of the Data from Studies of Protein Catabolism in Fibroblasts**

A complete copy of the program used in processing the data obtained from the release of radioactivity from fibroblasts appears in Appendix 2. This section contains a brief explanation of the programs and a summary of the calculations used.

1. **Program 1--Data**

   The amounts of TCA-soluble and TCA-insoluble radioactivity released at each time point are entered into the computer along with the total radioactivity remaining in the monolayer by the end of the experiment, \([\text{CPM}]_{\text{monolayer}}\). With this data, the program calculates the cumulative radioactivities released at each time for the two products, \([\text{CPM}_{\text{sol}}]_t\) and \([\text{CPM}_{\text{ins}}]_t\). The program next calculates the rates of release of TCA-soluble and -insoluble radioactivity at late times by linear regression analysis of \([\text{CPM}_{\text{sol}}]_t\) vs. \(t\) and \([\text{CPM}_{\text{ins}}]_t\) vs. \(t\) at late times. These rates are then used to calculate the ratio of TCA-soluble to total radioactivity released at late times, \(R_2\), from the relationship \(R_2 = \text{rate } [\text{CPM}_{\text{sol}}]/\text{rate } [\text{CPM}_{\text{total}}]\) at late times. The program next partitions
the radioactivity remaining in the monolayer between the two expected products according to the relationships

\[ [\text{CPM}_{\text{monolayer}}]_{\text{sol}} = R_2 \times [\text{CPM}_{\text{monolayer}}] \text{ and} \]

\[ [\text{CPM}_{\text{monolayer}}]_{\text{ins}} = (1-R_2) \times [\text{CPM}_{\text{monolayer}}]. \]

The total TCA-soluble and -insoluble radioactivity released by the monolayer were the reaction to proceed to completion, \([\text{CPM}_{\text{sol}}]_{\infty}\) and \([\text{CPM}_{\text{ins}}]_{\infty}\), should then be

\[ [\text{CPM}_{\text{sol}}]_{\infty} = [\text{CPM}_{\text{sol}}]_{\text{total released by}} + R_2 \times [\text{CPM}_{\text{monolayer}}] \]

last time point

\[ [\text{CPM}_{\text{ins}}]_{\infty} = [\text{CPM}_{\text{ins}}]_{\text{total released by}} + (1-R_2) \times [\text{CPM}_{\text{monolayer}}] \]

last time point

The fractions of TCA-soluble and TCA-insoluble remaining in the cells at each time point are then given by

\[ F_{\text{sol}} = \{[\text{CPM}_{\text{sol}}]_{\infty} - [\text{CPM}_{\text{sol}}]_t]/[\text{CPM}_{\text{sol}}]_{\infty} \text{ and} \]

\[ F_{\text{ins}} = \{[\text{CPM}_{\text{ins}}]_{\infty} - [\text{CPM}_{\text{ins}}]_t]/[\text{CPM}_{\text{ins}}]_{\infty} \]

The program next calculates the natural logs (ln) of \( F_{\text{sol}} \) and \( F_{\text{ins}} \). The program also calculates \( R_T \), the ratio of total TCA-soluble to total soluble and insoluble label released were the reaction to go to completion. \( R_T \) is given by

\[ R_T = [\text{TCAsol}]_{\infty}/\{[\text{TCAsol}]_{\infty} + [\text{TCAins}]_{\infty}\} \]

Lastly, a data file is created which stores the number of data points (N), \( R_2 \), \( R_T \), \( [\text{CPM}]_{\text{monolayer}}, \text{time}, [(\text{CPM})_{\text{sol}}]_t, [(\text{CPM})_{\text{ins}}]_t, \ln(F_{\text{sol}})_t \) and \( \ln(F_{\text{ins}})_t \).

2. Program 2--Enzfitter
This is a non-linear regression, data analysis program for IBM PC and compatible computers (Elsevier-Biosoft, Cambridge, UK). The best fit curve is calculated using the algorithm of Marquart (Bevington, 1969). The equations to be fitted to the data can be selected from a menu of available equations, and new equations can be added to the program using the integral equation editor. The operator can enable or disable an algorithm for eliminating "outliers" (data points which fall well outside the error range of the majority of the observations (Mostellei & Tukey, 1977; Duggilby, 1981). The operator can also choose from among different methods of data weighting, depending on how the error is distributed about the measured values. In order to help the operator select from among the weighting schemes available (simple weighting when the error is due to instrumental uncertainties and pipetting errors, proportional weighting, or statistical weighting), the program will also plot residual error versus the observed value ($\Delta y$ vs. $y$) and relative residual error versus the observed value ($\Delta y/y$ vs. $y$). It was observed that progress curves for the appearance of TCA/PTA-soluble and -insoluble radioactivity in the culture medium could be described as a sum of two exponential equations. Therefore, plots of $[(CPM)_{\infty} - (CPM)_t]$ vs. time were fitted to an equation for Double Exponential Decay selected from the equation menu. Furthermore, as the residual error, $\Delta y$, was found to be independent of the observed value, $y$, simple weighting was used in fitting the data to this equation (Leatherbarrow, 1987). The program then calculated the extent of each of the two phases of the decay curve, the corresponding first order rate constants, and their standard errors. The standard errors
were generally less than 10%, indicating "good" experimental data sets (Leatherbarrow, 1987).
CHAPTER III
RESULTS

PART I. LIPOSOME-MEDIATED MICROINJECTION OF PROTEINS INTO FIBROBLASTS

Characterization of Large Unilamellar Vesicles

The recovery of phospholipid in the liposome preparations was determined by measuring their phosphorus content as described in Methods. Table 1 lists Ag₃₀ and phosphorus (P) concentrations for three liposome preparations. With the exception of the first preparation, 1 ml of the final liposome suspension contained approximately 6 µmol P, which represented a recovery of approximately 60% of total lipid used for each of the preparations.

Samples were taken at the different stages in the preparation of the PS-LUV—the initial SUV, the Ca²⁺-cochleate intermediate, and the final LUV—and analyzed by electronmicroscopy (see Figures 1, 2, 3). Scales were made for the photographs, so that liposome sizes could be measured directly from the prints with a metric ruler.

SUV (Figure 1) were actually a heterogeneous collection of structures. The vesicles were often multilamellar and displayed a wide range of sizes, with the average diameter being about 65 nm. Very little could be seen in the electronmicrographs obtained from the Ca⁺⁺-cochleates (Figure 2). They appeared as irregular, lattice-like structures, possibly formed by the aggregation of smaller spherical components. Freeze-
Table 1. Phosphorus assay of liposomes.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$A_{830}$&lt;sup&gt;1&lt;/sup&gt;</th>
<th>[P]&lt;sup&gt;2&lt;/sup&gt; (mM)</th>
<th>µmol P recovered</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.199</td>
<td>1.00</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>0.263</td>
<td>6.40</td>
<td>6.4</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>0.237</td>
<td>6.10</td>
<td>6.1</td>
<td>61</td>
</tr>
</tbody>
</table>

<sup>1</sup>Average of 3 determinations.

<sup>2</sup>Determined from phosphorus standard curve obtained at the time of the analysis.
Figure 1. Electronmicrograph of PS-SUV, phosphotungstate negative staining, magnification 92,000 X. Ten µmol of bovine brain phosphatidylserine was sonicated for 30 minutes in buffered saline containing 1% phosphotungstate as described in MATERIALS AND METHODS. The vesicles show heterogeneity in size, shape and lamination. Average diameter was 65 nm.
Figure 2. Electron micrograph of "Ca++-chocleates" prepared by the precipitation of PS-SUV with Ca++, magnification 80,000 X. PS-SUV were precipitated by the addition of CaCl₂, final concentration 6.5mM, in the presence of 1% phosphotungstate. See MATERIALS AND METHODS for details. These "chocleates" appear as tightly aggregated complexes arranged in a lattice-like structure.
fracture electronmicroscopic studies (Staples et al., 1975) indicate that the calcium coehleste resembles a bilayer wound around a central axis to form a "jellyroll"-like structure with an average diameter of 500 nm. Following the addition of cholesterol, which falls within the space of two liposomes, the surface area of each particle was increased by 230 µl. Trapping efficiency for these preparations ranged from 0.36 to 2.77%.
fracture electronmicroscopic studies (Papahadjopoulos et al., 1975) showed that the calcium cochleate resembles a bilayer wound around a central axis to form a "jellyroll"-like structure. Such structures were not evident in any of our photographs.

LUV (Figure 3) had sizes ranging from 200-300 nm in diameter, with an average diameter of about 250 nm. Their appearance was somewhat translucent and spherical with electron opaque centers. The dark centers were assumed to be the entrapped phosphotungstate stain.

Molecular sieve chromatography was also used in this laboratory to determine LUV size (Thyagarajan, 1985). Figure 4 shows a calibration curve obtained following chromatography of monodispersed polystyrene latex beads of known diameter on a 1.5 x 50 cm column of Sephacryl S-1000. The arrows correspond to the elution volume of two liposome preparations. Liposome diameter was determined to be 0.26-0.28 µm, which falls within the range obtained by electron microscopy.

The Trapping Efficiency of Phosphatidylinerine LUV

[14C]-Labeled sucrose was used to determine the percent of aqueous space entrapment within LUV, and to estimate liposome size, while [14C]-labeled cholesterol was used as a marker for lipid incorporation into liposomes. Table 2 lists the results for 3 separate liposome samples prepared in the presence of 2 µCi [14C]-sucrose. Five µl of each preparation was removed for measuring radioactivity. The total volume of each sample was 830 µl. Trapping efficiency for these preparations ranged from 0.28-2.77%.
Figure 3. Electronmicrograph of PS-LUV, phosphotungstate negative staining, magnification 23,000 X. PS-LUV were prepared by adding EDTA, final concentration 7.9 mM, to a suspension of Ca^{++} -choleates in buffered saline containing 1% phosphotungstate. The vesicles are aggregate in small groups: Vesicle diameters ranged from 200-300 nm with an average of 250 nm.
Figure 4. Molecular sieve chromatography of LUV on Sephacryl S-1000. Open circle are the sieve coefficients of monodispersed polystyrene latex beads of known diameters eluted with 0.15 M NaCl, 0.02 M NaHCO₃, and 3 mM SDS, pH 8. Arrows correspond to the sieve coefficients of two preparations of LUV chromatographed in the absence of SDS.
Table 2. Percent incorporation of $^{14}$C-sucrose$^1$ into liposomes.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total activity recovered (cpm)</th>
<th>Aqueous space entrapment (%)</th>
<th>Total internal volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>122,840</td>
<td>2.77</td>
<td>23.0</td>
</tr>
<tr>
<td>2</td>
<td>16,770</td>
<td>0.38</td>
<td>3.15</td>
</tr>
<tr>
<td>3</td>
<td>12,450</td>
<td>0.28</td>
<td>2.32</td>
</tr>
</tbody>
</table>

$^1$The initial concentration [$^{14}$C]-sucrose used in each preparation of PS-LUV was 2µCi/ml.
The volume of the internal space per mole of liposome phospholipid is also presented.

Other LUV preparations were made in this laboratory to entrap \(^{14}\text{C}\)-sucrose, fluorescyl-dextran and \(^{125}\text{I}\)-phosphorylase b (Thyagarajan, 1985). LUV preparations typically entrapped 3-4% of the aqueous medium and had an internal volume ranging from 6.5-10 µl/µmol PS (Table 3). Assuming that LUV are spheres, and 1 µmol PS can form a bilayer with a surface area of 1.89 x 10\(^{17}\) nm\(^2\), (Levine & Wilkens, 1971) it is possible to calculate the average radius of the LUV from the entrapped volume as described in Materials and Methods according to the equation:

\[
D = \frac{6 \times (\mu l \text{ trapped}) \times 10^{18} \text{ nm}^3/\mu l}{(\mu mol \text{ PS}) \times 1.89 \times 10^{17} \text{ nm}^2/\mu mol}
\]

The internal volume can be determined from the entrapped radioactivity and the phosphorus content of the liposomes. The calculated diameters listed in Table 3 fall within the range obtained by electron microscopy.

**Incorporation of \(^{14}\text{C}\)-Cholesterol into Phosphatidylyserine LUV**

\(^{14}\text{C}\)-Cholesterol was incorporated into LUV more efficiently (33%) than \(^{14}\text{C}\)-sucrose (Table 4). This was expected since cholesterol would be incorporated directly in the PS bilayer of the liposomes. Typical recoveries of PS in LUV were 60 to 70%. The 33% incorporation of labeled cholesterol was thus half of expected. However, some spillage of the liposome preparation had occurred during the dialysis step, thereby decreasing the amount of \(^{14}\text{C}\) label recovered.
Table 3. Characterization of LUV from internal volume.

<table>
<thead>
<tr>
<th>Aqueous space marker</th>
<th>% Entrapment</th>
<th>Internal volume (µl/µmol PS)</th>
<th>Av. diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescyl-dextran</td>
<td>3.3</td>
<td>8.7</td>
<td>276</td>
</tr>
<tr>
<td>[(^{14})C] sucrose</td>
<td>2.9</td>
<td>7.4</td>
<td>234</td>
</tr>
<tr>
<td>[(^{125})I] phosphorylase b</td>
<td>3.1</td>
<td>8.5</td>
<td>270</td>
</tr>
</tbody>
</table>
Table 4. Percent incorporation of $^{14}$C-cholesterol into liposomes.

<table>
<thead>
<tr>
<th>$^{14}$C-cholesterol added (µCi)</th>
<th>Sample size (µl)</th>
<th>$^{14}$C activity (cpm)</th>
<th>Total activity</th>
<th>Entrapment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
<td>7100</td>
<td>534,000</td>
<td>32.8$^1$</td>
</tr>
</tbody>
</table>

$^1$Some loss of material occurred during recovery from the dialysis tubing.
Cultured fibroblasts (GM 3440) were grown as a confluent monolayer as described in Methods. Prior to the experiment, the cells were washed with PBS and then incubated for 2.5 hours at 37°C with 3.0 ml of PBS containing phosphatidylserine LUV (6 µmoles P) in which [14C]-sucrose had been entrapped. In order to make comparisons, control cells were incubated in the same way with 3.0 ml of PBS containing free sucrose (0.2 µCi). Both experimental and control cells were then washed 3 times with 5.0 ml PBS, the cells detached from the culture flask (Methods), and the cellular radioactivity measured. The results are shown in Table 5. It can be seen that fibroblasts incorporated 16 to 17.5 times more sucrose as the percent of the total label in the medium, when the sucrose was presented entrapped in liposomes than when it was presented in free form and internalized by pinocytosis.

The incorporation of [14C]-cholesterol into cells which had been exposed to liposomes containing labeled cholesterol was also examined. From Table 6, it can be seen that the percent incorporation of labeled liposome membrane markers into cells was very similar to the percent incorporation of the fluid phase marker [14C] sucrose. This supports the conclusion that the liposome-mediated delivery of sucrose to cells involves a physical contact between liposomes and the cells. Not unexpectedly, when cells were incubated with an insoluble dispersion of free cholesterol, a larger percentage of the cholesterol was delivered to cells than by liposomes alone (Table 6).
Table 5. Comparison of liposomes with pinocytosis in the delivery of $^{14}$C-sucrose to human fibroblasts.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Lipsome $^{14}$C-sucrose$^1$(cpm)</th>
<th>Free $^{14}$C-sucrose$^2$ activity (cpm)</th>
<th>Cellular radioactivity activity (cpm)</th>
<th>Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82,000</td>
<td>--</td>
<td>3984</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>440,000</td>
<td>1218</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>12,450</td>
<td>--</td>
<td>291</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>440,000</td>
<td>600</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^1$Total $^{14}$C-sucrose entrapped in PS-LUV. Approximately 6 moles phospholipid were added per culture dish.

$^2$Total $^{14}$C-sucrose added directly to the media.

Table 6. Percent incorporation of $^{14}$C-cholesterol into cells from liposomes.

<table>
<thead>
<tr>
<th>Liposome $^{14}$C-cholesterol (cpm)</th>
<th>Free $^{14}$C-cholesterol (cpm)</th>
<th>Cellular radioactivity (cpm)</th>
<th>Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>470,160$^1$</td>
<td>--</td>
<td>11,554</td>
<td>2.5</td>
</tr>
<tr>
<td>--</td>
<td>220,000$^2$</td>
<td>25,442</td>
<td>11.6</td>
</tr>
</tbody>
</table>

$^1$Total $^{14}$C-cholesterol associated with PS-LUV. Approximately 6 µmoles of phospholipid added.

$^2$Total cholesterol added as an insoluble dispersion.
Quantification of Phosphatidylserine LUV-Cell Fusion

The previous results suggest that the transfer of an aqueous space marker from PS liposomes to cells involves a direct interaction between them. However, the results do not prove that liposomes fuse with the plasma membrane of cells and inject their contents directly into the cytosol.

The pH-sensitive fluorescent probe fluorescein was utilized as a marker for liposome-cell fusion. This probe was theoretically convenient, since it could be quantified by fluorometry and visualized by fluorescence microscopy. Three fluorescein-labeled compounds were used: fluorescein-dextran (FD), 5(6)-carboxyfluorescein [5(6)-CF], and fluorescein-labeled bovine serum albumin (FITC-BSA).

The fluorescence of 5(6)-CF is quenched at high concentrations of this fluorophor; dilution causes large increases in the fluorescence of 5(6)-CF. This is illustrated in Part II. Table 7, where phosphatidylcholine (PC)-SUV prepared by sonicating PC in the presence of 0.388 M 5(6)-CF exhibited little fluorescence above the buffer blank. However, when 0.1 ml 10% Triton X-100 was added to the cuvette to disrupt the liposomes, fluorescence increased more than 20-fold. Consequently, if one was to observe a large increase in cellular fluorescence on incubating PS-LUV containing 5(6)-CF with fibroblasts, this could be evidence for liposome-mediated injection of 5(6)-CF into the cytosol with concommitant dilution of the fluorophore. Unfortunately, as shown in Part I. of Table 7, the preparation of PS-LUV had a much higher initial fluorescence (10 compared with 1), while the addition of Triton X-100 caused only a 2-
Table 7. Fluorescence of liposomes containing 5(6)-carboxy fluorescein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-LUV</td>
<td></td>
</tr>
<tr>
<td>1. Tris/NaCl buffer, pH 7.4^2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>2. Buffer + PS-LUV^3</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>3. Buffer + PS-LUV + Triton X-100^4</td>
<td>22.6 ± .5</td>
</tr>
<tr>
<td>PC-SUV</td>
<td></td>
</tr>
<tr>
<td>1. Tris/NaCl buffer, pH 7.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2. Buffer + PC-SUV</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>3. Buffer + PC-SUV + Triton X-100^4</td>
<td>21.7 ± 0.2</td>
</tr>
</tbody>
</table>

1 Each value represents the average of 3 different samples.

2 The buffer contained 100 mM NaCl, 20 mM Tris, 2 mM His, pH 7.4.

3 10 µl of a preparation of PS-LUV (6 µmol/ml, assuming a 60% recovery of PS) was added.

4 The final concentration of Triton X-100 was 0.33%.
fold increase in fluorescence. It was also observed that on overnight storage, 5(6)-CF leached into the buffer from the liposomes. Therefore, it was postulated that the PS-LUV were permeable to 5(6)-CF, and that 5(6)-CF was not efficiently incorporated into PS-LUV.

Further experimentation proved that the fibroblasts were also permeable to 5(6)-CF. This was determined in the following way. Cells are freely permeable to the non-fluorescent derivative 5(6)-CF diacetate. On incubation with 5(6)-CF diacetate, the cells became fluorescent as a consequence of its hydrolysis to 5(6)-CF by nonspecific cytoplasmic esterases. When fluorescent cells were suspended in buffers of varying pH, it was found that the fluorescence of such suspensions was due to 2 components: extracellular 5(6)-CF which had leaked from the cells and intracellular 5(6)-CF. The two could be independently quantified by measuring the fluorescence of cell suspensions before and after the cells were pelleted by centrifugation. The results are shown in Figure 5. From the squares in Figure 5, it can be seen that the cellular fluorescence was largely independent of the external pH, indicating that the cells were either impermeable to protons or else were able to maintain their cytosolic pH over a range of external pH's. In contrast, the fluorescence associated with the extracellular medium was markedly pH dependent (circles). From the point at which the 2 curves crossed, it was possible to estimate that the intracellular pH was about 6.6 and that it was largely unaffected by the external pH between 5.5 and 7.5.

Cell permeability to 5(6)-CF was also seen microscopically. Cells incubated with
Figure 5. Effect of pH on the fluorescence of intra- and extracellular 5(6)-carboxyfluorescein [5(6)-CF]. Fluorescence was observed at 519 nm following excitation at 495 and 450 nm. Circles, extracellular 5(6)-CF fluorescence; squares, intracellular 5(6)-CF fluorescence. See MATERIALS AND METHODS for details.
5(6)-CF (free in solution) and 5(6)-CF-containing liposomes were more fluorescent than control cells. However, since the liposomes were found to leak 5(6)-CF and cells were permeable to free 5(6)-CF, it was not certain whether cellular fluorescence was due to liposome-cell fusion or from free 5(6)-CF which diffused into the cells.

Fluorescein isothiocyanate-dextran (FD) was used as a non-permeant, pH-sensitive fluorescent probe to quantify liposome-cell fusion. The ratio of FD fluorescence at two different excitation wavelengths (F495/F450) is observed to change with pH (Okuma & Poole, 1978). The results of fluorescence measurements at different pH's are given in Table 8 and the resulting standard curve in Figure 6. The curve is sigmoid with the lowest absorbance ratios at pH 4.0 and the highest at pH 8.5. Results of replicate measurements between pH 5.5 and 8.0 are also presented in Figure 7 (Thyagarajan, 1985) at somewhat different instrument settings.

In order to determine whether PS-LUV are permeable to protons, liposomes containing entrapped FD were suspended in buffers of varying pH and their fluorescence measured at the two different excitation wavelengths as described above. The ratio F495/F450 as a function of pH is presented in Table 9. From Table 9, it is evident that the buffer alone and a suspension of empty liposomes had almost no fluorescence at the wavelengths tested. Thus, almost all the fluorescence seen in FD-containing liposomes is due to entrapped FD. The results also show that, PS-LUV were permeable to protons, since the fluorescence ratio of the PS-LUV-entrapped FD was extremely sensitive to the pH of the external buffer. Thus, the fluorescence ratios obtained at external buffer pH's
Table 8. pH vs fluorescence standard curve for fluoresceyl dextran.

<table>
<thead>
<tr>
<th>pH</th>
<th>F$_{450}^1$</th>
<th>F$_{495}^2$</th>
<th>F$<em>{495}/F</em>{450}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>3.2</td>
<td>1.6</td>
<td>0.50</td>
</tr>
<tr>
<td>4.5</td>
<td>6.3</td>
<td>4.4</td>
<td>0.70</td>
</tr>
<tr>
<td>4.75</td>
<td>6.7</td>
<td>5.3</td>
<td>0.79</td>
</tr>
<tr>
<td>5.0</td>
<td>7.3</td>
<td>7.5</td>
<td>1.03</td>
</tr>
<tr>
<td>5.5</td>
<td>8.8</td>
<td>16.4</td>
<td>1.86</td>
</tr>
<tr>
<td>6.0</td>
<td>11.6</td>
<td>32.5</td>
<td>2.80</td>
</tr>
<tr>
<td>6.3</td>
<td>15.2</td>
<td>57.7</td>
<td>3.80</td>
</tr>
<tr>
<td>6.5</td>
<td>15.2</td>
<td>53.6</td>
<td>3.53</td>
</tr>
<tr>
<td>6.8</td>
<td>17.2</td>
<td>65.1</td>
<td>3.78</td>
</tr>
<tr>
<td>7.0</td>
<td>17.8</td>
<td>71.5</td>
<td>4.02</td>
</tr>
<tr>
<td>7.4</td>
<td>18.6</td>
<td>80.6</td>
<td>4.33</td>
</tr>
<tr>
<td>8.0</td>
<td>18.8</td>
<td>82.1</td>
<td>4.37</td>
</tr>
<tr>
<td>8.5</td>
<td>19.8</td>
<td>90.1</td>
<td>4.55</td>
</tr>
</tbody>
</table>

$^1$Fluorescence at 520 nm when excited at 450 nm.

$^2$Fluorescence at 520 nm when excited at 495 nm.
Figure 6. Effect of pH on the fluorescence of fluorescyle dextran (FD) (standard curve). Ex wavelengths = 495 and 450 nm; Em wavelength = 519 nm. Fluorescyle dextran (300 µg/ml) was added to various buffers at the indicated pH values. Fluorescence was then measured at excitation wavelengths of 495 and 450 nm, and the emission wavelength of 519 nm.
Figure 7. Effect of pH on the fluorescence of fluoresceyl dextran (FD). The excitation wavelengths were 495 nm and 450 nm, and the emission wavelength was 519 nm. Open circles, free FD; closed circles, FD trapped in PS-LUV at pH 7.4 and transferred to buffers at the indicated pH's.
Table 9. pH dependency of the fluorescence of Fluoresceyl dextran entrapped in PS-LUV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>External pH</th>
<th>F\textsubscript{450}\textsuperscript{1}</th>
<th>F\textsubscript{495}\textsuperscript{2}</th>
<th>Ratio (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>4.75</td>
<td>1.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>4.75</td>
<td>1.4</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Liposomes c/FD</td>
<td>4.75</td>
<td>4.3</td>
<td>3.3</td>
<td>0.72</td>
</tr>
<tr>
<td>Buffer</td>
<td>6.8</td>
<td>1.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>6.8</td>
<td>1.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Liposomes c/FD</td>
<td>6.8</td>
<td>8.0</td>
<td>30.5</td>
<td>4.33</td>
</tr>
<tr>
<td>Buffer</td>
<td>8.5</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>8.5</td>
<td>1.3</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Liposomes c/FD</td>
<td>8.5</td>
<td>8.6</td>
<td>34.0</td>
<td>4.47</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Fluorescence at 520 nm when excited at 450 nm.
\textsuperscript{2} Fluorescence at 520 nm when excited at 495 nm.
of 4.75 and 8.5 are similar to values with free FD alone (compare Tables 8 and 9). At pH 6.8, the absorbance ratio indicated an internal pH of 7.4 (that of the entrapment buffer). Figure 7 shows fluorescence titration curve for PS-LUV-entrapped FD. It is evident that the PS-LUV were leaky to protons, such that a complete equilibration between the internal and external pH was obtained (Thyagarajan, 1985).

As shown above, FD-containing PS-LUV were permeable to protons, whereas the pH of the cell cytosol was relatively independent of the pH of the medium. These observations were used to develop a method to distinguish between the passive adsorption of PS-LUV to the plasma membrane and membrane-vesicle fusion. The fluorescence of FD in plasma membrane-bound PS vesicles should show a similar dependency on external pH as does FD in PS-LUV. In contrast, fluorescence of FD injected into the cytosol following membrane-vesicle fusion should be independent of the pH of the medium as was seen for 5(6)-CF. It should, therefore, be possible to distinguish between liposome binding to the membrane and vesicle-membrane fusion. Figure 8 shows theoretical curves calculated for various degrees of fusion of bound vesicles with the cell membrane according to the equation:

$$(FR)_{calc} = f \times (FR)_{pH \, 6.6} + (1-f) \times (FR)_{pH \, of \, medium}$$

In this equation, FR is the ratio of fluorescence at the excitation wavelengths of 495 and 450, and f is the fraction of membrane-bound vesicles which fuse, injecting the fluorophor into the cytosol. $(FR)_{pH \, 6.6}$ is the fluorescence ratio at pH 6.6, the apparent pH of the cytosol as determined from the intersection of the curves in Figure 5.
Figure 8.  Theoretical effect of vesicle-membrane fusion on the pH dependency of cell-associated FD. Curve 1, calculated for no fusion of membrane-bound LUV; curve 2, calculated for 25% fusion; curve 3, 50% fusion; curve 4, 75% fusion; curve 5, 100% fusion. See text for the details of these calculations.
Figure 9 shows the experimental data obtained with FD-containing PS-LUV. The open circles represent the experimental values of FR, the dashed line was calculated assuming 25% fusion of membrane-bound vesicles, and the solid line was calculated for 0% fusion. The results indicate that much less than 25% and probably closer to 0% of the cell associated vesicles have actually fused with the plasma membrane (Thyagarajan, 1985).

Figures 10 and 11 show the appearance of fluorescence intracellularly after endocytosis of FD dissolved in the media. Figure 10 was photographed with phase-contrast lighting, while 11 was photographed with U.V. light to show the fluorescence. Although not apparent from the black and white photograph, there was a marked contrast between cellular autofluorescence (gold) and FD fluorescence (green). The fluorescein fluorescence appeared to be confined within discrete vesicle-like structures in the cytosol, presumably lysosomes, giving the cell a stippled appearance. These results show that intracellular FD could have been visualized by PS-LUV.

Preparation of Liposomes Containing Lysolecithin and Stearylamine

The failure of PS-LUV to fuse with cells as judged by the fluorescence assay prompted the examination of other liposome preparations as potential vehicles for the microinjection of proteins into cultured cells. The rationale for incorporating lysolecithin and stearylamine into liposomes is as follows. Lysolecithin is reported to destabilize a lipid bilayer and to promote liposome membrane fusion (Martin & MacDonald, 1976a; 1976b). Stearylamine is a positively-charged detergent which intercalates into the
Figure 9. Interaction between human fibroblasts and fluorescein dextran-containing PS-LUV. Circles are the observed fluorescence ratios (excitation at 495 nm and 450 nm emission at 519 nm) at the given pH. Solid line, calculated for 0% fusion of membrane-bound LUV; dashed line, calculated for 25% fusion.
Figure 10. Uptake of free fluoresceyl dextran (FD) by cultured fibroblasts. Fibroblasts were grown in medium containing fluoresceyl dextran (1 mg/ml), fixed on glass coverslips; photographed with phase-contrast lighting (Tri-X film).
Figure 11. Fibroblasts grown in medium containing fluoresceyl dextran (FD), fixed on glass coverslips; photographed with U.V. light (Tri-X film). See Figure 10 for further details.
membrane of liposomes, causing them to acquire a positive charge. As the plasma membrane of cultured cells is negatively charged, stearylamine-containing liposomes should have a good affinity and specificity for these lipids. As described in the Methods section, fluorescence microscopy was used to observe a FITC-BSA mixture.
membrane of liposomes, causing them to acquire a positive charge. As the plasma membrane of cultured cells is negatively charged, stearylamine-containing liposomes should have a high affinity for the cell surface.

Small unilamellar vesicles (SUV) were prepared from PC, lysolecithin and stearylamine. FITC-bovine serum albumin (BSA) was used as the fluorescent probe for these liposomes. The pH dependency of FITC-BSA is given in Table 10. From the fluorescence ratios, it is apparent that FITC-BSA is not useful as a pH-sensitive probe, although it could be used as a fluorescent marker for fusion. Percent entrapment of FITC-BSA was determined by fluorescence and was found to be 1.03%.

The cells were incubated with the liposomes containing FITC-BSA as described in the Methods section. After the cells were fixed, they were examined microscopically. None of the cells showed any fluorescence other than the gold-colored autofluorescence characteristic of the cell line. It was, therefore, concluded that PC/Lyso-PC-stearylamine-containing liposomes did not fuse with cells.
Table 10. Effect of pH on the fluorescence of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA).

<table>
<thead>
<tr>
<th>pH</th>
<th>F_{450}^1</th>
<th>F_{492}^2</th>
<th>F_{492}/F_{450}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>2.8</td>
<td>17.8</td>
<td>6.36</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>25.5</td>
<td>6.38</td>
</tr>
<tr>
<td>6.2</td>
<td>4.0</td>
<td>26.6</td>
<td>6.65</td>
</tr>
<tr>
<td>6.4</td>
<td>4.2</td>
<td>28.0</td>
<td>6.67</td>
</tr>
<tr>
<td>6.8</td>
<td>4.8</td>
<td>31.0</td>
<td>6.46</td>
</tr>
<tr>
<td>7.0</td>
<td>4.4</td>
<td>31.2</td>
<td>7.09</td>
</tr>
<tr>
<td>7.2</td>
<td>3.9</td>
<td>25.8</td>
<td>6.62</td>
</tr>
<tr>
<td>7.4</td>
<td>5.2</td>
<td>35.2</td>
<td>6.77</td>
</tr>
<tr>
<td>8.3</td>
<td>5.8</td>
<td>36.8</td>
<td>6.34</td>
</tr>
</tbody>
</table>

1Fluorescence at 520 nm when excited at 450 nm.
2Fluorescence at 520 nm when excited at 492 nm.
Models for the Release of Radiolabeled Products from Metabolically-Labeled Fibroblasts

Cultured human fibroblasts, labeled in the presence of $[^3H-4,5]\text{L-Leucine}$ for 48 hours, were be observed to release both TCA/PTA-soluble and TCA/PTA-insoluble radioactivity into the culture medium. The TCA/PTA-insoluble radioactivity represented about 30% of the total labeled products released in serum- and amino acid-supplemented medium (Table 11.). Figure 12 reveals that semilog plots of the appearance of both products with time were biphasic. The rapid phase generally represented less than 15% of the initial cellular radioactivity when rate constants and extents of reaction were determined by a non-linear least squares analysis of the data. These rate constants, ± standard deviation (N = 3), were found to be $1.20 \pm 0.37 \times 10^{-3} \text{ min}^{-1}$ ($t_{1/2} = 9.6 \text{ h}$) and $6.85 \pm 1.59 \times 10^{-3} \text{ min}^{-1}$ ($t_{1/2} = 1.7 \text{ h}$) for the rapid phase of release of TCA/PTA-soluble label and TCA/PTA-insoluble label, respectively. The non-identity of these rate constants indicates that the substrate pools which contributed to each of these products were not identical (Buktenica et al., 1987; Buktenica & Frankfater, 1987). The half-life for the rapid phase of release of TCA/PTA-soluble products fell within the range of values previously reported for the degradation of a small fraction of cellular proteins which are observed to turn over rapidly (Poole & Wibo, 1973). It has been suggested that the degradation of such proteins does not involve the participation of lysosomes (Seglen et al., 1979). This material was designated Pool I.
Table 11. Rate constants and relative pool sizes for cellular protein turnover in the presence of 13% fetal bovine serum.

Part A. Fast phase.

<table>
<thead>
<tr>
<th>Addition</th>
<th>$(k_1)_{sol}$ $10^3 \text{ min}^{-1}$</th>
<th>$(k_1)_{ins}$ $10^3 \text{ min}^{-1}$</th>
<th>$P_1/P_0$</th>
<th>$P_2/P_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM Leu (control 1)</td>
<td>$0.88 \pm 0.23$</td>
<td>$4.99 \pm 0.70$</td>
<td>0.076</td>
<td>0.044</td>
</tr>
<tr>
<td>0 mM Leu</td>
<td>$1.60 \pm 0.50$</td>
<td>$8.29 \pm 1.58$</td>
<td>0.046</td>
<td>0.054</td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>$1.12 \pm 0.36$</td>
<td>$7.28 \pm 1.50$</td>
<td>0.064</td>
<td>0.047</td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>$2.78 \pm 0.35$</td>
<td>$7.81 \pm 2.02$</td>
<td>0.036</td>
<td>0.050</td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>$2.46 \pm 0.09$</td>
<td>$3.08 \pm 0.36$</td>
<td>0.072</td>
<td>0.068</td>
</tr>
<tr>
<td>10 mM NH$_4$Cl</td>
<td>$0.77 \pm 0.17$</td>
<td>$4.21 \pm 0.33$</td>
<td>0.073</td>
<td>0.071</td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>$1.06 \pm 0.15$</td>
<td>$2.95 \pm 0.46$</td>
<td>0.061</td>
<td>0.058</td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>$1.71 \pm 0.30$</td>
<td>$4.74 \pm 0.48$</td>
<td>0.039</td>
<td>0.059</td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>$1.81 \pm 0.38$</td>
<td>$5.27 \pm 0.85$</td>
<td>0.041</td>
<td>0.056</td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>$1.74 \pm 0.36$</td>
<td>$5.79 \pm 0.76$</td>
<td>0.050</td>
<td>0.083</td>
</tr>
<tr>
<td>2 mM Leu (control 2)</td>
<td>$0.63 \pm 0.16$</td>
<td>$4.47 \pm 0.78$</td>
<td>0.165</td>
<td>0.041</td>
</tr>
<tr>
<td>6.67 µg/ml Cytochalasin B</td>
<td>$0.56 \pm 0.05$</td>
<td>$6.73 \pm 1.80$</td>
<td>0.156</td>
<td>0.028</td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>$2.17 \pm 0.05$</td>
<td>$1.43 \pm 0.34$</td>
<td>0.044</td>
<td>0.037</td>
</tr>
<tr>
<td>26°C C</td>
<td>$0.58 \pm 0.24$</td>
<td>$7.30 \pm 0.49$</td>
<td>0.096</td>
<td>0.077</td>
</tr>
</tbody>
</table>
Table 11. Rate constants and relative pool sizes for cellular protein turnover in the presence of 13% fetal bovine serum.

Part B. Slow phase.

<table>
<thead>
<tr>
<th>Addition</th>
<th>( (k_2)_\text{sol} ) (10^3 \text{min}^{-1} )</th>
<th>( (k_2)_\text{ins} ) (10^3 \text{min}^{-1} )</th>
<th>( P_3/P_0 )</th>
<th>( (P_3)_\text{sol} )</th>
<th>( (P_3)_\text{total} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM Leu (control 1)</td>
<td>7.87±0.67</td>
<td>8.49±0.38</td>
<td>0.88</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>0 mM Leu</td>
<td>8.57±0.43</td>
<td>10.01±0.50</td>
<td>0.90</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>8.27±0.66</td>
<td>10.16±0.57</td>
<td>0.89</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>5.99±0.23</td>
<td>4.29±0.34</td>
<td>0.91</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>4.96±0.19</td>
<td>4.38±0.55</td>
<td>0.86</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>5.58±0.64</td>
<td>6.26±0.23</td>
<td>0.86</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>6.04±0.46</td>
<td>6.91±0.32</td>
<td>0.88</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>9.37±0.19</td>
<td>10.00±0.39</td>
<td>0.90</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>8.74±0.37</td>
<td>10.26±0.55</td>
<td>0.90</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>10.25±0.31</td>
<td>12.08±0.69</td>
<td>0.87</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>2 mM Leu (control 2)</td>
<td>8.71±1.63</td>
<td>14.13±0.53</td>
<td>0.79</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>6.67 µg/ml Cytochalasin B</td>
<td>9.07±0.53</td>
<td>14.65±0.42</td>
<td>0.82</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>13.58±0.66</td>
<td>12.03±0.41</td>
<td>0.92</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>26°C</td>
<td>5.32±0.21</td>
<td>5.32±0.57</td>
<td>0.83</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>
Figure 12. Release of Protein Products from Human Fibroblasts Cultured in Serum and Leucine Supplemented Medium. Near confluent monolayers of GM3440 human fibroblasts were preincubated for 48 hrs with [3H-4,5]-L-leucine. After washing the monolayer the cells were incubated in medium supplemented with 13% fetal calf serum and 2 mM unlabeled L-leucine. At intervals the amount of TCA/PTA-soluble and -insoluble radioactivity released into the medium was measured (see METHODS). The quantity (1-F) is the fraction of TCA/PTA-soluble (closed circles) and -insoluble (open circles) radioactivity remaining in the monolayer at each time point. The data was analyzed by nonlinear least squares regression analysis using the program Enzfitter. The lines are the calculated best fit using the parameters in Table 11. (k1)_{sol} = 0.88±0.23 \times 10^{-3} \text{ min}^{-1}, (k1)_{ins} = 4.99±0.70 \times 10^{-3} \text{ min}^{-1}, (k2)_{sol} = 7.87±0.67 \times 10^{-5} \text{ min}^{-1}, (k2)_{ins} = 8.49±0.38 \times 10^{-5} \text{ min}^{-1}. Each data point is the average of three determinations.
The half-life for the rapid appearance of TCA/PTA-insoluble radioactivity is similar to the transit times reported for some proteins from the endoplasmic reticulum through the Golgi apparatus to the cell surface (Dong et al., 1989; Mainferme et al., 1985). Consequently, the rapid phase of appearance of TCA/PTA-insoluble radioactivity could represent the secretion of proteins synthesized during the period immediately preceding the chase. This material was designated as Pool 2.

In some experiments, the composition of the culture medium used in labeling the cells differed from that of the subsequent chase medium. As the response of the cells to such changes are not expected to be instantaneous, any associated lag phase would be superimposed on the initial rapid phases of release of TCA/PTA-soluble and -insoluble products, complicating their interpretation.

The slow phase of products released corresponded to greater than 85% of the initial cellular radioactivity and was characterized by apparently identical rate constants, \(8.24 \pm 0.35 \times 10^{-5} \text{ min}^{-1}\) and \(9.55 \pm 0.92 \times 10^{-5} \text{ min}^{-1}\) (\(t_{1/2} \approx 5\) and 5.8 days) for TCA/PTA-soluble and TCA/PTA-insoluble products. These fell within the range of values previously reported for the turnover of the majority of cellular proteins in human tissues (Bradley, 1977). Identical rate constants for the two products imply that both were derived from the same pool of cellular substrate (Buktenica et al., 1987; Buktenica & Frankfater, 1987), and/or that the processes which contributed to the appearance of both products shared a common rate limiting step.

Any explanation of the slow phases of release of TCA/PTA-soluble and -insoluble
products must account for their near identical rate constants. Models which assume that tritiated leucine arising from the degradation of cellular proteins is reutilized in the synthesis of secreted proteins can be eliminated as explanations of the near identity of the rate constants for the slow release of both products. Thus, when the chase medium contained increasing concentrations of unlabeled leucine---0 mM, 2 mM, and 10 mM—in order to dilute the intracellular amino acid pool of free tritiated leucine, no significant reduction was observed in the amount of TCA/PTA-insoluble label released during the slow phase \([(P_3)_\text{sol}/(P_3)_\text{total} \text{ is unchanged in Table 11; Figures 13, 14}].\] In addition, 10 µM and 50 µM cycloheximide, concentrations which inhibited protein synthesis 34% and greater than 90% (Table 12), failed to inhibit or abolish the slow phase of release of the TCA/PTA-insoluble label \([(P_3)_\text{sol}/(P_3)_\text{total} \text{ is not increased in Table 11}].\] However, consistent with prior observations of inhibition of protein degradation by cycloheximide (Goldberg & St. John, 1976), both concentrations of cycloheximide reduced \(k_{\text{obs}}\) for the slow phases of release of TCA/PTA-soluble and TCA/PTA-insoluble label by about 40% (Table 11).

Three models can be proposed to explain identical rate constants for the release of TCA/PTA-soluble and -insoluble radioactivity from labeled fibroblasts. The derivations of equations which permit calculation of rate constants and pool sizes for all 3 models appear in complete form in Appendix 1. Only brief summaries of each model and the final rate equations will be discussed in this section.

Model 1 is depicted in Figure 15. It posits that the slow phase of loss of label
Figure 13. Release of products vs. time for cells incubated in serum-supplemented medium containing 0 added Leu. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. (k₁)_{sol} = 1.60±0.50 \times 10^{-3} \text{ min}^{-1}, (k₁)_{ins} = 8.29±1.58 \times 10^{-3} \text{ min}^{-1}, (k₂)_{sol} = 8.57 \times 10^{-5} \text{ min}^{-1}, and (k₂)_{ins} = 10.01±0.50 \times 10^{-5} \text{ min}^{-1}. See Figure 12 for further details.
Figure 14. Release of products vs. time for cells incubated in serum-supplemented medium containing 10 mM added Leu. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. $(k_1)_{sol} = 1.12 \pm 0.36 \times 10^{-3} \text{min}^{-1}$, $(k_1)_{ins} = 7.28 \pm 1.50 \times 10^{-3} \text{min}^{-1}$, $(k_2)_{sol} = 8.27 \pm 0.66 \times 10^{-5} \text{min}^{-1}$, $(k_2)_{ins} = 10.16 \pm 0.57 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for further details.
Table 12. Incorporation of $^3$H-Leu into fibroblasts in the presence of cycloheximide.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Medium (cpm)</th>
<th>Cell (cpm)</th>
<th>Total (cpm)</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1</td>
<td>854,112</td>
<td>220,504</td>
<td>1,074,616</td>
<td>20.5</td>
</tr>
<tr>
<td>10 µM cycloheximide</td>
<td>908,368</td>
<td>145,833</td>
<td>1,054,201</td>
<td>13.8</td>
</tr>
<tr>
<td>Control-2</td>
<td>604,155</td>
<td>85,309</td>
<td>725,464</td>
<td>11.76</td>
</tr>
<tr>
<td>50 µM cycloheximide</td>
<td>697,548</td>
<td>8,014</td>
<td>705,562</td>
<td>1.14</td>
</tr>
</tbody>
</table>

1Each value represents the average of 3.

2Each value represents the average of 2, rather than 3.
Figure 15. Illustration of Model 1 for the release of label from fibroblasts. $P_1$ represents a protein pool destined for rapid turnover (rate constant = $k_D$). $P_2$ represents newly synthesized proteins destined for secretion (rate constant = $k_S$). $P_3$ represents a group of proteins which may either "leak" from the cell (rate constant = $k_L$) or are transferred to lysosomes (rate constant = $k_T$) where they are degraded (rate constant = $k_D'$).
from the cell is due to a third pool of cellular proteins, pool P3, which can either "leak" from the cell (TCA-insoluble) or be transferred to the lysosomes. Within the lysosome (pool P3'), degradation to TCA/PTA-soluble products ensues. The rate constants are explained as follows:

1. \( k_D \) = degradation rate constant for the unstable proteins in P1.
2. \( k_S \) = exocytosis (secretion) rate constant for proteins in P2.
3. \( k_T \) = rate constant for the transfer of proteins in P3 from the cytosol to the lysosomes.
4. \( k_D' \) = rate constant for the degradation of proteins (P3) within the lysosomes.
5. \( k_L \) = rate constant for leakage of proteins (P3) from the cells.

\( k_D \) and \( k_S \) are "fast-phase" rate constants, while \( k_T \), \( k_L \), and \( k_D' \) are rate constants relevant to the "slow phase".

The integrated rate equations for TCA-soluble and TCA-insoluble products, assuming a steady-state between P3 and P3', are as follows:

\[
[(TCA)_{\text{sol}}]_t = (P_1)_0[1 - e^{-kDt}] + \frac{k_T(P_3)_0}{k_L + k_T} [1 - e^{-(k_L + k_T)t}]
\]

\[
[(TCA)_{\text{ins}}]_t = (P_2)_0[1 - e^{-kSt}] + \frac{k_L(P_3)_0}{k_L + k_T} [1 - e^{-(k_L + k_T)t}]
\]

Note that identical rate constants are predicted for the slow phase of release of TCA-soluble and TCA-insoluble products, \( k_{\text{obs}} \) = \( k_L \) and \( k_T \). Fractional pool sizes are calculated from the extents of each phase and a knowledge of the total amount of protein.
which was labeled with tritiated leucine during the preincubation period. Thus

\[ P_0 = [(TCA)_{sol}] + [(TCA)_{ins}] \]

\((P_1)_0\) and \((P_2)_0\) are determined experimentally by fitting the above equations to the experimental data for TCA/PTA-soluble and -insoluble radioactivity \((P_3)_0\) in turn is given by the sum

\[ (P_3)_0 = \frac{k_T(P_3)_0}{k_T + k_L} + \frac{k_L(P_3)_0}{k_T + k_L} \]

Pool sizes are also collected in Table 11.

Since \(k_{obs} = k_L + k_T\), and

\[ k_T \quad \frac{(P_3)_{sol}}{k_T + k_L} = \frac{(P_3)_{total}}{k_T + k_L} \]

\(k_T\) and \(k_L\) can be calculated. It is important to note that under steady state conditions, the rate constant for degradation within the lysosome, \(k_D\), cannot be determined.

In Model 2 (Figure 16), cellular leakage is thought to be insignificant; \((TCA)_{ins}\) radioactivity arises solely from exocytosis from vacuolar compartments in steps \(k_S\) and \(k_R\). \(P_1\) and \(P_2\) are unchanged from Model 1. The proteins in \(P_3\) are transferred to the lysosomes \((P_3')\), where they may be degraded \((k_D')\) or regurgitated \((k_R)\) (Buktenica et al., 1987).

Assuming a steady-state between \(P_3\) and \(P_3'\), the integrated rate equations for Model 2 are as follows:
Figure 16. Illustration of Model 2 for the release of label from fibroblasts. $P_1$, $P_2$, $k_D$, and $k_S$ are explained in Figure 15. Proteins in $P_3$ are transferred ($k_T$) to the lysosomes ($P_3'$), where they are either degraded ($k_D'$) or exocytosed ($k_R$).
\[
[(TCA)_{\text{sol}}]_t = (P_1)_0 \left[1 - e^{-k_D t}\right] + \frac{k_D'(P_3)_0}{k_D' + k_R} \left[1 - e^{-k_T t}\right]
\]

\[
[(TCA)_{\text{ins}}]_t = (P_2)_0 \left[1 - e^{-k_S t}\right] + \frac{k_R(P_3)_0}{k_D' + k_R} \left[1 - e^{-k_R t}\right]
\]

\[
(P_1)_0, (P_2)_0, \text{ and } (P_3)_0 \text{ are determined as described in Model 1.}
\]

\[
\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}} = \frac{k_D'}{k_D' + k_R}
\]

Now, however, \[
\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}} = \frac{k_D'}{k_D' + k_R}
\]

It is important to note that the absolute values of the intrinsic degradation and exocytosis rate constants, \(k_D'\) and \(k_R\) respectively, cannot be determined, since \(k_{\text{obs}}\) for the slow phase is equal to \(k_T\) rather than \((k_D' + k_R)\).

According to Model 3 (Figure 17), there is no significant protein degradation in the lysosomes. Instead, protein degradation occurs wholly within the cytosol. Furthermore, there are two cytosolic degradative systems, such that one protein pool \((P_1)\) is degraded rapidly, while the other \((P_3)\) is degraded slowly. The differences in degradation rates for these two populations may depend on the presence or absence of signals which are recognized by cytosolic degradative systems to designate proteins for rapid destruction. The slow phase of release of TCA/PTA-insoluble radioactivity is due to leakage.

The integrated rate equations for Model 3 are as follows:
Figure 17. Illustration of Model 3 for the release of label from fibroblasts. $P_1$, $P_2$, $k_D$, and $k_S$ are explained in Figure 15. Proteins in $P_3$ may be either degraded by a non-lysosomal degradative system ($k_D'$) or "leak" from the cell ($k_L$).
\[
[(TCA)_{\text{sol}}]_t = (P_1)_0 \frac{k_D'(P_3)_0}{k_D' + k_L} [1 - e^{-(k_D' + k_L)t}] + \frac{1}{k_L} \frac{(P_3)_0}{(TCA)_{\text{insl}}} [1 - e^{-(k_D' + k_L)t}]
\]

The procedures described for Model 1 may be used to determine \((P_1)_0\), \((P_2)_0\), and \((P_3)_0\). As \(k_{\text{obs}} = k_D' + k_L\), and

\[
\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}} = \frac{k_D'}{k_D' + k_L},
\]

\(k_D'\) and \(k_L\) can now be calculated. The rate equations for Model 3 are similar to Model 1, except that the intrinsic rate constant calculated for the appearance of \((TCA)_{\text{sol}}\) radioactivity in the slow phase is \(k_D'\), rather than \(k_T\). Moreover, \(k_D'\) in Model 3 would depend on the activities of cytosolic degradation systems and would be expected to be insensitive to inhibition by inhibitors of lysosomal proteinases. In addition, if peptide bond cleavage steps are not rate determining for cytosolic protein degradative systems, then \(k_D'\) may also be insensitive to other protease inhibitors.

These derivations yield an interesting result which is not usually stressed in protein turnover studies. Peptide bond cleavage steps are not rate determining in steady state models of lysosomal proteolysis (Models 1 and 2), and consequently \(k_D'\) cannot be determined from measurements of the slow phase of release of TCA/PITA-soluble products. What can be measured is \(k_T\), the rate constant for the transfer of proteins from other compartments into the lysosome (Models 1 and 2), and \(k_L\), the rate constant for leakage of proteins from the cell (Model 1). The rate constants \(k_D'\) and \(k_L\) can only be
determined as a ratio, and then only in the case where the source of TCA/PTA-insoluble radioactivity is regurgitation of lysosomal contents (Model 2). Although $k_D'$ appears to be determinable for nonlysosomal degradative models (Model 3), even in this case $k_D'$ may not correspond to a proteolytic step. Instead, protein unfolding or covalent modification is likely to be rate-limiting for cytosolic proteolysis.

Table 13 summarizes the identities of the observed rate constants for the fast and slow phases of products released for each model. For Model 1, $k_D'$ cannot be determined; $k_L$ and $k_T$ can be calculated from $k_{obs}$ and $[(P_3)_{sol}/(P_3)_{total}]$. In Model 2, $k_T = k_{obs}$; $k_D'$ and $k_R$ cannot be independently calculated. However, the ratio of $k_D'/(k_D' + k_R)$ is equal to $[(P_3)_{sol}/(P_3)_{total}]$. In Model 3, $k_D'$ and $k_L$ can be calculated. However, $k_D'$ is due to a cytoplasmic degradative system and is insensitive to lysosomal proteinase inhibitors and lysosomotropic agents. Moreover, peptide bond cleavage may not be rate limiting, and $k_D'$ could correspond to a nonproteolytic step.

Tables 11, 14, and 15 present the results for the turnover of cellular proteins under a variety of experimental conditions. These tables show the experimentally observed rate constants for the biphasic release of TCA/PTA-soluble and -insoluble radioactivity, the fractional sizes of the three substrate pools, and the ratio of TCA/PTA-soluble to total products released from pool 3 $[(P_3)_{sol}/(P_3)_{total}]$.

**Lack of Re-utilization of [3H-4,5] L-Leucine in Metabolically-Labeled Fibroblasts**

The possible re-utilization of tritiated leucine in labeled fibroblasts during a chase in unlabeled medium may be expected to result in the underestimation of the rates of
Model 2 and Model 3 also differ in that $k_D'$ in the former is due to the contribution of lysosomal proteinases, while $k_D'$ in the latter is due to contributions of extralysosomal degradative systems.
Table 14. Rate constants and pool sizes calculated from experimental progress curves obtained in the absence of serum.

Part A. Fast Phase

<table>
<thead>
<tr>
<th>Addition</th>
<th>( (k_1)_{\text{sol}} ) ( 10^3\text{ min}^{-1} )</th>
<th>( (k_1)_{\text{ins}} ) ( 10^3\text{min}^{-1} )</th>
<th>( P_1/P_0 )</th>
<th>( P_2/P_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.26±0.06</td>
<td>1.92±0.30</td>
<td>0.136</td>
<td>0.088</td>
</tr>
<tr>
<td>2 mM Leu + 13% FCS (control 2)</td>
<td>0.63±0.16</td>
<td>4.74±0.72</td>
<td>0.165</td>
<td>0.041</td>
</tr>
<tr>
<td>1% FCS, 1.23 mM Leu</td>
<td>0.86±0.13</td>
<td>3.57±0.47</td>
<td>0.174</td>
<td>0.056</td>
</tr>
<tr>
<td>100 µg/ml Antipain</td>
<td>10.15±1.46</td>
<td>2.70±0.64</td>
<td>0.020</td>
<td>0.038</td>
</tr>
<tr>
<td>5 µM Chloroquine</td>
<td>5.56±0.64</td>
<td>3.38±0.39</td>
<td>0.032</td>
<td>0.060</td>
</tr>
<tr>
<td>Sat. Pepstatin</td>
<td>3.77±0.41</td>
<td>0.75±0.10</td>
<td>0.054</td>
<td>0.149</td>
</tr>
<tr>
<td>26°C</td>
<td>0.75±0.33</td>
<td>3.52±0.33</td>
<td>0.015</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Part B. Slow Phase

<table>
<thead>
<tr>
<th>Addition</th>
<th>( (k_2)_{\text{sol}} ) ( 10^3\text{ min}^{-1} )</th>
<th>( (k_2)_{\text{ins}} ) ( 10^3\text{min}^{-1} )</th>
<th>( P_3/P_0 )</th>
<th>( (P_3)_{\text{total}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.07±0.06</td>
<td>5.16±0.38</td>
<td>0.77</td>
<td>0.23</td>
</tr>
<tr>
<td>2 mM Leu + 13% FCS (control 2)</td>
<td>8.71±1.63</td>
<td>14.13±0.53</td>
<td>0.79</td>
<td>0.74</td>
</tr>
<tr>
<td>1% FCS, 1.23 mM Leu</td>
<td>8.20±1.00</td>
<td>11.37±0.63</td>
<td>0.77</td>
<td>0.71</td>
</tr>
<tr>
<td>100 µg/ml Antipain</td>
<td>5.49±0.16</td>
<td>5.74±0.21</td>
<td>0.94</td>
<td>0.17</td>
</tr>
<tr>
<td>5 µM Chloroquine</td>
<td>10.23±0.38</td>
<td>9.03±0.22</td>
<td>0.91</td>
<td>0.20</td>
</tr>
<tr>
<td>Sat. Pepstatin</td>
<td>9.67±0.63</td>
<td>5.00±0.59</td>
<td>0.80</td>
<td>0.21</td>
</tr>
<tr>
<td>26°C</td>
<td>2.99±0.97</td>
<td>3.52±0.33</td>
<td>0.96</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Table 15. Average rate constant and % change for the slow phase of cellular protein turnover under various experimental conditions.

Part A. In the presence of serum and amino acids.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_{obs}$ (10^5 min^-1)</th>
<th>% Change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM Leu (control 1)</td>
<td>8.05±0.59 (15)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>None</td>
<td>9.00±0.45 (14)</td>
<td>+11.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>8.80±0.63 (14)</td>
<td>+9.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>4.94±0.30 (15)</td>
<td>-38.6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>4.76±0.32 (14)</td>
<td>-40.9</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>5.88±0.45 (15)</td>
<td>-27.0</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>6.54±0.38 (15)</td>
<td>-18.8</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>9.56±0.25 (14)</td>
<td>+18.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>9.18±0.42 (15)</td>
<td>+14.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>10.78±0.42 (15)</td>
<td>+33.9</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>2 mM Leu (control 2)</td>
<td>10.12±1.34 (15)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6.67 µg/ml Cytochalasin B</td>
<td>10.68±0.50 (15)</td>
<td>+5.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>12.93±0.56 (12)</td>
<td>+27.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>26°C</td>
<td>6.27±0.30 (6)</td>
<td>-38.0</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Table 15. Average rate constant and % change for the slow phase of cellular protein turnover under various experimental conditions.

Part B. In the absence of leucine, glutamine and serum.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_{obs}^b$ $(10^5 \text{ min}^{-1})$</th>
<th>% Change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.45±0.43 (15)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1% FCS</td>
<td>3.89±0.46 (15)</td>
<td>-12.6 n.s. c</td>
<td></td>
</tr>
<tr>
<td>1 mM Leu, 1% FCS</td>
<td>9.12±0.89 (15)</td>
<td>+104.9 &lt;0.005</td>
<td></td>
</tr>
<tr>
<td>2.8 mM Leu, 13% FCS</td>
<td>10.12±1.34 (15)</td>
<td>+127.4 &lt;0.005</td>
<td></td>
</tr>
<tr>
<td>5 µM Chloroquine</td>
<td>9.28±0.25 (14)</td>
<td>+108.5 &lt;0.005</td>
<td></td>
</tr>
<tr>
<td>100 µg/l Antipain</td>
<td>5.70±0.20 (15)</td>
<td>+28.1 &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Sat. Pepstatin</td>
<td>5.98±0.60 (15)</td>
<td>+34.4 &lt;0.025</td>
<td></td>
</tr>
<tr>
<td>7.5 µg/ml Cytochalasin B</td>
<td>3.29±4.79 (5)</td>
<td>-26.1 n.s. c</td>
<td></td>
</tr>
<tr>
<td>12 µM Vinblastine</td>
<td>4.92±4.05 (8)</td>
<td>+10.6 n.s. c</td>
<td></td>
</tr>
<tr>
<td>26°C</td>
<td>3.25±0.25 (15)</td>
<td>-26.9 &lt;0.025</td>
<td></td>
</tr>
</tbody>
</table>

aThe average rate constant is a weighted average of $k_{obs}$ for the release of TCA/PTA-soluble and TCA/PTA-insoluble radioactivity from labeled fibroblasts. $k_{obs}$ is calculated as being equal to

$$
\frac{(P_3)_{sol}}{(k2)_{sol} x (P_3)_{total}} + \frac{(P_3)_{ins}}{(k2)_{ins} x (P_3)_{total}}
$$

bValues are the mean ± the standard error. The numbers in parentheses are the number of data points on which the calculations of $k_{obs}$ are based.

cn.s. is not statistically significant ($p > 0.05$).
turnover of cellular proteins based on measurements of release of radioactivity from the cellular monolayer. Re-utilization of tritiated leucine might also be expected to result in the continued release of labeled secretory proteins during the chase. Therefore, we examined the efficacy of adding increasing concentrations of cold leucine—0, 2 and 10 mM—in preventing the re-utilization of the \(^3\text{H}\)-Leu label when added to the complete growth medium during the chase period. From Table 11, it is evident that the complete growth medium (0 added leucine) contained a sufficient concentration of endogenous leucine (0.8 mM) and added serum proteins (13%) to prevent re-utilization of the label, since the rate constants, extents of reaction, and the ratio of soluble to total activity released remained essentially unchanged when additional cold leucine was added. This is further illustrated in Figures 12-14, where the progress curves obtained at all three concentrations of added unlabeled leucine are relatively superimposable (within experimental error). The mean value of \(\left[\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}}\right]\) was 0.71 with a standard deviation of 0.010.

To further demonstrate that tritiated leucine reutilization is unimportant under the conditions of these experiments, 10 µM and 50 µM cycloheximide were added to the chase medium (Figures 18 and 19). These concentrations were shown to reduce the incorporation of tritiated leucine into cellular proteins by 30% and greater than 90%, respectively (Table 12). No increase in the ratio of TCA/PTA-soluble to TCA/PTA-insoluble label released was noted at either concentration \(\left[\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}}\right]\) in Table 11. Instead, \(\left[\frac{(P_3)_{\text{sol}}}{(P_3)_{\text{total}}}\right]\) actually decreased, indicating that TCA/PTA-insoluble
Figure 18. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 10 µM cycloheximide. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. \( (k_1)_{\text{sol}} = 2.78 \pm 0.35 \times 10^{-3} \text{min}^{-1} \), \( (k_1)_{\text{ins}} = 7.81 \pm 2.02 \times 10^{-3} \text{min}^{-1} \), \( (k_2)_{\text{sol}} = 5.99 \pm 0.23 \times 10^{-5} \text{min}^{-1} \), \( (k_2)_{\text{ins}} = 4.29 \pm 0.34 \times 10^{-5} \text{min}^{-1} \). See Figure 12 for details.
Figure 19. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM added Leu. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. Arrow indicates the time point (1470 min) at which 50 µM cycloheximide was added to the medium. The solid lines were calculated using the parameters in Table 11. $(k_1)_{sol} = 2.46\pm0.09 \times 10^{-3} \text{min}^{-1}$, $(k_1)_{ins} = 3.08\pm0.36 \times 10^{-3} \text{min}^{-1}$, $(k_2)_{sol} = 4.96\pm0.19 \times 10^{-5} \text{min}^{-1}$, $(k_2)_{ins} = 4.38\pm0.55 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for details.
radioactivity did not arise from secretory proteins synthesized during the chase. Instead, the source of TCA/PTA-insoluble radioactivity was proteins synthesized during the labeling period. In addition, the rate constant for the loss of label from the cellular monolayer was not increased as one might expect were reutilization of tritiated leucine important. Instead, there was a nearly 50% inhibition of the rate of turnover of cellular proteins, suggesting that protein synthesis and degradation are coordinately regulated. Table 16 shows that were Model 1 correct, the effect of both concentrations of cycloheximide was to decrease by about 60% the rate of transport of cellular substrates into lysosomes. Were Model 3 correct, then the cycloheximide effect was to decrease the activity of (a) cytoplasmic degradative system(s). According to both models, cycloheximide has no effect on the rates of cell leakage and/or cell death.

According to model 2, cycloheximide has two effects: (1) it decreases the rate of transport of cellular proteins into lysosomes by about 40%, and (2) it decreases protein degradation within the lysosome, or increases protein regurgitation from the lysosome, or both, thereby resulting in a 25 to 75% decrease in the ratio $k_D/k_R$.

**Effects of Lysosomotropic Amines and Lysosomal Proteinase Inhibitors on Intracellular Protein Turnover**

Ammonium chloride and chloroquine are lysosomotropic amines which disrupt the intravacuolar pH in lysosomes (Maxfield, 1982), thereby inhibiting many of the lysosomal hydrolases which have acidic pH optima. These agents also interfere with the transport of newly synthesized enzymes to lysosomes (Nishimura et al., 1988). Ten mM
Table 16. Effects of addition of cold leucine and cycloheximide on the rate constants for the slow phase of release of TCA/PTA-soluble and -insoluble radioactivity.

I. Model 1. Lysosomal degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T^a$</th>
<th>$k_L^a$</th>
<th>$k_D^a$</th>
<th>$k_R^a$</th>
<th>$k_T/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.30±0.71</td>
<td>2.70±0.31</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.33</td>
</tr>
<tr>
<td>2 mM Leu</td>
<td>5.72±0.71d</td>
<td>2.33±0.31d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>6.33±0.76d</td>
<td>2.47±0.30d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.57</td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>1.88±0.28e</td>
<td>3.06±0.46d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.61</td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>3.09±0.46e</td>
<td>1.67±0.35d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>1.85</td>
</tr>
</tbody>
</table>

II. Model 2. Lysosomal degradation plus regurgitation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.00±0.45</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.33</td>
</tr>
<tr>
<td>2 mM Leu</td>
<td>8.05±0.59d</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>8.80±0.63d</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.57</td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>4.94±0.30e</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.61</td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>4.76±0.32e</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.85</td>
</tr>
</tbody>
</table>
Table 16. Continued.

III. Model 3. Cytosolic degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N.A.</td>
<td>2.70±0.31</td>
<td>6.30±0.71</td>
<td>N.A.</td>
<td>2.33</td>
</tr>
<tr>
<td>2 mM Leu</td>
<td>N.A.</td>
<td>2.33±0.31</td>
<td>5.72±0.71</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>10 mM Leu</td>
<td>N.A.</td>
<td>2.47±0.30</td>
<td>6.33±0.76</td>
<td>N.A.</td>
<td>2.57</td>
</tr>
<tr>
<td>10 µM Cycloheximide</td>
<td>N.A.</td>
<td>3.06±0.46</td>
<td>1.88±0.23</td>
<td>N.A.</td>
<td>0.61</td>
</tr>
<tr>
<td>50 µM Cycloheximide</td>
<td>N.A.</td>
<td>1.67±0.35</td>
<td>3.09±0.46</td>
<td>N.A.</td>
<td>1.85</td>
</tr>
</tbody>
</table>

$a_{10^5} \times \text{min}^{-1}$

$b$ N.D.--not determinable

$c$ N.A.--not applicable

$d$ Not significantly different from control

e Differs from control at a level of $p < 0.005$.  

---
NH₄Cl caused small but significant decreases (31%) in the rate constants for the slow phases of release of TCA/PTA-soluble and -insoluble radioactivity (Tables 11, 15; Figure 20). \[ (P₃)_{sol}/(P₃)_{total} \] was also decreased to 0.56, which was more than 3 standard deviations below the mean value for the control. Twenty-five µM chloroquine (Figure 21) produced similar decreases in the rate constants for the slow phase (Tables 11 and 15). The value of \[ (P₃)_{sol}/(P₃)_{total} \] with 25 µM chloroquine (0.43) was also significantly reduced with respect to the control (Table 11).

Table 17 contains the rate constants calculated from the data in Tables 11 and 15 for each of the three models for the slow phase of protein turnover. If Model 1 is correct, then the respective effects of chloroquine and ammonium chloride was to reduce by 53% and 45% the rate of transfer of cellular proteins into lysosomes. According to Model 1, it is not possible to predict the effects of chloroquine and ammonium chloride on the rate of proteolysis within the lysosome. If Model 3 is correct, then chloroquine and ammonium chloride inhibited the activity of (a) cytoplasmic degradative pathway(s) by 53% and 45%, respectively. According to both models, ammonium chloride has no effect on cell death and/or cell leakage. Chloroquine, however, may increase the fractional rate constant for cellular leakage from \[ 2.46 \times 10^{-5} \text{ min}^{-1} \] to \[ 3.73 \times 10^{-5} \text{ min}^{-1} \] (p < 0.05). If Model 2 is correct, then chloroquine and ammonium chloride produced small decreases (23% and 31%, respectively) in the rates of transfer of cellular proteins into lysosomes while resulting in more substantial (69% and 48%) decreases in the ratio of \[ k_{D}/k_{R} \]. Such a result would be expected if chloroquine and ammonium chloride were to inhibit
Figure 20. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 10 mM NH₄Cl. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. \( (k_1)_{sol} = 0.77 \pm 0.17 \times 10^{-3} \text{ min}^{-1}, \ (k_1)_{ins} = 4.21 \pm 0.33 \times 10^{-3} \text{ min}^{-1}, \ (k_2)_{sol} = 5.58 \pm 0.64 \times 10^{-5} \text{ min}^{-1}, \ (k_2)_{ins} = 6.26 \pm 0.23 \times 10^{-5} \text{ min}^{-1}. \) See Figure 12 for details.
Figure 21. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 25 µM chloroquine. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. (k₃)₈ol = 1.06±0.15 x 10⁻³ min⁻¹, (k₄)₈ins = 2.95±0.46 x 10⁻³ min⁻¹, (k₅)₈ol = 6.04±0.46 x 10⁻⁵ min⁻¹, (k₆)₈ins = 6.91±0.32 x 10⁻⁵ min⁻¹. See Figure 12 for details.
Table 17. Effects of lysosomotropic agents on the rate constants for the slow phases of release of TCA/PTA-soluble and TCA/PTA-insoluble radioactivity.

I. Model 1. Lysosomal degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T^g$</th>
<th>$k_L^g$</th>
<th>$k_D^g$</th>
<th>$k_R^g$</th>
<th>$k_T/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.72±0.71</td>
<td>2.33±0.31</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>2.81±0.30c</td>
<td>3.73±0.40d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.75</td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>3.29±0.42c</td>
<td>2.59±0.33e</td>
<td>N.D.</td>
<td>N.A.</td>
<td>1.27</td>
</tr>
</tbody>
</table>

II. Model 2. Lysosomal degradation plus regurgitation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.05±0.59</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>6.54±0.38f</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.75</td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>5.88±0.45c</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.27</td>
</tr>
</tbody>
</table>

III. Model 3. Cytosolic degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N.A.</td>
<td>2.33±0.31</td>
<td>5.72±0.71</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>25 µM Chloroquine</td>
<td>N.A.</td>
<td>3.73±0.40d</td>
<td>2.81±0.30c</td>
<td>N.A.</td>
<td>0.75</td>
</tr>
<tr>
<td>10 mM NH₄Cl</td>
<td>N.A.</td>
<td>2.59±0.33c</td>
<td>3.29±0.42c</td>
<td>N.A.</td>
<td>1.27</td>
</tr>
</tbody>
</table>
Table 17. (Continued)

aN.D.--not determinable
bN.A.--not applicable
cDiffers from control at a level of $p < 0.005$
dDiffers from control at a level of $p < 0.05$
eNot significantly different from control
fDiffers from control at a level of $p < 0.010$
g($x \times 10^5$ min$^{-1}$)
lysosomal proteolysis without significantly altering the rate of substrate regurgitation from the lysosome. While it is not possible to eliminate any of the three models, Model 3 is least consistent with the data, since there is no reason to suspect that chloroquine and ammonium chloride should inhibit cytoplasmic degradative systems which function at pH's near neutrality. From the inhibitions observed with 25 µM chloroquine (53% according to Model 1 and 69% according to Model 2), we might conclude that extralysosomal (chloroquine-insensitive) degradative pathways could contribute no more than 30 to 50% to the turnover of long-lived cellular proteins in human fibroblasts.

Effects of Lysosomal Proteinase Inhibitors on Cellular Protein Turnover

Leupeptin and antipain are specific cysteine protease inhibitors. Both inhibit cathepsins B, H, and L, known to be present in lysosomes. In addition, leupeptin inhibits cytoplasmic calcium-dependent cysteine proteinases, calpains I and II in vitro, but may be unable to penetrate to the cytosol of cultured cells (Mehdi, 1991). Leupeptin, at 20 µM and 200 µM, had no effect on the rate constants for both fast and slow phases or on \[(P_3)_{s0}/(P_3)_{total}\] (Tables 11 and 15; Figures 22 and 23). Antipain, at 40 µg/ml, had no effect on the rate constants for the fast phase of protein turnover, but did produce a small (27%) but significant (p < 0.010) increase in the rate of the slow phase of turnover (Tables 11 and 15; Figure 24). \[(P_3)_{s0}/(P_3)_{total}\] was also unaffected by antipain.

Table 18 contains the rate constants calculated from the data in Tables 11 and 15 for each of the three models. It can be seen that leupeptin and antipain had no effect on any of the kinetic parameters calculated for Models 1 and 3. Antipain did seem to
Figure 22. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 20 µM leupeptin. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. \((k_1)_{\text{sol}} = 1.71 \pm 0.30 \times 10^{-3} \text{ min}^{-1}\), \((k_1)_{\text{ins}} = 4.74 \pm 0.48 \times 10^{-3} \text{ min}^{-1}\), \((k_2)_{\text{sol}} = 9.37 \pm 0.19 \times 10^{-5} \text{ min}^{-1}\), \((k_2)_{\text{ins}} = 10.00 \pm 0.39 \times 10^{-5} \text{ min}^{-1}\). See Figure 12 for details.
Figure 23. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 200 µM leupeptin. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. $(k_1)_\text{sol} = 1.81 \pm 0.38 \times 10^{-3} \text{min}^{-1}$, $(k_1)_\text{ins} = 5.27 \pm 0.85 \times 10^{-3} \text{min}^{-1}$, $(k_2)_\text{sol} = 8.74 \pm 0.37 \times 10^{-5} \text{min}^{-1}$, $(k_2)_\text{ins} = 10.26 \pm 0.55 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for details.
The graph shows the relationship between $\ln(1-F)$ and time (in minutes). The data points and the fitted lines indicate a decrease in $\ln(1-F)$ over time, suggesting a exponential decay process.
Figure 24. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 40 µg/ml antipain. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. \( (k_1)_{sol} = 1.74\pm0.36 \times 10^{-3} \text{min}^{-1}, \) \( (k_1)_{ins} = 5.79\pm0.76 \times 10^{-3} \text{min}^{-1}, \) \( (k_2)_{sol} = 10.25\pm0.31 \times 10^{-5} \text{min}^{-1}, \) \( (k_2)_{ins} = 12.08\pm0.69 \times 10^{-5} \text{min}^{-1}. \) See Figure 12 for details.
Table 18. Effects of proteinase inhibitors on the rate constants for the slow phases of release of TCA/PTA-soluble and TCA/PTA-insoluble radioactivity.

I. Model 1. Lysosomal degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D$</th>
<th>$k_R$</th>
<th>$k_T/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.30±0.71</td>
<td>2.70±0.31</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>6.69±0.47</td>
<td>2.87±0.22</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.33</td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>6.52±0.62</td>
<td>2.66±0.25</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>7.65±0.68</td>
<td>3.13±0.28</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
</tbody>
</table>

II. Model 2. Lysosomal degradation plus regurgitation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.05±0.59</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>9.56±0.25</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.33</td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>9.18±0.42</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>10.78±0.42</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
</tbody>
</table>
### Table 18. (Continued)

#### III. Model 3. Cytosolic degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_{D'}$</th>
<th>$k_R$</th>
<th>$k_{D'}/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N.A.</td>
<td>2.70±0.31</td>
<td>6.30±0.71</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>20 µM Leupeptin</td>
<td>N.A.</td>
<td>2.87±0.22</td>
<td>6.69±0.47</td>
<td>N.A.</td>
<td>2.33</td>
</tr>
<tr>
<td>200 µM Leupeptin</td>
<td>N.A.</td>
<td>2.66±0.25</td>
<td>6.52±0.62</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>40 µg/ml Antipain</td>
<td>N.A.</td>
<td>3.13±0.28</td>
<td>7.65±0.68</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
</tbody>
</table>

aN.D.--Not determinable
bN.A.--Not applicable
cNot significantly different from control
dDifference statistically significant at a level of $p < 0.010$
e($x \times 10^5$ min$^{-1}$)
produce a small but apparently significant increase in $k_T$ for Model 2. **Model 1** predicts that the rate determining step in the lysosomal degradation of cellular proteins is the transfer of substrates into the lysosome, $k_T$. The intrinsic degradation rate constant, $k_D'$, is undeterminable. Consequently, if a steady state is maintained in the presence of leupeptin and antipain, and if $k_T$ is unaffected by these agents, then the results in Table 18 are not inconsistent with Model 1. **Model 2** is least consistent with the results in Table 18. Although $k_T$ is expected to be rate determining for the lysosomal degradation of cellular proteins, the relative contributions of lysosomal degradation and regurgitation, $k_D'/k_R$, is related to $[(P_3)_{sol}/(P_3)_{total}]$, the ratio of TCA/PTA-soluble to total radioactivity released during the second slow phase of protein turnover. From Tables 11 and 18, it can be seen that $[(P_3)_{sol}/(P_3)_{total}]$ and $k_D'/k_R$ are unaffected by both leupeptin and antipain. This is unexpected since these inhibitors should have decreased both $[(P_3)_{sol}/(P_3)_{total}]$ and $k_D'/k_R$ as was seen with chloroquine and ammonium chloride. The lack of an effect of leupeptin and antipain on the ratio $k_D'/k_R$ argues strongly against Model 2 for intracellular protein catabolism. It cannot be due to failure of leupeptin to gain entry to lysosomes, since leupeptin can be internalized by fluid phase endocytosis. In addition, leupeptin inhibits the degradation of endocytosed proteins and causes the accumulation of cellular protein in autophagic lysosomes in rat liver (Kominami et al., 1991). In contrast, the failure of leupeptin to inhibit the slow and fast phases of protein degradation may not argue against the participation of cytosolic leupeptin-sensitive enzymes such as calpain, since evidence suggests that leupeptin may not penetrate to the
Effects of Temperature and Agents which Affect the Cytoskeleton of Cells

A new batch of GM 3440 fibroblasts was received in July, 1986. A second control experiment was performed in the presence of 2 mM Leu in complete growth medium (Figure 25) in order to compare these cells with the fibroblasts used in earlier work. The rate constants were similar to the first controls (Table 11). $k_1(\text{sol})$ and $k_1(\text{ins})$ were slower for the new cells, although these differences do not appear to be significantly different. In addition, values for $k_2(\text{sol})$ and $k_2(\text{ins})$ were higher. For $k_2(\text{sol})$, the difference was not significant. In contrast, $k_2(\text{ins})$ was much higher (14.13 as compared with 10.01, $p < 0.005$). In addition, $[(P_3)_{\text{sol}}(P_3)_{\text{total}}]$ was higher for the new batch of fibroblasts (0.74 as compared with 0.71). As a result, the weighted averages of the observed rate constants for the slow phase of protein turnover were not significantly different for the two batches of cells ($10.12 \pm 1.34 \times 10^{-5} \text{ min}^{-1}$ as compared with $8.49 \pm 0.62 \times 10^{-5} \text{ min}^{-1}$). The new control was used for purposes of comparison in subsequent experiments.

Vinblastine, a microtubule inhibitor, increased $k_1(\text{sol})$, $k_1(\text{ins})$, and $k_2(\text{sol})$ (Table 11; Figure 26). However, $k_2(\text{ins})$ was unaffected. Also, from Table 11, it can be seen that the substrate pool for the fast secretory phase of release of TCA-insoluble label was greatly decreased (0.044 as compared with 0.165 in the control). $[(P_3)_{\text{sol}}(P_3)_{\text{total}}]$ was substantially decreased (Table 11) from 0.74 to 0.58. In addition, it can be seen that the observed rate constants for the slow turnover of cellular protein ($k_{\text{obs}}$ in Table 15)
Figure 25. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu (7-86). F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. \((k_1)_{\text{sol}} = 0.63 \pm 0.16 \times 10^{-3} \text{min}^{-1},\) \((k_1)_{\text{INS}} = 4.47 \pm 0.72 \times 10^{-3} \text{min}^{-1},\) \((k_2)_{\text{sol}} = 8.71 \pm 1.63 \times 10^{-5} \text{min}^{-1},\) \((k_2)_{\text{INS}} = 14.13 \pm 0.53 \times 10^{-5} \text{min}^{-1}.\)

See Figure 12 for details.
Figure 26. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 55 µM vinblastine. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. $(k_1)_{\text{sol}} = 2.17\pm0.05 \times 10^{-3} \text{min}^{-1}$, $(k_1)_{\text{ins}} = 11.43\pm0.34 \times 10^{-3} \text{min}^{-1}$, $(k_2)_{\text{sol}} = 13.58\pm0.66 \times 10^{-5} \text{min}^{-1}$, $(k_2)_{\text{ins}} = 12.03\pm0.41 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for details.
was increased from $10.12 \times 10^{-5} \text{ min}^{-1}$ to $12.93 \times 10^{-5} \text{ min}^{-1}$ ($p < 0.05$).

Table 19 shows the intrinsic rate constants calculated for the slow phase of metabolism of cellular protein in the presence of vinblastine. According to Models 1 and 3, vinblastine-induced increase in $k_{\text{obs}}$ for the slow phase [$k_2(\text{sol})$ or $k_2(\text{ins})$] is largely due to an increase in the rate of leakage of protein from the cellular monolayer. Thus, $k_L$ in Table 19 doubles from $2.63 \pm 0.48 \times 10^{-5} \text{ min}^{-1}$ to $5.43 \pm 0.49 \times 10^{-5} \text{ min}^{-1}$ ($p < 0.005$). In contrast, $k_T$ (or $k_D'$ for Model 3) is unchanged. If Model 2 is correct, then the major effect of vinblastine is to either decrease lysosomal degradation ($k_D'$), increase lysosomal regurgitation ($k_R$), or both, such that $k_D'/k_R$ decreases by more than 50%, from 2.85 to 1.38.

Cytochalasin B, an actin inhibitor, had little if any effect on any of the kinetic parameters: $k_1(\text{sol}), k_1(\text{ins}), k_2(\text{sol}), k_2(\text{ins})$, and $k_{\text{obs}}$ (Tables 11 and 15). Pool sizes and the fraction of the slow turnover pool degraded to TCA-soluble products, $[(P_3)_\text{sol}/(P_3)_\text{total}]$, were also unaffected. The various intrinsic rate constants calculated for Models 1 through 3 were substantially similar to those of the control (Table 19; Figure 27).

The effect of temperature on intracellular protein turnover was next examined. The lowest temperature that could be attained with the incubator was $26^\circ \text{C}$, though the original proposal stated $19^\circ$. At $26^\circ$, the cells under control conditions (2 mM Leu in complete growth medium) began to contract after 6 h. Therefore, they were kept at $37^\circ$ overnight, during which time they appeared to recover. After the $37^\circ$ overnight
Table 19. Effects of reduced temperature and agents which disrupt microfilaments and microtubules.

I. Model 1. Lysosomal degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>( k_T )</th>
<th>( k_L )</th>
<th>( k_D )</th>
<th>( k_R )</th>
<th>( k_T/k_L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.49±1.37</td>
<td>2.63±0.48</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.85</td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>7.50±0.67c</td>
<td>5.43±0.49f</td>
<td>N.D.</td>
<td>N.A.</td>
<td>1.38</td>
</tr>
<tr>
<td>6.6 µg/ml Cyto. B</td>
<td>7.58±0.73c</td>
<td>3.09±0.30c</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>Temperature 26°C</td>
<td>4.70±0.39d</td>
<td>1.57±0.13d</td>
<td>N.D.</td>
<td>N.A.</td>
<td>3.00</td>
</tr>
</tbody>
</table>

II. Model 2. Lysosomal degradation plus regurgitation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>( k_T )</th>
<th>( k_L )</th>
<th>( k_D )</th>
<th>( k_R )</th>
<th>( k_D/k_R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.12±1.34</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.85</td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>12.93±0.51d</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.38</td>
</tr>
<tr>
<td>6.6 µg/ml Cyto. B</td>
<td>10.68±0.50c</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>Temperature 26°C</td>
<td>6.27±0.21e</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3.00</td>
</tr>
</tbody>
</table>
III. Model 3. Cytosolic degradation plus leakage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_T$</th>
<th>$k_L$</th>
<th>$k_D'$</th>
<th>$k_R$</th>
<th>$k_D'/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N.A.</td>
<td>$2.63 \pm 0.48$</td>
<td>$7.49 \pm 1.37$</td>
<td>N.A.</td>
<td>2.85</td>
</tr>
<tr>
<td>55 µM Vinblastine</td>
<td>N.A.</td>
<td>$5.43 \pm 0.49^f$</td>
<td>$7.50 \pm 0.67^e$</td>
<td>N.A.</td>
<td>1.38</td>
</tr>
<tr>
<td>6.6 µg/ml Cyto. B</td>
<td>N.A.</td>
<td>$3.09 \pm 0.30^c$</td>
<td>$7.50 \pm 0.73^c$</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>Temperature 26°C</td>
<td>N.A.</td>
<td>$1.57 \pm 0.13^d$</td>
<td>$4.70 \pm 0.39^d$</td>
<td>N.A.</td>
<td>3.00</td>
</tr>
</tbody>
</table>

\( ^a \)Not determinable  
\( ^b \)Not applicable  
\( ^c \)Not significantly different from control  
\( ^d \)Difference statistically significant at a level of \( p < 0.05 \)  
\( ^e \)Difference statistically significant at a level of \( p < 0.01 \)  
\( ^f \)Difference statistically significant at a level of \( p < 0.005 \)  
\( g(x \times 10^5 \text{ min}^{-1}) \)
Figure 27. Release of products vs. time for cells incubated in serum-supplemented medium containing 2 mM Leu and 6.6 µg/ml cytochalasin B. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 11. $(k_1)_\text{sol} = 0.56 \pm 0.05 \times 10^{-3} \text{min}^{-1}$, $(k_1)_\text{ins} = 6.73 \pm 1.80 \times 10^{-3} \text{min}^{-1}$, $(k_2)_\text{sol} = 9.07 \pm 0.53 \times 10^{-5} \text{min}^{-1}$, $(k_2)_\text{ins} = 14.65 \pm 0.42 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for details.
incubation, the initial rapid phases of release of TCA/PTA-soluble and -insoluble radioactivity had ended. The cells were then returned to 26° C and data was collected between 1620 and 1800 min. The cycle of warming and cooling was repeated and data was collected between 3060 and 3240 min. From the slopes of the two line segments obtained for both TCA/PTA-soluble and TCA/PTA-insoluble radioactivity released, \( k_2(\text{sol}) \) and \( k_2(\text{ins}) \) were calculated (\( \Delta \text{CPM}_{\text{sol}}/\text{Total CPM}_{\text{sol}} \) remaining and \( \Delta \text{CPM}_{\text{ins}}/\text{Total CPM}_{\text{ins}} \) remaining). \( [(P_3)_{\text{sol}}/(P_3)_{\text{total}}] \) was calculated from the ratio of TCA/PTA-soluble to total radioactivity between 3060 and 3240 min. The results are presented in Tables 11, 15, and Figure 28. It can be seen that \( k_1(\text{sol}) \) and \( k_1(\text{ins}) \) were unaffected. However, \( k_2(\text{sol}) \), \( k_2(\text{ins}) \) and \( k_{\text{obs}} \) were significantly decreased. The size of Pool 3 and the fraction of Pool 3 degraded to TCA/PTA-soluble products was also unaffected.

The intrinsic rate constants calculated for each of the three models are collected in Table 19. According to Model 1, decreasing the temperature to 26° C produced a proportional decrease in the rate of transfer of proteins into lysosomes and the rate of leakage of proteins from the cells such that \( k_T/k_L \) is essentially unaffected. Model 2 predicts that decreasing temperature decreases the rate of transfer of proteins into lysosomes. Any effects on degradation in the lysosome and regurgitation from the lysosome are similar so that \( k_D'/k_R \) is not significantly changed. Model 3 also predicts similar effects of decreasing temperature on cytosolic degradation and leakage so that \( k_D'/k_L \) is not affected. Both Models 1 and 3 predict that decreasing temperature 11° C
Figure 28. Release of Protein Products from Human Fibroblasts Cultured in Serum and Leucine Supplemented Medium at 26°C. Media samples were collected at intervals between 30 min and 6 hr. The fibroblasts were then transferred to 37°C overnight. The cells were returned to 26°C and at intervals between 1520 min and 1800 min media samples were obtained. After recovering at 37°C overnight the cells were again returned to 26°C and media samples collected between 2940 min and 3240 min. $(k_1)_{sol}$, $(k_1)_{ins}$, $(k_2)_{sol}$ and $(k_2)_{ins}$ were estimated from a least squares regression analysis of the initial data at 26°C. Values of $(k_2)_{sol}$ and $(k_2)_{ins}$ were also estimated from the slopes of the lines obtained by plotting $CPM_{sol}/Total\ CPM_{sol}$ remaining and $CPM_{ins}/Total\ CPM_{ins}$ remaining during the two intervals at 26°C at late times. Other details are similar to Figure 12. Each point is the average of three determinations ($N = 3$).
reduces leakage from fibroblasts by 40%.

From the effect of temperature on the rate constants in Table 19, it was possible to estimate activation energies (E_a) for the putative steps in each of the models. E_a was calculated from the relationship

\[ \frac{E_a}{R} = \frac{\ln k_2 - \ln k_1}{1/T_2 - 1/T_1} \]

These activation energies were found to be similar, ranging from 7.8 kcal/mol for kT (Model 1) or k_D' (Model 3) to 7.8 kcal/mol for k_L (Models 1 and 3). E_a for k_T (Model 2) was similarly calculated to be 8.0 kcal/mol. These values are much smaller than 27 ± 5 kcal/mol reported for the degradation of proteins microinjected into HeLa cells using red cell ghosts as a vehicle (Rechsteiner et al., 1984).

**Protein Turnover during Amino Acid Deprivation and Protein Starvation**

I was initially concerned by the failure of the lysosomal proteinase inhibitors leupeptin and antipain to inhibit intracellular protein turnover in cultured fibroblasts. I now believe that this may be due to the fact that proteolysis in the lysosome is not rate-determining for lysosomal protein catabolic pathways. Rather, the rate-determining step would be the transfer of cellular proteins to the lysosomes. To explore this question further, I sought to examine the effects of amino acid deprivation and/or protein starvation on the turnover of cellular proteins. These conditions are reported to increase autophagy in rodent liver. This is manifested by an accumulation of cytoplasmic constituents in lysosomes and an increase in protein catabolism (Mortimore & Ward,
1981; Mortimore et al., 1993). Starvation has also been reported to increase the turnover of a subset of cytosolic proteins by a lysosome-dependent pathway in fibroblasts (Dice, 1990).

Conditions which were intended to depict protein starvation were very difficult to attain with this cell line. Even at 1% FCS, the rate constants for the slow phase of protein turnover did not change appreciably (Tables 15, 20; Figure 29). However, major changes did occur in Leu-free/Gln-free medium containing no (0%) FCS (Figure 30). Contrary to an expected increase in protein breakdown, $k_2(\text{sol})$ and $k_2(\text{ins})$ were decreased to 2.07E-5 and 5.16E-5, respectively (Table 14). In addition, the ratio of TCA/PTA-soluble to total radioactivity, $(P_3)_{\text{sol}}/(P_3)_{\text{total}}$, (Table 14) was greatly decreased from about 0.74 for the control to 0.23. The effects of this amino acid and protein deprivation were similar to those produced by the protein synthesis inhibitor cycloheximide. As starvation is also expected to lead to an inhibition of protein synthesis, these results again point to a coordinated regulation of protein synthetic and degradative pathways in fibroblasts. However, starvation did increase the rate of degradation of short-lived proteins, $k_D$ $[(k_1)_{\text{sol}}$ in Table 14], from 0.63E-3 min$^{-1}$ to 1.26E-3 min$^{-1}$ without great effect on the size of the pool of short-lived proteins $(P_1/P_0)$. Starvation did appear to increase the extent of the initial rapid phase of protein secretion $(P_2/P_0)$, but decreased its rate by a comparable amount $[(k_1)_{\text{ins}}$ in Table 14].

In Table 20 are collected the intrinsic rate constants calculated for each of the 3 models of protein catabolism. If Model 1 is correct, then the principal effect of protein
Table 20. Effects of serum and amino acid deprivation on the rate constants for the slow phases of release of TCA-soluble and TCA-insoluble radioactivity in the presence of inhibitors.

I. Model 1

<table>
<thead>
<tr>
<th>Additions</th>
<th>( k_T^m )</th>
<th>( k_L^m )</th>
<th>( k_D'^m )</th>
<th>( k_R^m )</th>
<th>( k_T/k_L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None(^a)</td>
<td>1.04±0.10(^f)</td>
<td>3.43±0.33(^h)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.30</td>
</tr>
<tr>
<td>2 mM Leu + 13% FCS(^b)</td>
<td>7.49±1.32</td>
<td>2.63±0.40</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.85</td>
</tr>
<tr>
<td>1% FCS, 1.2 mM Leu(^c)</td>
<td>6.48±0.73(^h, i)</td>
<td>2.64±0.28(^h, i)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>100 µg/ml Antipain(^d)</td>
<td>0.97±0.10(^f, j)</td>
<td>4.73±0.17(^f, k)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.20</td>
</tr>
<tr>
<td>Sat. Pepstatin(^d)</td>
<td>1.26±0.13(^f, j)</td>
<td>4.72±0.50(^f, l)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.27</td>
</tr>
<tr>
<td>5 µM Chloroquine(^d)</td>
<td>1.86±0.19(^f, k)</td>
<td>7.42±0.21(^f, i)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>0.25</td>
</tr>
<tr>
<td>Temperature, 26(^o)C(^e)</td>
<td>1.48±0.07(^f, k)</td>
<td>1.42±0.06(^g, i)</td>
<td>N.D.</td>
<td>N.A.</td>
<td>1.04</td>
</tr>
</tbody>
</table>

II. Model 2

<table>
<thead>
<tr>
<th>Additions</th>
<th>( k_T )</th>
<th>( k_L )</th>
<th>( k_D' )</th>
<th>( k_R )</th>
<th>( k_D'/k_R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None(^a)</td>
<td>4.45±0.43(^e)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.30</td>
</tr>
<tr>
<td>2 mM Leu + 13% FCS(^b)</td>
<td>10.12±1.34</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.85</td>
</tr>
<tr>
<td>1% FCS, 1.2 mM Leu(^c)</td>
<td>9.12±0.89(^h, i)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.45</td>
</tr>
<tr>
<td>100 µg/ml Antipain(^d)</td>
<td>5.70±0.20(^f, l)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.20</td>
</tr>
<tr>
<td>Sat. Pepstatin(^d)</td>
<td>5.98±0.60(^g, l)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.27</td>
</tr>
<tr>
<td>5 µM Chloroquine(^d)</td>
<td>9.28±0.25(^h, i)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.25</td>
</tr>
<tr>
<td>Temperature, 26(^o)C(^e)</td>
<td>2.90±0.13(^f, k)</td>
<td>N.A.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.04</td>
</tr>
</tbody>
</table>
Table 20. Continued.

III. Model 3

<table>
<thead>
<tr>
<th>Additions</th>
<th>$k_Tm$</th>
<th>$k_Lm$</th>
<th>$k_Dm$</th>
<th>$k_Rm$</th>
<th>$k_D/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None$^a$</td>
<td>N.A.</td>
<td>$3.43\pm0.33^h$</td>
<td>$1.04\pm0.10^f$</td>
<td>N.A.</td>
<td>0.30</td>
</tr>
<tr>
<td>2 mM Leu + 13% FCS$^b$</td>
<td>N.A.</td>
<td>$2.63\pm0.40$</td>
<td>$7.49\pm1.32$</td>
<td>N.A.</td>
<td>2.85</td>
</tr>
<tr>
<td>1% FCS, 1.2 mM Leuc$^c$</td>
<td>N.A.</td>
<td>$2.64\pm0.23^h$</td>
<td>$6.48\pm0.73^h$</td>
<td>N.A.</td>
<td>2.45</td>
</tr>
<tr>
<td>100 µg/ml Antipain$^d$</td>
<td>N.A.</td>
<td>$4.73\pm0.17^f,k$</td>
<td>$0.97\pm0.10^f,j$</td>
<td>N.A.</td>
<td>0.20</td>
</tr>
<tr>
<td>Sat. Pepstatin$^d$</td>
<td>N.A.</td>
<td>$4.72\pm0.50^f,i$</td>
<td>$1.26\pm0.13^f,j$</td>
<td>N.A.</td>
<td>0.27</td>
</tr>
<tr>
<td>5 µM Chloroquine$^d$</td>
<td>N.A.</td>
<td>$7.42\pm0.21^f,i$</td>
<td>$1.86\pm0.19^f,k$</td>
<td>N.A.</td>
<td>0.25</td>
</tr>
<tr>
<td>Temperature, 26°C$^e$</td>
<td>N.A.</td>
<td>$1.42\pm0.06^f,g,i$</td>
<td>$1.48\pm0.07^f,k$</td>
<td>N.A.</td>
<td>1.04</td>
</tr>
</tbody>
</table>

$^a$Serum and amino acid deprived control.

$^b$Control performed 7/86 in complete medium.

$^c$1% FCS and 1.2 mM leucine added to serum-free medium containing glutamine.

$^d$Inhibitors added to serum-free medium from which leucine and glutamine have been omitted.

$^e$Temperature reduced to 26°C in serum-free medium from which leucine and glutamine have been omitted.

$^f$Differs from control in complete medium at a level of $p < 0.005$.

$^g$Differs from control in complete medium at a level of $p < 0.010$.

$^h$Not significantly different from control in complete medium.

$^i$Differs from serum and amino acid deprived control at a level of $p < 0.005$.

$^j$Not significantly different from serum and amino acid deprived control.

$^k$Differs from serum and amino acid deprived control at a level of $p < 0.01$. 


Differs from serum and amino acid deprived control at a level of $p < 0.05$.

$m_{10^5}$ min$^{-1}$
Figure 29. Release of products vs. time for cells incubated in medium containing 1% serum and 1.23 mM Leu. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14. $(k_1)_{sol} = 0.86±0.13 \times 10^{-3} \text{ min}^{-1}$, $(k_1)_{ins} = 3.57±0.47 \times 10^{-3} \text{ min}^{-1}$, $(k_2)_{sol} = 8.20±1.00 \times 10^{-5} \text{ min}^{-1}$, $(k_2)_{ins} = 11.37±0.63 \times 10^{-5} \text{ min}^{-1}$. See Figure 12 for details.
Figure 30. Release of products vs. time for cells incubated in serum-free, Leu/Gln-free medium. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14. $(k_1)_{\text{sol}} = 1.26 \pm 0.06 \times 10^{-3} \text{min}^{-1}$, $(k_1)_{\text{ins}} = 1.92 \pm 0.03 \times 10^{-3} \text{min}^{-1}$, $(k_2)_{\text{sol}} = 2.07 \pm 0.06 \times 10^{-5} \text{min}^{-1}$, $(k_2)_{\text{ins}} = 5.16 \pm 0.38 \times 10^{-5} \text{min}^{-1}$. See Figure 12 for details.
and amino acid deprivation is to decrease by about a factor of 6-7 the rate of transfer of substrates to the lysosome, from $6.5-7.5 \times 10^{-5} \text{ min}^{-1}$ to $1.0 \times 10^{-5} \text{ min}^{-1}$. The rate of leakage is not significantly affected, as $k_L$ changes from $2.6 \times 10^{-5} \text{ min}^{-1}$ to $3.4 \times 10^{-5} \text{ min}^{-1}$. If Model 2 is correct, then starvation results in a 2-fold decrease in the rate of transfer of substrates to lysosomes, while at the same time either decreasing lysosomal proteolysis, increasing lysosomal protein regurgitation, or both, such that $k_D'/k_R$ dramatically decreases from 2.4-3.2 to 0.30. If Model 3 is correct, the main effect of starvation is to decrease the activity of extralysosomal degradative pathways by 6-7 fold.

I next examined the effects on cellular proteolysis in the starved state of many of the same agents previously studied in the fed state (Figures 31-34). The results are collected in Tables 14 and 20. Antipain--at 100 µg/ml under conditions of serum and amino acid deprivation--caused a large increase in the observed rate constant for the initial rapid phases of protein degradation, from $1.26E-3 \text{ min}^{-1}$ to $10.2E-3 \text{ min}^{-1}$. However, the size of the substrate pool subject to rapid degradation was diminished proportionately from 0.14 to 0.020 (Table 14; Figure 31). The amount of cell-associated radioactivity released as TCA/PTA-insoluble material during the initial rapid phase was not significantly affected (from $1.92 \pm 0.30 \times 10^{-3} \text{ min}^{-1}$ to $2.70 \pm 0.64 \times 10^{-3} \text{ min}^{-1}$). Antipain did not cause further decreases in the observed rate constants for the slow phase of appearance of TCA/PTA-soluble and -insoluble products (Table 15). When the intrinsic rate constants for the slow phases were calculated for each of the three models, these were found to be similar to the corresponding values for the serum- and amino acid-
Figure 31. Release of products vs. time for cells incubated in serum-free, Leu/Gln-free medium containing 100 µg/ml antipain. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14. (k₁)_{sol} = 10.15\pm 1.46 \times 10^{-3} \text{min}^{-1}, (k₁)_{ins} = 2.70\pm 0.64 \times 10^{-3} \text{min}^{-1}, (k₂)_{sol} = 5.49\pm 0.16 \times 10^{-5} \text{min}^{-1}, (k₂)_{ins} = 5.74\pm 0.21 \times 10^{-5} \text{min}^{-1}. See Figure 12 for details.
deprived controls (Table 20). The small increase in the rate of leakage appears statistically significant. For Model 2, the small effect of antipain on the ratio $k_D/k_R$ (decreases from 0.30 to 0.20) is similar to the result obtained with complete media.

I next examined the effect of chloroquine on intracellular protein turnover under starvation conditions (Figure 32). The concentration of chloroquine had to be reduced to 5 µM, because cell death occurred within 24 hours at higher concentrations under these conditions. The effect of 5 µM chloroquine was to increase the rate constants for the initial phases of protein degradation and secretion by factors of 4.4 and 1.8, respectively, relative to the serum- and amino acid-deprived control (Table 15). Chloroquine also caused an increase in the observed rate constants for the slow phase of release of TCA/PTA-soluble and -insoluble products (from $2.07 \times 10^{-5}$ min$^{-1}$ to $10.23$ and $9.03 \times 10^{-5}$ min$^{-1}$, respectively, Table 14). However, when intrinsic rate constants were calculated (Table 20), it was found that for Models 1 and 3 that a substantial part of this increase could be explained by a highly significant increased leakage of proteins from the cell and/or increased cell death, although smaller increases in $k_T$ (Model 1) and $k_D'$ (Model 3) were also observed. The rate constants calculated for Model 2 indicate that chloroquine increased the rate of transfer of proteins into the lysosome to a value which approached that of the control in the presence of amino acids and serum. However, the ratio $k_D'/k_R$ resembled that of the serum- and amino acid-deprived control (0.25 as compared with 0.30, Table 20) and was much less than the control in the presence of complete medium (Table 20). These results for chloroquine were totally unexpected, as
Figure 32. Release of products vs. time for cells incubated in serum-free, Leu/Gln-free medium containing 5 µM chloroquine. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14. \((k_1)_{\text{sol}} = 5.56\pm0.64 \times 10^{-3}\ \text{min}^{-1}\), \((k_1)_{\text{ins}} = 3.38\pm0.39 \times 10^{-3}\ \text{min}^{-1}\), \((k_2)_{\text{sol}} = 10.23\pm0.38 \times 10^{-5}\ \text{min}^{-1}\), \((k_2)_{\text{ins}} = 9.03\pm0.22 \times 10^{-5}\ \text{min}^{-1}\). See Figure 12 for details.
earlier studies on protein catabolism in rodent liver and cultured fibroblasts had suggested that serum and amino acid deprivation increases the contributions of lysosomal (and thus cloroquine-sensitive) degradative pathways in cellular protein turnover. Instead, in human fibroblasts, serum and amino acid deprivation abolishes the chloroquine sensitivity of intracellular proteolysis.

Pepstatin A, an aspartyl protease inhibitor, produced opposite effects on the rapid phase of appearance of TCA/PTA-soluble and -insoluble products, increasing \( k_1 \) \( \text{sol} \) and decreasing \( k_1 \) \( \text{ins} \). The soluble fraction in the fast phase \( (P_1/P_0) \) was decreased more than 2-fold, and the insoluble fraction \( (P_2/P_0) \) was increased nearly 2-fold (Table 14; Figure 33). The observed rate constants for the slow phase, \( k_2(\text{sol}) \) and \( k_2(\text{ins}) \), were actually increased moderately over serum-deprived controls (Table 14), though not to the extent seen with chloroquine. When intrinsic rate constants were calculated (Table 20), this small increase was principally reflected in \( k_L \) for Models 1 and 3. If Model 2 is correct, then the moderate increases in \( k_2(\text{sol}) \) and \( k_2(\text{ins}) \) may be due to the increased transfer of substrate to the lysosomes. \( k_D/k_R \) was unaffected. Since pepstatin inhibits lysosomal protein degradation, this result indicates that either Model 2 is incorrect or lysosomes do not participate in protein turnover under serum- and amino acid-deprived conditions. It should be noted that pepstatin A was not very soluble, and its actual concentration could not be determined.

Vinblastine (12 µM) and cytochalasin B (7.5 µg/ml) caused cellular detachment and death within 6 h and 3 h, respectively, under serum-deprived conditions. Therefore,
Figure 33. Release of products vs. time for cells incubated in serum-free, Leu/Gln-free medium containing saturated pepstatin A. F is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14. \((k_1)_{\text{sol}} = 3.77 \pm 0.41 \times 10^{-3} \text{min}^{-1}\), \((k_1)_{\text{ins}} = 0.75 \pm 0.10 \times 10^{-3} \text{min}^{-1}\), \((k_2)_{\text{sol}} = 9.67 \pm 0.63 \times 10^{-5} \text{min}^{-1}\), \((k_2)_{\text{ins}} = 5.00 \pm 0.59 \times 10^{-5} \text{min}^{-1}\). See Figure 12 for details.
the kinetic parameters were not determined for these experimental conditions.

At 26°, the cells in serum-deprived media fared better than under control conditions. The cells were able to remain at 26° throughout the entire experiment (Figure 34). The rate constants for the fast phase, $k_1$(sol) and $k_1$(ins) did not vary appreciably from those of serum-deprived controls (Table 14). However, the pool of substrates which were degraded in the rapid phase ($P_1/P_0$) or which were secreted ($P_2/P_0$) were decreased 9-fold and 3-fold from control values (Table 14). The slow phase rate constants, $k_2$(sol), $k_2$(ins), and $k_{obs}$, were somewhat lower than the serum- and amino acid-deprived control (Tables 14 and 15).

When intrinsic rate constants (Table 20) were calculated for the three models of intracellular protein catabolism, it was found that the decreases in $k_2$(sol) and $k_2$(ins) at 26°C, relative to the serum- and amino acid-deprived controls, were largely due to decreases in $k_L$ (Models 1 and 3). For Model 2, these decreases were due to $k_T$. According to this model, the decrease in the rate of release of TCA/PTA-insoluble radioactivity during the slow phase is due to a decrease in the rate of lysosomal substrate regurgitation, such that $k_D/k_R$ is increased to 1.04 from 0.30.

An interesting observation from these data is that reducing the temperature decreases the intrinsic rate constants for protein catabolism in the fed state but not in the deprived state. For the fed state, $k_T$ (Model 1) or $k_D'$ (Model 3) declines 40% from a value of $7.49 \times 10^{-5}$ min$^{-1}$ at 37°C to $4.70 \times 10^{-5}$ min$^{-1}$ at 26°C. For the deprived state, the corresponding rate constants at 37°C and 26°C, if anything, have changed in
Figure 34. Release of products vs. time for cells incubated in serum-free, Leu/Gln-free medium at 26°C. \( F \) is the fraction of total TCA/PTA-soluble (closed circles) or TCA/PTA-insoluble (open circles) radioactivity remaining by each time. The solid lines were calculated using the parameters in Table 14.

\[
(k_1)_{\text{sol}} = 0.75 \pm 0.33 \times 10^{-3} \text{min}^{-1}, \quad (k_1)_{\text{ins}} = 3.52 \pm 0.33 \times 10^{-5} \text{min}^{-1}, \quad (k_2)_{\text{sol}} = 2.99 \pm 0.97 \times 10^{-5} \text{min}^{-1}, \quad (k_2)_{\text{ins}} = 3.52 \pm 0.33 \times 10^{-5} \text{min}^{-1}.
\]
See Figure 12 for details.
the opposite direction from $1.04 \times 10^{-5}$ min$^{-1}$ to $1.48 \times 10^{-5}$ min$^{-1}$. For Model 2, the effect of reducing the temperature is a 35% decrease in $k_T$ under both conditions. Lowering the temperature also appears to reduce leakage (Models 1 and 3) or regurgitation (Model 2) under deprived conditions.

**Lactate Dehydrogenase Assay**

LDH was used as a marker enzyme to determine whether the TCA-insoluble radioactivity released was due to cell leakage. In preliminary experiments, it was noted that LDH activity appeared in the cell culture medium within 1 h of adding media to cells, and that a plateau activity was attained within 2 h.

Table 21 gives the LDH activity over an incubation period of 29 h using the first procedure given in the Methods section. LDH activity increased over the first 2 h and thereafter, reached a plateau value of $1.03 \times 10^{-2}$ µmol lactate/ml medium (average of 2-5 h). These results implied that LDH was released for only the first 2 hours and remained active in the medium, or that LDH was released continually, and the plateau was the point of steady state between cellular release and inactivation in the medium.

Table 22 gives the LDH activity using the second procedure [the entire medium was removed every hour and replaced with fresh medium]. In this assay, the release of LDH was constant (average = $4.40 \times 10^{-3}$ µmol lactate/ml/h) over a 5-hour time period. This amounted to the release of 0.422% of cellular LDH activity into the medium each hour ($0.00422$ h$^{-1}$) and was of similar magnitude to the intrinsic rate constant for leakage.
Table 21. LDH assay, procedure 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>µmol lactate/ml medium</th>
<th>Corrected activity&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>6.53 x 10^-3</td>
<td>--</td>
</tr>
<tr>
<td>1 h</td>
<td>5.93 x 10^-3</td>
<td>-6 x 10^-4</td>
</tr>
<tr>
<td>2 h</td>
<td>1.62 x 10^-2</td>
<td>9.70 x 10^-3</td>
</tr>
<tr>
<td>3 h</td>
<td>1.74 x 10^-2</td>
<td>1.08 x 10^-2</td>
</tr>
<tr>
<td>4 h</td>
<td>1.60 x 10^-2</td>
<td>9.47 x 10^-3</td>
</tr>
<tr>
<td>5 h</td>
<td>1.79 x 10^-2</td>
<td>1.14 x 10^-2</td>
</tr>
<tr>
<td>25 h</td>
<td>1.02 x 10^-2</td>
<td>3.67 x 10^-3</td>
</tr>
<tr>
<td>29 h</td>
<td>1.45 x 10^-2</td>
<td>7.97 x 10^-3</td>
</tr>
</tbody>
</table>

<sup>1</sup>Obtained by subtracting 0 h activity from the activity at each time point.

Table 22. LDH assay, procedure 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>µmol lactate/ml medium</th>
<th>Corrected activity&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>7.58 x 10^-3</td>
<td>--</td>
</tr>
<tr>
<td>FCS (full strength)</td>
<td>7.98 x 10^-2</td>
<td>--</td>
</tr>
<tr>
<td>1 h</td>
<td>1.20 x 10^-2</td>
<td>4.42 x 10^-3</td>
</tr>
<tr>
<td>2 h</td>
<td>1.23 x 10^-2</td>
<td>4.72 x 10^-3</td>
</tr>
<tr>
<td>3 h</td>
<td>1.22 x 10^-2</td>
<td>4.62 x 10^-3</td>
</tr>
<tr>
<td>4 h</td>
<td>1.20 x 10^-2</td>
<td>4.42 x 10^-3</td>
</tr>
<tr>
<td>5 h</td>
<td>1.14 x 10^-2</td>
<td>3.82 x 10^-3</td>
</tr>
<tr>
<td>Whole cell</td>
<td>5.29 x 10^-1</td>
<td>1.042</td>
</tr>
</tbody>
</table>

<sup>1</sup>Obtained by subtracting 0 h activity from the activity at each time point.
calculated for Models 1 and 3 (kL = 0.0015 to 0.0018 h\(^{-1}\)).

Electrophoresis and Autoradiography

In order to further assess the source and nature of the TCA/PTA-insoluble radioactivity released into the culture medium, cell lysates and media samples from metabolically-labeled fibroblasts were subjected to SDS-PAGE. Photographs of the stained gels appear in Figures 35a and 35b. The lanes are read from left to right such that the left-most lane is 2 and the right-most lane 14. Lane identities are given in Table 23. Molecular weight (M\(_r\)) standards and their corresponding R\(_f\) values appear in Table 24; the standard curve, log\(_{10}\) M\(_r\) vs. R\(_f\), appears in Figure 36. The standard curve is linear up to M\(_r\) = 66,000, then becomes curvilinear at higher M\(_r\)'s.

Photographs of the developed films from autoradiography appear in Figures 37 and 38. Figure 37 is 1 month exposure; Figure 38 is 1 week exposure. The numbers indicate the lanes of the gel. [Lanes 7 and 8 are blank, because the M\(_r\) standards were non-radioactive.]

For the whole cell lysate obtained after a 42-hour labeling period and a 72-hour chase period (lane 14), the one month exposure proved too long to permit individual bands to be identified. For the remaining lanes, a one month exposure was necessary for the visualization of the protein bands. The unique bands, their calculated R\(_f\)'s and the lanes in which they appear are presented in Table 25 for some of the lanes.

---

3 The activity at 0 h in Table 24 was due to an endogenous LDH activity in the fetal calf serum, as shown by the activity obtained with 100% FCS.
Figure 35a. Fractionation of cell lysates and media samples from cultured human fibroblasts by SDS-PAGE. Gels were stained with coomassie blue and photographed. The content of each line is identified in Table 23.
Figure 35b. Fractionation of cell lysates and media samples from cultured human fibroblasts by SDS-PAGE. Gels were stained with Coomassie blue and photographed. The molecular weights x $10^{-3}$ of the standards (lanes 7 and 8) appear in the right margin. The remaining lanes are identified in Table 23.
Table 23. Lane identities for electrophoresis.

<table>
<thead>
<tr>
<th>Lane #</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
</tr>
<tr>
<td>2</td>
<td>30-min labeling medium (S)(^1)</td>
</tr>
<tr>
<td>3</td>
<td>Incubation medium 0-60 min (S)</td>
</tr>
<tr>
<td>4</td>
<td>Whole cell after 60 min incubation (S)</td>
</tr>
<tr>
<td>5</td>
<td>42-h labeling medium (L)(^2)</td>
</tr>
<tr>
<td>6</td>
<td>Incubation medium 90-180 min (L)</td>
</tr>
<tr>
<td>7</td>
<td>Low Mr standards</td>
</tr>
<tr>
<td>8</td>
<td>High Mr standards</td>
</tr>
<tr>
<td>9</td>
<td>Incubation medium 0-90 min (L)</td>
</tr>
<tr>
<td>10</td>
<td>Incubation medium 180-360 min (L)</td>
</tr>
<tr>
<td>11</td>
<td>Incubation medium 360-1620 min (L)</td>
</tr>
<tr>
<td>12</td>
<td>Incubation medium 1620-3030 min (L)</td>
</tr>
<tr>
<td>13</td>
<td>Incubation medium 3030-4250 min (L)</td>
</tr>
<tr>
<td>14</td>
<td>Whole cell after a 42 h pulse and 72 h chase (L)</td>
</tr>
<tr>
<td>15</td>
<td>Blank</td>
</tr>
</tbody>
</table>

\(^1\)S = short-term labeling period (30 min)

\(^2\)L = long-term labeling period (42 h)
Table 24. Electrophoresis $M_r$ standards and $R_f$ values.

<table>
<thead>
<tr>
<th>Mr Standard</th>
<th>$M_r$</th>
<th>Distance traveled (cm)</th>
<th>$R_f$</th>
<th>log $M_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin (rabbit muscle, subunit)</td>
<td>205,000</td>
<td>1.2</td>
<td>0.108</td>
<td>5.31</td>
</tr>
<tr>
<td>B-galactosidase (E. coli, subunit)</td>
<td>116,000</td>
<td>2.4</td>
<td>0.216</td>
<td>5.06</td>
</tr>
<tr>
<td>Phosphorylase B (rabbit muscle, subunit)</td>
<td>97,400</td>
<td>3.5</td>
<td>0.315</td>
<td>4.99</td>
</tr>
<tr>
<td>Albumin (bovine plasma)</td>
<td>66,000</td>
<td>4.25</td>
<td>0.383</td>
<td>4.82</td>
</tr>
<tr>
<td>Albumin (egg)</td>
<td>44,000</td>
<td>5.9</td>
<td>0.532</td>
<td>4.64</td>
</tr>
<tr>
<td>Glyceraldehyde Dehydrogenase (rabbit muscle)</td>
<td>36,000</td>
<td>7.0</td>
<td>0.631</td>
<td>4.56</td>
</tr>
<tr>
<td>Carbonic Anhydrase (bovine RBC)</td>
<td>29,000</td>
<td>8.4</td>
<td>0.757</td>
<td>4.46</td>
</tr>
<tr>
<td>Trypsinogen (PMSF treated)</td>
<td>24,000</td>
<td>8.9</td>
<td>0.802</td>
<td>4.38</td>
</tr>
<tr>
<td>Trypsin Inhibitor (soybean)</td>
<td>20,100</td>
<td>10.55</td>
<td>0.950</td>
<td>4.30</td>
</tr>
</tbody>
</table>

gel length = 11.1 cm
Figure 36. Standard curve for the determination of molecular weights by SDS-PAGE. Graphed are the $\log_{10}$ of the molecular weights vs. the relative mobility ($R_f$) of the standard.
Figure 37. Fractionation of all lysates and media samples from cultured human fibroblasts by SDS-PAGE after incorporation of $^{35}$S - methionine. Radioactive products were identified by autoradiography after a one month exposure. [Note: lanes 7 and 8 are blank, as they contain the $M_r$ standards.] The remaining lanes are identified in Table 23.
Figure 38. Fractionation of cell lysates and media samples from cultured human fibroblasts by SDS-PAGE. Same as Figure 37 except after autoradiography for one week.
Table 25. Rf values of protein bands detected following electrophoresis and autoradiographs of cell lysates and media samples from pulse-chase experiments with 35S-methionine.
Table 25. Rf values of protein bands detected following electrophoresis and autoradiographs of cell lysates and media samples from pulse-chase experiments with 35S-methionine.

<table>
<thead>
<tr>
<th>Cells after 42-h pulse and 72-h chase</th>
<th>Cells after 60-min pulse</th>
<th>Labeling medium after 42 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>0.11</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.13</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>0.15</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>0.17</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>0.22</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>0.26</td>
<td>0.27</td>
<td>0.28</td>
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<tr>
<td>0.29</td>
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<td>--</td>
</tr>
<tr>
<td>0.31</td>
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<tr>
<td>0.34</td>
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<td>0.35</td>
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<tr>
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<tr>
<td>0.46</td>
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</tr>
<tr>
<td>0.49</td>
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<tr>
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<tr>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>--2</td>
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</tr>
<tr>
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<td>0.58</td>
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<tr>
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<td>0.61</td>
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<td>0.66</td>
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<td>0.86</td>
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<tr>
<td>--</td>
<td>0.96</td>
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</table>
Table 25. Continued.

<table>
<thead>
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<th>Chase medium 3</th>
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<td>--</td>
<td>--</td>
<td>0.96</td>
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</table>
1 One week exposure.

2 A broad smear appears on this region of the gel.

3 One month exposure.

4 Detected in the one week exposure; in the one month exposure, this band appears to be obscured by the intense band at $R_f = 0.17$.

5 These bands are present in the chase medium between 0 and 180 min after the pulse, but are absent from the chase medium in samples obtained after 360 min.
Approximately 32 distinct bands could be detected in the autoradiograms, of which at least 29 were visible in the cell lysate obtained from cells pulsed for 42 hours and chased for 72 hours (lane 14, Figure 38). Three additional bands, which appear in the lysates of cells pulsed for 60 min, at \( R_f \) equal to 0.55, 0.58 and 0.96 were probably also present in lysates of cells pulsed for 42 hours, but were either poorly resolved or were obscured by an intense neighboring band.

A comparison of the cell lysates obtained from cells pulsed for 42 hours and cells pulsed for 30 min (lanes 14 and 4) reveal that proteins with \( R_f \) values greater than 0.55 incorporated less \( ^{35} \text{S}-\text{methionine} \) than did proteins which migrated more slowly. This is in agreement with earlier claims that large proteins or proteins composed of larger subunits turnover more rapidly, and therefore incorporate label more quickly, than do smaller proteins (Neff et al., 1981).

Lanes 9 and 6 (Figures 38 and Table 25) represent the incubation medium collected 90 minutes and 180 minutes after the start of the chase during the rapid phase of appearance of TCA/PTA-insoluble radioactivity. In contrast, lanes 12 and 13 (Figure 38 and Table 25) are media samples collected at late times during the slow phase of product release. Three protein bands were detected in the culture medium at early times after the chase, which were not present in the media samples obtained at late times. These proteins had \( R_f \) values of 0.13 (\( M_r = 182,000 \)), 0.15 (\( M_r = 158,000 \)) and 0.50-0.52 (\( M_r = 49,500-45,700 \)). These are presumed to be secreted proteins which contribute to the rapid phase of appearance of TCA/PTA-insoluble material during the initial rapid phase of
product release. In addition, in the media samples (lanes 5-13) larger proteins (R_f values less than 0.55) incorporated more radioactivity than smaller proteins, as was observed for the cell lysates.

The nature of the TCA/PTA-insoluble material released into the culture medium during the slow phase of appearance of products is of considerable interest. Such proteins are expected to be present in the sample corresponding to the 42-hour labeling medium (lane 5 in Figure 38 and Table 25) and in samples of the incubation medium collected at late times (lanes 12 and 13 in Figure 38 and Table 25). Three possible sources of TCA/PTA-insoluble material released during the slow phase have been considered. First, this material could arise from the reincorporation of labeled amino acids into secretory proteins during the slow phase of cellular protein turnover. If this was the case, one might expect to see only a limited subset of protein bands in the 42-hour labeling medium and the samples of incubation medium collected at late times when compared with the cell lysates. This was not the case, as virtually all prominent proteins seen in the whole cell lysates (e.g. bands with R_f values of 0.07, 0.17, 0.22, 0.26, 0.29, 0.31, 0.45, 0.49, and 0.53 in Figures 37 and 38, and Table 25) were also released into the culture medium during the labeling period (Figure 37, lane 5, and Table 25) and during the subsequent chase (Figure 37, lanes 6-13, and Table 25). Alternatively, TCA/PTA-insoluble material might arise through the sequestration of cellular proteins into lysosomes with subsequent regurgitation of the lysosomal contents (Buktenica et al., 1987). However, were cellular protein to pass through a degradative compartment prior
to secretion, then one might expect to see evidence of proteolysis having occurred on analysis of the 42-hour labeling medium and samples of the incubation medium collected at late times. This might be reflected in a disappearance of higher molecular weight bands and the appearance of new bands in the lower molecular weight range of the gels. This, too, was not seen. Finally, a possible third source of TCA/PTA-insoluble products is cell leakage and/or cell death. In this case, one should see similar protein patterns for the cell lysate after 42 hours of labeling and the 42-hour labeling medium. A comparison of lane 14 (Figure 37 and Table 25) and lane 5 (Figure 38 and Table 25) shows that virtually every major band seen in the cell lysate is also represented in the 42-hour labeling medium, with the conspicuous exception of 2 or 3 bands with Rf values between 0.78 and 0.86. Nearly two thirds of the protein bands seen in the cell lysate are also detectable in the 42-hour labeling medium. In addition, many of these bands are also present in incubation media collected at late times after the beginning of the chase period (lanes 12 and 13, Figure 38 and Table 25).

**Distribution of Radioactivity after Centrifugation**

The media containing radioactivity released from fibroblasts was centrifuged at low and high speeds, as described in the Methods section, to determine whether the TCA-insoluble material was due to dead cells, vesicular structures shed from cells, or was free in solution. The low-speed pellet contained detached and dead cells, the high-speed pellet was presumed to contain vesicles, and the high-speed supernatant contained soluble
proteins and the TCA/PTA-soluble degradation products. The high-speed supernatant as treated with TCA-PTA to separate TCA-soluble and -insoluble radioactivity.

Table 26 shows the partitioning of the radioactivity in the culture medium after a three-hour incubation (fast phase) and after a three-day incubation (slow phase) under control conditions (2 mM Leu in complete growth medium). After 3 hours, 91% of the radioactivity released into the culture medium was non-sedimentable at 108,000 x g. Of that material, 71% was TCA/PTA-soluble. Dead or detached cells represented only 2.7% of the radioactivity in the medium, and vesicles represented 6.3% of the total radioactivity in the medium. On day 3, 92.8% of the radioactivity could not be sedimented at 108,000 x g, and 80% of it was TCA/PTA soluble. Dead cells and vesicles accounted for 3.3% and 3.9%, respectively, of the total radioactivity released.

Table 27 shows the partitioning of the radioactivity released under serum-deprived conditions (Leu-free/Gln-free medium, 0% FCS) after centrifugation. After 3 h, 76.1% could not be sedimented at 108,000 x g, and of that a little more than half (58%) was TCA/PTA soluble. Vesicles accounted for 17.8%, and dead cells, 6.0% of the total radioactivity released. On day 3, only 59.1% of the radioactivity was not sedimentable, and of that only 29.8% was in the TCA/PTA-soluble fraction of the high-speed supernatant. Significant proportions of the radioactivity released were due to dead or detached cells (15.1%, low speed pellet), vesicles (25.8%, high speed pellet), and soluble but non- or incompletely degraded proteins (41.6% TCA/PTA precipitable).
Table 26. Release of soluble and particulate radioactivity from cultured human fibroblasts after labeling with $^3\text{H}$-leucine and incubating in complete medium.

<table>
<thead>
<tr>
<th>Day</th>
<th>g force (x g)</th>
<th>Sample</th>
<th>$^3\text{H}$ (cpm)</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14,116</td>
<td>P$^1$</td>
<td>208</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>108,920</td>
<td>SS</td>
<td>4977</td>
<td>64.5</td>
</tr>
<tr>
<td>1</td>
<td>108,920</td>
<td>SP</td>
<td>2045</td>
<td>26.5</td>
</tr>
<tr>
<td>1</td>
<td>108,920</td>
<td>PP</td>
<td>483</td>
<td>6.3</td>
</tr>
<tr>
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<td>P</td>
<td>245</td>
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</tr>
<tr>
<td>3</td>
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<td>SS</td>
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<td>74.6</td>
</tr>
<tr>
<td>3</td>
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<td>SP</td>
<td>1348</td>
<td>18.2</td>
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<tr>
<td>3</td>
<td>108,920</td>
<td>PP</td>
<td>268</td>
<td>3.9</td>
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</table>

$^1$P = pellet (14,117 x g)

SS = TCA soluble fraction of high speed supernatant

SP = TCA insoluble fraction of high speed supernatant

PP = pellet (108,920 x g)
Release of soluble and particulate radioactivity from cultured human fibroblasts after labeling with \(^3\text{H}\)-leucine and incubating in medium lacking serum and amino acids.

<table>
<thead>
<tr>
<th>Day</th>
<th>g force (x g)</th>
<th>Sample</th>
<th>(^3\text{H}) (cpm)</th>
<th>% Total</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>14,116</td>
<td>P(^1)</td>
<td>340</td>
<td>6.0</td>
</tr>
<tr>
<td>1</td>
<td>108,920</td>
<td>SS</td>
<td>2480</td>
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<tr>
<td>1</td>
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<td>SP</td>
<td>1792</td>
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<tr>
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<td>SS</td>
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</tr>
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<td>SP</td>
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<tr>
<td>3</td>
<td>108,920</td>
<td>PP</td>
<td>1744</td>
<td>25.8</td>
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</table>

\(^1\)Same footnote as Table 26.
To the extent that cells shed vesicles or become detached from the culture dish, measurements of TCA/PTA-precipitable radioactivity in the incubation medium will overestimate the occurrence of protein leakage (Models 1 and 3) or protein exocytosis (Model 2) from the cellular monolayer. However, the intrinsic rate constants which characterize the rate-determining step in intracellular protein degradation, \( k_T \) (Model 1) and \( k_D' \) (Model 3), will not be affected because their calculation is not dependent on the manner by which TCA/PTA-insoluble radioactivity is released into the incubation medium. In contrast, Model 2 is badly impacted by the occurrence of cell leakage (see results of lactate dehydrogenase measurements), cell detachment and vesicle shedding, since it assumes that all the radioactivity released into the culture medium (both TCA/PTA-soluble and -insoluble) arises from a vacuolar degradative compartment. Thus, this model presumes that substrates must first be sequestered in lysosomes or other vesicular compartments before being released into the culture medium. Consequently, the existence of non-lysosomal sources of TCA/PTA-precipitable radioactivity in the culture medium will result in a serious overestimation of \( k_T \) for this model. In addition, the ratio \( k_D'/k_R \) will not describe the partition of substrates within the lysosome between degradation and exocytosis under conditions where leakage or similar processes occur.

**Cathepsin B Assay**

The results of the cathepsin B assay are given in Table 2B. The slope of \( A_{300} \) vs. time was 2.6 for the cells incubated under control conditions; this value corresponds to 22 nmol/min/plate of confluent fibroblasts. The serum-deprived cells had a slope of 2.2,
Table 28. Effects of serum and amino acid deprivation, and antipain pretreatment, on cellular levels of Cathepsin B in human fibroblasts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>A300 ± S.E.</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM Leu²</td>
<td>2.6 ± 0.10</td>
<td>22</td>
</tr>
<tr>
<td>0 Leu/Gln</td>
<td>2.2 ± 0.06</td>
<td>18</td>
</tr>
<tr>
<td>100 µg/ml Antipain</td>
<td>2.9 ± 0.17</td>
<td>24</td>
</tr>
</tbody>
</table>

1Activity expressed as nmol/min/cell plate

2Cells were incubated for 66 hours in the indicated medium prior to measuring the enzyme activity. See MATERIALS AND METHODS for details.
which was more than 2 standard deviations below that for the control value. The cells incubated with antipain had a slope of 2.9, which was just within 2 standard deviations of the control value.
CHAPTER IV

DISCUSSION

PART I. LIPOSOMES

Liposomes have been prepared from a variety of phospholipid species employing a number of techniques. The addition of calcium chelating agents to the hydrated calcium salt of PS which produces PS-LUV (Papahadjopoulos, 1970; Papahadjopoulos et al., 1975) was the method selected to prepare liposome-encapsulated protein substrates. LUV typically have relatively large aqueous interiors compared to LMV and SUV (Table 3). In addition, liposomes prepared from fluid-charged lipids such as PS were reported to be incorporated into cultured cells by fusion rather than by endocytosis (Poste & Papahadjopoulos, 1976). Of the two methods for preparing PS-LUV, reverse phase evaporation and calcium chelation, the former, while producing higher yields and greater trapping efficiencies (Szoka & Papahadjopoulos, 1978), was thought to be less suitable for the preservation of the tertiary structure of the encapsulated protein substrates.

In this study, electron microscopy was used to monitor the individual steps in the formation of PS-LUV. Sequentially, these steps are: (1) hydration and sonication of dried phospholipid films to produce small vesicles; (2) addition of calcium, causing progressive aggregation and rupture of the small vesicles producing calcium chelate cylinders in which the phospholipid bilayer is wound around a central axis in the manner
of a "jelly roll"; (3) addition of the calcium chelator, which results in dissociation of the
choclate structures and LUV formation (Papahadjopoulos, 1970; Papahadjopoulos et al.,
1975). A number of staining procedures were tried, including uranyl acetate and osmium
tetroxide (results not shown), in order to visualize these steps by electron microscopy.
Encapsulation of a phosphotungstate negative stain provided the best resolution of vesic­
ular structures, particularly with regard to the SUV formed during the first step of PS­
LUV formation (Figure 1). In contrast, choclate-type structures, the reported
intermediate in PS-LUV preparation, could not be clearly identified by phosphotungstate
negative staining. Instead, I observed an apparent lattice-like structure composed of
aggregates of ill-defined individual units (Figure 2). PS-LUV appeared as aggregates of
vesicles with electron-dense centers (Figure 3); resolution of the lipid bilayer was less
distinct than for PS-SUV initially produced by sonication. The effect of the
phosphotungstate negative stain on aggregation is not known. In addition, the vesicle-
vesicle interfaces in PS-LUV aggregates were not well-visualized in this study.
Estimates of PS-LUV size gave diameters in the range of 200 to 300 nm, in excellent
agreement with values obtained by gel exclusion chromatography (0.26-0.28 μm)
(Figure 4) and values calculated from determinations of captured volume to phospholipid
ratios (234-276 nm) (Table 3). These latter calculations are significant because sizable
quantities of large multilamellar structures would have resulted in a significant
underestimation of liposome size based on trapped volume measurements.

While PS-LUV were relatively impermeable to 14C-sucrose, FD, fluorescein
isothiocyanate-labeled BSA, and a number of $^{125}$I-labeled proteins, they appeared permeable to protons. Thus, the fluorescence of PS-LUV-encapsulated FD responded to changes in the pH of the external buffering medium in a similar fashion as free FD (Figure 10). In addition, PS-LUV were either unable to efficiently trap 6-CF or were permeable to this fluorophore. Thus, PS-LUV prepared in the presence of high, self-quenching concentrations of 6-CF were nevertheless highly fluorescent and yielded only a small increment in fluorescence on addition of detergent (Table 7). In contrast, PC-SUV prepared in the presence of similar high concentrations of 6-CF had very little intrinsic fluorescence, but became highly fluorescent after lysis and dilution of the entrapped fluorophore.

Contrary to implications in the literature regarding liposome-cell fusion, the work from this laboratory showed that while liposomes prepared from PS or PC/LL/SA adhered tightly to cells, they rarely fused with cellular membranes to deliver their contents to the cytosol. There has been a considerable decrease in recent literature involving liposome-cell fusion experiments. One report claims to have shown fusion between 3T3 cells and liposomes composed of various combinations of phosphatidic acid (PA), PC and PS (Siegmann, 1987). Carboxyfluorescein (CF) was used as an intracellular marker, and cellular fusion was determined by fluorescent microscopic examination. In that study, cells were incubated with either CF-containing liposomes or with CF free in solution. The authors reported no uptake of free CF, while the liposome-encapsulated CF was incorporated into the cells. This is in contrast to our findings,
which showed rapid cellular uptake of free CF (Figure 7). Furthermore, Siegmann's study did not show any photomicrographs of fluorescent cells, so it is impossible to evaluate whether the fluorescence was located diffusely within the cytoplasm (consistent with fusion or diffusion) or in vesicular structures (more consistent with endocytosis or adherence of liposomes to the cell membranes).

It is apparent that a careful re-examination of the earlier liposome-cell fusion studies is necessary in view of our results. It should be reiterated that fusion was rarely proven by direct evidence, nor was it ever shown to be quantitatively significant. One study describing the use of endocytosis inhibitors (Poste & Papahadjopoulos, 1976) was particularly hampered by dependence on presumptive evidence. Fusion was determined by the presence of cell-associated radioactivity after washing to "remove" free liposomes. However, we found that approximately 2.5% of the PS liposomes adhered tightly to the cells after numerous washings (Tables 5, 6) so that cell-associated radioactivity cannot be used to assess fusion. These authors may have been correct, however, in proposing that fluid-neutral (PC) and solid-charged (PS/DSPC/DPPC) vesicles were incorporated into cells primarily through endocytosis. They also implied that a significant fraction (30-40%) of charged-fluid vesicles (PC/PS) were endocytosed as well. The differences in behavior of the liposome species presumably involved surface charge and surface tension properties (Kachar et al., 1986). PS/PC liposomes were perhaps capable of forming stable bilayer interfaces with the cell membranes, while PC and PS/DSPC/DPPC vesicles were not.
A lipid uptake study (Papahadjopoulos, 1974) showed that cholesterol and DPPC were incorporated into cells in the same molar ratio as was present in liposomes. In addition to fusion, another equally plausible explanation of these results is liposome-cell adhesion. The electron-micrograph of a RBC ghost incubated with MLV was re-examined. There appeared to be an interface between the cell membrane and the vesicles, which would be more consistent with vesicle adherence than with fusion. Similar objections can also be raised about electron micrographs of PC, lysolecithin and stearylamine SUV which are purported to demonstrate liposome cell fusion (Martin & MacDonald, 1976b).

The enzyme repletion study of Gregoriadis and Buckland (1973) is less questionable, since endocytosis was shown to be the mechanism of cellular delivery. An interesting experiment to confirm this conclusion would have included cytochalasin B in the incubation medium. A decrease in the release of radioactivity coupled with retention of the phase-lucent vacuoles would be additional evidence for endocytosis of the liposomes.

The results obtained with liposome-encapsulated viral polynucleotides and whole viruses (Mayhew et al., 1977; Taber et al., 1978; Wilson et al., 1977; Wilson et al., 1979) to demonstrate liposome fusion may be more difficult to discount. The observation that liposome-encapsulated naked nucleotides remain functional after delivery to cells implies that they are not significantly degraded upon entry as would be expected for endocytosis (Mayhew et al., 1977; Wilson et al., 1979). Similarly, CHO cells, which have no
membrane receptors for poliovirus, can be infected by encapsulated poliovirus, but not by the free virus (Taber et al., 1978). However, in both cases the adherence of PS liposomes to cellular membranes may act to promote nucleotide/viral delivery. It may be possible for membrane-associated nucleotides to enter the cell by some presently unknown mechanism which does not involve endocytosis or fusion. With whole viruses, liposome-mediated binding to the cell surface may obviated the need for receptors, and viral entry may be due to the presence of viral proteins.

A more important objection to the above assays of liposome-cell fusion is their employment of potent biological agents which exert their effects at very low concentrations through (an) amplification step(s) associated with their actions. Agents used in such assays include plant and bacterial toxins which possess enzymatic (ADP-ribosylation) activities, viruses which can undergo replication, and oligonucleotides which can function as templates in biosynthetic reactions. Consequently, such assays magnify the effects of rare events and are unsuitable for quantifying the extent to which cell surface-bound liposomes fuse with the plasma membrane.

In contrast, we have developed an assay which provides quantitative information about liposome-cell fusion with PS-LUV. When the pH sensitive, non-permeant probe, FD, was used as an aqueous space marker, about 2.5% of the aqueous space entrapped in PS-LUV becomes associated with the cellular monolayer under our experimental conditions. In addition, the majority of the cellular fluorescence remained within a compartment which was sensitive to manipulations of the pH of the medium. Only very
little, if any, of the FD was delivered to a cellular compartment which was insensitive to changes in the external pH. Similar conclusions were reached independently in our laboratory from studies of the delivery and degradation of $^{125}$I-labeled proteins to fibroblasts using PS/PC-LUV prepared by a freeze-thaw method (Thyagarajan, 1985). In these studies, between 2 and 3% of the entrapped radiolabeled proteins became associated with the cellular monolayer. However, these proteins were remarkably inert. Very little degradation could be detected based on the appearance of TCA/PTA-soluble radioactivity ($t_{1/2} > 30$ days). When Ca$^{++}$ was added to cells preincubated with PS/PC-LUV-encapsulated substrates to effect fusion, degradation of the radiolabeled proteins ensued. However, the time course of appearance of TCA/PTA-soluble products was qualitatively similar to that seen with endocytosed protein substrates (Thyagarajan, 1985). This suggested that Ca$^{++}$ treatment either induced the uptake of liposomes by endocytosis or that the $^{125}$I-labeled proteins had leaked from the liposomes and were then internalized by endocytosis. In agreement, when cells were preincubated with PS/PC-LUV-containing FD, addition of Ca$^{++}$ caused a progressive quenching of the cellular fluorescence (Ex495; Em519) consistent with the delivery of FD to an acidic (lysosomal/endoosomal) compartment (Thyagarajan, unpublished results).

During the past decade, a number of fusion studies have been performed using complex systems (Arvinte, 1987). Fusogenic proteins have been well-described and characterized from enveloped viruses (Stegmann et al., 1985; White & Helenius, 1980; White et al., 1982), mitochondria (Schneider et al., 1980), chromatophores (Snozzi &
Crofts, 1984), and bacterial membranes (Duzgunes et al., 1985). Fusion of cells or vesicles may also be induced by high electric field impulses (1-20 kV/cm, 1-20 µs) (electrofusion) (Sugar et al., 1987). It is possible that fusogenic proteins exist in eukaryotic intracellular organelle membranes, since intracellular vesicular fusion is a widely-accepted phenomenon. Whether or not fusogenic proteins exist in the plasma membrane is currently unknown.

Liposomes, regardless of their ability to fuse with cell membranes, may cause profound cellular changes. Phosphatidic acid (PA), whether incorporated into liposomes or suspended in solution, was shown to stimulate DNA synthesis in quiescent cells (Siegmann, 1987). SUV prepared from PC/PS caused an increase in aberrant mitoses in both diploid and heteroploid cell lines (Nuzzo et al., 1987). This effect was previously demonstrated with MLV (Nuzzo et al., 1985). When combined with the fusogenic agent polyethylene glycol (PEG), the mixture was highly cytotoxic (Nuzzo et al., 1987). The highest level of chromosome abnormalities was seen with liposome-encapsulated DNAase. The mechanism may be related to the interaction of the liposomes or PEG with cell membranes with the production of free radicals, or their interaction with lysosomes and the intracellular release of endogenous nucleases (Nuzzo et al., 1987).

The failure of PS-LUV to deliver proteins to the cytosol of cultured cells and reports of profound cellular changes caused by liposomes prompted alternative approaches to the study of cellular protein degradation. The results are discussed in the next section.
PART II. INTRACELLULAR PROTEIN TURNOVER

The unsuitability of liposomes, either PS-LUV prepared from Ca$^{++}$-PS, PS/PC-LUV prepared by freeze-thawing (Thyagarajan, 1985), or PC/LL/SA-SUV, as vehicles for the microinjection of radiolabeled proteins directly into the cytosol of cells prompted a reexamination of cellular protein turnover in cultured fibroblasts using a standard method. In this procedure, cellular proteins were labeled by preincubating fibroblasts with $[4, 5-^3H]$ L-leucine for 40-48 hours. Protein degradation was then studied by monitoring the release of TCA/PTA-soluble and -insoluble radioactivity into the culture medium. This study differed from a number of earlier studies of protein turnover in cultured cells in that I monitored the appearance of products over a longer time period, encompassing as much of the reaction as feasible. In addition, in analyzing the data I have attempted to account for the observation that, up to 75% of the radioactivity released by the cellular monolayer appears as TCA/PTA-insoluble material, depending on conditions. This is presumed to be undigested or incompletely degraded cellular proteins released by cell leakage, cell death, and/or exocytosis.

Although rate constants were calculated from the amount of substrate remaining in the cellular monolayer at each time point, what was actually measured was the amount of TCA/PTA-soluble and TCA/PTA-insoluble products released into the culture medium. It was therefore necessary to convert products released to substrate remaining. This was done with the aid of the conservation equation: 

$\text{(substrate remaining at time } t') = (\text{total substrate}) - (\text{products released at time } t')$. Total substrate was calculated at the end of
the experiment with a second conservation equation: \((\text{total substrate}) = (\text{substrate remaining in the monolayer}) + (\text{total products released by the end of the experiment})\).

This analysis was complicated by the observation that the labeled proteins in the cellular monolayer gave rise to two products, TCA/PTA-soluble and TCA/PTA-insoluble radioactivity. For parallel (competitive) first order reactions which convert substrate to two different products, it has been shown that rate constants can be calculated by analyzing the appearance of each product separately (Buktenica et al., 1987). For this, it is necessary to predict the amount of radioactivity remaining in the cellular monolayer at the end of the experiment which is destined to give rise to the TCA/PTA-soluble product and the amount which will appear as TCA/PTA-insoluble material. This is accomplished by calculating \(R_2\), the ratio of the rates of TCA/PTA-soluble to total products released from the data obtained in the time period immediately preceding the termination of the experiment. The amount of cellular radioactivity remaining in the monolayer at the end of the experiment which is destined to give rise to the TCA/PTA-soluble product is \(R_2 \times [\text{CPM}]_{\text{monolayer}}\). For the TCA/PTA-insoluble product the corresponding value is \((1 - R_2) \times [\text{CPM}]_{\text{monolayer}}\). The validity of this method and the "goodness" of the calculated value of \(R_2\) is strongly inferred from the observation that identical rate constants were obtained for the appearance of both products, which is exactly what is expected for parallel first order reactions (Buktenica et al., 1987; Frost & Pearson, 1961).

The source of the TCA/PTA-insoluble radioactivity released by metabolically-labeled fibroblasts is of considerable interest, as it contributes 25 to 75% of the total
products released by these cells depending on the experimental conditions. This TCA/PTA-insoluble material could represent extracellular matrix proteins which are synthesized and secreted by fibroblasts. (Clark, 1993). It is possible that tritiated leucine, arising from the degradation of cellular proteins, is continually reincorporated into secretory proteins during the chase in unlabeled medium, giving rise to a continued release of TCA/PTA-insoluble material. Extensive reutilization of tritiated leucine would also result in a considerable underestimation of the observed rate constants for cellular protein turnover. However, when the intracellular pool of tritiated leucine was diluted by the addition of unlabeled leucine to the medium, the relative amount of TCA/PTA-insoluble products released (given by \(1 - R_T\)) was not decreased, and \(k_{\text{obs}}\) for the appearance of TCA/PTA-soluble products was not increased (Table 11 and Figures 12-14). In addition, concentrations of cycloheximide which inhibited protein synthesis by 90% (Table 12) failed to decrease \(1 - R_T\) or increase \(k_{\text{obs}}\). Instead, \(k_{\text{obs}}\) was actually decreased by 50% (discussed later). Thus, no evidence could be obtained that tritiated leucine reutilization was significant under the experimental conditions employed. Thus, the source of the TCA/PTA-insoluble radioactivity released by fibroblasts during the slow phase could not be due to labeled secreted proteins whose syntheses reutilized tritiated leucine generated during the chase in unlabeled medium.

Two additional sources have been considered for the TCA/PTA-insoluble radioactivity released during the slow phase of cellular protein catabolism. These are cell disruption (e.g. cell detachment, cell leakage and/or cell death) (Models 1 and 3), and
exocytosis (regurgitation) of cellular proteins following their transfer to lysosomes (Buktenica et al., 1987) (Model 2). Models 1 and 3 agree with respect to the source of TCA/PTA-insoluble radioactivity (disruption/leakage) but disagree with respect to the site of cellular protein turnover. Model 1 presumes that cellular proteins are primarily degraded in lysosomes, whereas Model 3 supposes that the majority of cellular proteins are degraded by extralysosomal proteases. Possible candidates for extralysosomal degradative systems would include: (1) a high molecular weight oligomeric protein particle, which is composed of several distinct proteolytic activities (Bond & Butler, 1987), calcium-dependent proteinases or calpains which have been found in association with the soluble cytosolic fraction of cells (Bond & Butler, 1987), and a ubiquitin-independent proteolytic system which has been documented in rabbit reticulocytes and in yeast (Hersko & Ciechanover, 1982; Bachmair et al., 1986), that catalyzes the polyubiquitinylation of proteins prior to their degradation (Bond & Butler, 1987). Proteolytic and peptidyl-lytic activities have also been reported in association with ribosomes (Archambault de Vencay et al., 1991), endoplasmic reticulum (Urade & Kito, 1992), the Golgi apparatus (Amitay et al., 1992), mitochondria (Desautels, 1992), and the nucleus (Rivett et al., 1992). Models 1 and 2 agree with respect to the site of degradation of cellular protein—the lysosome—but disagree with respect to the source of TCA/PTA-insoluble products (leakage as opposed to regurgitation of cellular proteins from lysosomes). Not unexpectedly, the meaning of the rate constants calculated from the time course of release of TCA/PTA-soluble and -insoluble products depends on which model
is being considered. Before discussing the predicted consequences of each of these models, I will describe how the individual rate equations for each of these models were derived.

For Model 3, which presumes that proteins are degraded within the cellular compartment in which they initially reside, no additional assumptions are required to derive the respective rate equations for the appearance of TCA/PTA-soluble and -insoluble products. For Models 1 and 2, the additional assumption is made that a steady state exists between the rate of transfer of substrates into the lysosome and the rate of substrate degradation and egress from the lysosome. Consequently, after an initial pre-steady state period following some perturbation, the amount of cellular substrates within the lysosome \( (P_3') \), and thus the lysosomal space (volume), approaches a new constant level which remains unchanged over time. Support for this assumption has come from studies of protein degradation in perfused livers from rats and mice (Mortimore et al., 1983; Mortimore & Ward, 1981; Schworer et al., 1981). Thus, under conditions of basal proteolysis in liver, the lysosomal space and the fraction of cellular protein substrates residing in the lysosome is about 2% of the total. On removing essential amino acids from the liver perfusate, there is a significant increase in the rate of cellular protein degradation, which is accompanied by expansions of the lysosomal space and the lysosomal protein substrate pool to new values corresponding to about 7% of the total. On refeeding, the lysosomal space immediately shrinks to the initial value of 2%. Partial hepatectomy, which results in a dramatic decrease in protein turnover in perfused
liver, results in a decrease in the lysosomal space and lysosomal substrate pool to about 0.2% of the total cellular space and 0.2% of the total cellular substrate pool (Scornik & Botbol, 1976; Swick & Ip, 1974). Furthermore, a direct linear relationship was observed between the volume of the lysosomal space and the rate of cellular protein catabolism in liver, which extrapolated to zero space at zero proteolysis. In addition, it was observed that the rate of protein degradation in isolated lysosomes was always much greater than the overall rate of cellular protein degradation in rodent liver under all conditions. From these observations, it can be concluded that a steady state exists between protein uptake and degradation in lysosomes, with transfer to lysosomes being the rate-determining step in cellular protein catabolism in the perfused rodent liver (Mortimore & Ward, 1981).

A consequence of the steady state assumption in deriving the rate equations for Models 1 and 2 is that protein transfer to lysosomes, kT, becomes rate-determining; \( k_{obs} = k_T + k_L \) for Model 1 and \( k_{obs} = k_T \) for Model 2. Thus, both models for lysosomal degradation predict that the rate constant, \( k_{obs} \), corresponds to the disappearance of the extralysosomal substrate pool, \( P_3 \), rather than the disappearance of the lysosomal substrate pool, \( P_3' \).

A variety of evidence support the conclusion that proteolytic steps in the lysosome are not rate determining during the degradation of cellular and extracellular proteins. Dunn et al. (1980) has reported that the rate-limiting step in the degradation of asialoglycoproteins in the perfused rodent liver is their transfer from a pre-lysosomal compartment to lysosomes. Work in our laboratory to characterize endocytosis in human
fibroblasts has revealed a degradative compartment with the capacity to convert 20 to 50% of an endocytosed protein to TCA/PTA-soluble products with half-lives of 5 to 10 minutes. In contrast, the half-lives of the majority of cellular proteins in fibroblasts from my present work is about 5 days. Thus, the rate of lysosomal protein degradation could be nearly 1000 times faster than the rates of turnover of a large proportion of cellular proteins in fibroblasts. For the slow phase of cellular protein turnover to involve the participation of the same vacuolar degradative compartments as in endocytosis (Models 1 and 2) then the rate-determining step in cellular protein catabolism must come prior to peptide bond hydrolysis in the endosomal/lysosomal system.

Although Models 1 and 2 both predict that the transfer of substrates to lysosomes makes a major contribution to the observed rate constant for the appearance of TCA/PTA-soluble and -insoluble radioactivity, $k_{obs} = k_T + k_L$ (Model 1) and $k_{obs} = k_T$ (Model 2), they disagree as to the meaning of $R_2$, the ratio of TCA/PTA-soluble radioactivity to total radioactivity released by the cellular monolayer during the slow phase of protein catabolism. For Model 1, $R_2$ is the fraction of the cellular protein which is degraded, $1 - R_2$ is the fraction of the cellular protein which escapes degradation by leakage or other disruptive processes, and $R_2/(1 - R_2) = k_T/k_L$, the ratio of the rate constants (or rates) for transfer of proteins to lysosomes and leakage of proteins from cells. For Model 2, however, $R_2$ describes the partition of substrates within the lysosome, $P_3'$, between degradation and regurgitation. Thus, $R_2/(1 - R_2)$ gives directly $k_D/k_R$. Models 1 and 2 can therefore be distinguished by examining the effects of
lysosomal proteinase inhibitors at concentrations known to inhibit the degradation of endocytosed proteins. From Tables 11, 15, 18 and 20, it can be seen that leupeptin, antipain and pepstatin have no effect on the values of $R_2$ and $R_2/(1 - R_2)$ either under basal conditions or conditions of serum and amino acid deprivation. At the concentrations in which these agents were employed, substantial inhibitions of lysosomal cysteine or aspartic acid proteinases were expected (Choudhuri et al., 1992; Buktenica et al., 1987). The failure to observe a decrease in the values of $R_2/(1 - R_2)$ in the presence of these agents argues strongly against Model 2.

Other evidence for models where cell disruption or leakage is the source of TCA/PTA-insoluble radioactivity released into the culture medium is the finding of similar radiolabeled protein profiles in cell lysates and in the culture medium conditioned by the cells. Thus, nearly identical patterns of protein bands were observed in cells prelabeled for 42 hours with $^{35}$S-methionine and in the culture medium collected after the 42 hour labeling period (Figures 37 and 38, Table 25). This is exactly what one would expect for cell leakage. Were cellular proteins sequestered into a degradative compartment prior to their exocytosis (Model 2), then one might have expected to see released into the medium decreased amounts and numbers of high molecular weight proteins and increased amounts and numbers of lower molecular weight protein degradation products. Second, measurements of the fractional rate of release of LDH, a presumptive marker for cell leakage (Buktenica et al., 1987), gave leakage rates which were similar to those observed for the release of TCA/PTA-insoluble radioactivity into
the culture medium. Finally, the large increase in the amount of TCA/PTA-insoluble material released into the medium under conditions of serum and amino acid deprivation was accompanied by the recovery of significant amounts of radioactivity in the pellets obtained after low and high speed centrifugation of the medium. This was consistent with disruptive processes leading to cell detachment and release of particulate/vesicular structures under conditions of nutritional stress. Thus, of the three models which we have described, only Models 1 and 3—which identify cell leakage and/or other disruptive processes as the cause of the release of TCA/PTA-insoluble radioactivity into the culture medium during the slow phase of protein catabolism—seem to best explain the data. Consequently, further discussions will be largely confined to these two models.

A remarkable finding in this work is the complete insensitivity of cellular protein turnover to inhibitors of lysosomal proteinases. Although the intracellular concentrations of leupeptin, pepstatin and antipain cannot be easily determined, their concentrations in the medium were sufficient to have produced substantial inhibition of their target enzymes. Thus, leupeptin concentrations of 20 µM and 200 µM were 4000 and 40,000 times higher than the reported Kᵢ for leupeptin inhibition of cathepsin B (Schultz et al., 1989). Similarly, the Kᵢ for pepstatin inhibition of cathepsin D is reported to be 0.5 nM, and saturated concentrations of pepstatin were previously shown to inhibit the lysosomal degradation of endocytosed BSA by 50% in mouse peritoneal macrophages (Buktenica et al., 1987).

One explanation which can be proposed for the failure to see inhibition of
intracellular protein turnover by inhibitors of lysosomal proteinases is that lysosomes are not participants. Instead, protein degradation occurs in an unidentified degradative compartment by proteinases which are insensitive to the inhibitors which I employed, or degradation occurs within the same compartment (Model 3) in which the proteins initially reside by enzymes insensitive to these same agents (e.g. cytosolic proteins are degraded by inhibitor-insensitive cytosolic proteinases). By this reasoning, the calcium-dependent extralysosomal proteinases, calpains, may not be involved in cellular protein catabolism as they are leupeptin inhibitable. However, even for Model 3 where $k_{\text{obs}} = k_D' + k_L$ and $k_D'$ can be obtained explicitly, peptide bond hydrolysis may not occur in the rate determining step. Instead, $k_D'$ could represent a step in which the substrate undergoes a physical or chemical modification prior to degradation (Bachmair et al., 1986; Bond & Butler, 1987; Hershko & Ciechanover, 1982). In such a case, the rate determining step might be associated with a regulatory function of the proteolytic system, or a step associated with the assembly of a multiprotein degradative complex (Rechsteiner et al., 1984). Then, proteinase inhibitors could have little effect on $k_{\text{obs}}$. Arguing against Model 3 as an exclusive explanation is the observation of a marked sensitivity of the slow phase of protein catabolism to the lysosomotropic amines, $\text{NH}_4\text{Cl}$ and chloroquine.

A second explanation for the lack of inhibition by proteinase inhibitors is that protein degradation occurs within the lysosome (Model 1), but that peptide bond hydrolysis is not rate limiting. It has previously been proposed that the rate controlling step in lysosomal proteolysis is the ill-defined "sequestration of cellular proteins into
lysosomes or autophagic vacuoles" (Mortimore & Ward, 1981; Katunuma & Kominami, 1983; Bond & Aronson, 1983). In addition, lysosomal proteinase activity is notoriously difficult to abolish, even when using a combination of inhibitors (Ahlberg et al., 1985). This may be due to the fact that lysosomal proteinases are present in excess (Katunuma & Kominami, 1983), or else the lysosome contains a wide diversity of proteinase and peptidase activities with differing inhibitor specificities so as to make their complete inhibition difficult to accomplish. Thus, even were the individual inhibitors capable of producing a significant reduction in the activity of its target proteinases, \( k_D \) could still be one or more orders of magnitude greater than \( k_T \). A sample calculation may suffice to illustrate this point. Frankfater (1988) has observed that a major component of the endosomal/lysosomal system in fibroblasts is capable of degrading HSA to TCA/PTA-soluble products with a half-life of about 10 minutes. Mortimore and Ward (1981) have reported that lysosomes from rat liver labeled with \(^{14}\text{C}\) valine release TCA/PTA-soluble products in vitro with a half-life of about 30 minutes. Estimates of lysosomal proteolysis in rat liver in vivo from the rates of regression of the volume of autophagic vacuoles on refeeding yielded half-lives of about 8 minutes (Mortimore & Ward, 1981). Using the half-life for the slow phase of cellular protein turnover to calculate the rate of transfer of substrate to lysosomes yields a half-life of 7780 minutes (5.4 days) in this present work. \( P_3/P_3 \) can then be calculated \((k_T/k_D)\) to be 0.1 to 0.3%, which is similar to the fractional lysosomal space in rodent liver following partial hepatectomy. In addition, Steinman et al. (1983) have reported a value of about 0.5% in murine fibroblasts
(L-cells) by stereologic analysis. A 50% inhibition of lysosomal proteolysis, as seen with saturating concentrations of pepstatin in mouse macrophages (Buktenica et al., 1987), would result in an expansion of the initial substrate pool to 0.2 to 0.6% (or 1% based on the initial value reported for L-cells) without any discernable decrease in the overall rate of cellular protein turnover. If fibroblasts were able to tolerate an expansion of the lysosomal space to 7-10% of the cellular volume without impairing $k_T$, then agents which produce a 93 to 99% inhibition of lysosomal proteolysis might still not produce a detectable inhibition of intracellular protein turnover.

Based on the preceding discussions, it should be clear that the failure of the lysosomal proteinase inhibitors--leupeptin, pepstatin and antipain--to decrease $k_{obs}$ for the appearance of TCA/PTA-soluble products in metabolically-labeled fibroblasts may be in agreement with Model 3, which identifies inhibitor-insensitive extralysosomal degradative systems as primarily responsible for cellular protein turnover. This lack of inhibition is also not inconsistent with Model 1 under conditions where the rate determining step in cellular protein turnover is the transfer of substrates to lysosomes. In contrast, the failure of these inhibitors to alter the ratio $R_2$ is inconsistent with Model 2 which explicitly identifies $R_2$ as being equal to $k_D/k_R$. Inhibition of the slow phase of protein degradation by the lysosomotropic amines, NH$_4$Cl and chloroquine, provides further support for Model 1.

A putative lysosomal pathway of cellular protein turnover might be expected to include one or more of the following steps: (1) sequestration of cellular proteins in
vacuoles derived from existing membrane elements such as the endoplasmic reticulum, the Golgi apparatus, or components of the endosome/lysosome system; (2) transport of the newly formed vesicles from their site(s) of formation to the lysosome-rich perinuclear region of the cell; and (3) fusion of these vacuoles with newly formed or preexisting lysosomes. Similar kinds of steps have been observed during the degradation and endocytosis of extracellular proteins. Endosomes formed by invagination of the plasma membrane at the cell periphery migrate toward the perinuclear or trans-Golgi region and fuse with lysosomes (Gruenberg & Howell, 1989). Directed movement of membrane-bound vesicles and membrane-vesicle fusion have also been observed during exocytosis (Burgess & Kelly, 1987), during the biosynthetic transport of membrane proteins and lysosomal enzymes (ibid), and during fibroblast migration as plasma membrane is retrieved from the retreating edge of the cell and is inserted into the leading edge (Singer & Kupfer, 1986). Thus, agents which inhibit endocytosis, exocytosis, or cell migration might also be expected to inhibit cellular protein turnover.

Endocytosis is temperature dependent with a $Q_{10}$ of 2.7 (corresponding to an activation energy, $E_a$, of 17.6 kcal/mol) (Steinman et al., 1974), and fusion of endosomes with lysosomes is almost completely abolished at temperatures less than 18$^\circ$C (Dunn et al., 1980; Wall & Hubbard, 1981). Low temperatures (10-15$^\circ$C) also block the vesicular transport of proteins between the rough endoplasmic reticulum and the Golgi complex (Saraste & Kuisman, 1984; Saraste et al., 1986).

The cytoskeleton of cells has also been implicated in membrane associated
processes. Cytochalasin B, which binds to the "forked end" of actin filaments, prevents microfilament elongation (Sampath & Pollard, 1991). In cell cultures it inhibits endocytosis (Gottlieb et al., 1993), exocytosis, plasma membrane blebbing, and cell migration (Singer & Kupfer, 1986). In addition, cytochalasin B inhibits plasma membrane glucose and glucosamine transport and lysosomal membrane N-acetyl-D-glucosamine and N-acetyl-D-galactosamine transport (Jones et al., 1989). Microtubules radiate from a microtubule organizing center (MTOC) which is adjacent to the nucleus and Golgi complex outward toward the periphery of the cell. Most membraneous organelles are found in association with this microtubular network. These include lysosomes, mitochondria, the Golgi apparatus, secretory granules and the endoplasmic reticulum. Attachments of microtubules to cell organelles appear to be of two types. Static attachments of the endoplasmic reticulum and Golgi apparatus have a structural role in organizing and positioning these elements. Vinblastin and other microtubule depolymerizing agents cause connections between adjacent Golgi stacks to be broken and induce the ER to retract from the cell periphery and to coalesce in the central region of the cell (Singer & Kupfer, 1986; Terasaki et al., 1986). In contrast, mobile organelles such as mitochondria and small cytoplasmic vacuoles have dynamic attachments which permit movements in both directions along microtubule filaments. Colchicine, which also causes depolymerization of microtubules, inhibits such saltatory movements of vesicles in cultured cells (Freed & Lebowitz, 1970; Wang & Goldman, 1978).

In my study, cytochalasin B had virtually no effect on any of the kinetic
parameters for the slow phase of protein turnover in cultured cells (Table 11, part B and Table 15). In addition, cytochalasin B did not inhibit the release of TCA/PTA-insoluble radioactivity during the initial rapid phase (Table 11, part A). This is consistent with the recent consensus that actin microfilaments are not directly involved in vesicle transport in most animal cells (Burgess & Kelley, 1987).

Vinblastine had an unusual effect. It appeared to increase the rates of the fast phase of products released (Table 11). Both (k1)_{sol} and (k1)_{ins} increased by more than a factor of 2. In addition, the pool which gave rise to the fast phase of appearance of TCA-soluble products, P1, was also decreased under these conditions. P2, however, was largely unaffected. The failure of vinblastine to decrease P2, the labeled pool which gave rise to the rapid phase of release of TCA-insoluble radioactivity, may be inconsistent with our identification of P2 as newly synthesized secretory proteins, as inhibition of microtubule assembly would be expected to affect the transport of newly synthesized proteins from trans-Golgi elements to the plasma membrane (Burgess & Kelley, 1987). Therefore, vinblastine should have reduced either P2 or (k1)_{ins}.

The release of products in the slow phase was not inhibited by vinblastine. In fact, there was a small increase in (k2)_{sol} and k_{obs} (Table 11, part B and Table 15), which can be attributed to an increased release of TCA/PTA-insoluble products. This is also reflected in moderate decreases in (P3)_{sol}/(P3)_{total} from control values (Table 11, part B). According to Models 1 and 3, the increase in k_{obs} was entirely due to a 2-fold increase in k1 (Table 19). Increased cell leakage and/or death is certainly plausible, since
vinblastine is cytotoxic. \( k_T \) (Model 1) and \( k_D' \) (Model 3) appeared to be unaffected. Model 2 is again implausible since it attributes the vinblastine effects to an increase in regurgitation from the lysosome or a decrease in degradation in the lysosome. If either Models 1 or 3 are correct, it would appear that vinblastine-sensitive steps are either nonexistent or are not rate-determining for the slow phase of protein turnover in cultured fibroblasts.

The role of microtubules in vesicle transport is not entirely clear. Vinblastine inhibits locomotion in fibroblasts by preventing the directed transport of plasma membrane-derived vesicles from the trailing edge to the leading edge of the migrating cell. However, vinblastine does not block vesicle formation or vesicle-membrane fusion. Instead, vesicle membranes are inserted into the plasma membrane in a random fashion (Singer & Kupfer, 1986). Similarly, microtubule disruption in liver does not block albumin secretion but causes albumin to appear in the bile (Mullock et al., 1980). Thus, an intact microtubule network may not be required for vesicular transport per se, but is required for directional transport. In addition, cell free studies suggest microtubules are not involved in vesicular transport between the ER and the Golgi complex nor between the various cisternal elements of the Golgi stack (Pfeffer and Rothman, 1987). Thus, the failure of vinblastine to inhibit the slow phase of protein turnover does not preclude the participation of lysosomes.

The results in Tables 11, part B, Table 15, part A, and Figure 28 show that intracellular protein turnover is markedly temperature dependent in the fed state. Because
fibroblasts did not tolerate the 26°C incubation, it was necessary to limit the time of exposure to this temperature to periods of 3 hours between 1629 minutes and 1800 minutes and between 3060 minutes and 3240 minutes after the chase in unlabeled medium. From the slope of the lines obtained, \(k_{\text{obs}}\) was found to be reduced to about 2/3 of the control value of 37°C. \(k_T\) (Model 1) and \(k_p'\) (Model 3) were similarly decreased (Table 19). This was similar to the magnitude of the reductions in \(k_{\text{obs}}\), \(k_T\) and \(k_p'\) caused by the lysosomotropic amines, NH₄Cl and chloroquine. In view of the sensitivity of endosome-lysosome fusion and biosynthetic protein transport to decreasing temperature, my results are consistent with the participation of a lysosomal pathway in cellular protein turnover. A further observation is that the decrease in \(k_{\text{obs}}\) at 26°C is due equally to a decrease in \(k_T\) (or \(k_p'\) for Model 3) and \(k_L\) for cellular protein leakage. Consequently, the ratio \(R_2\) is essentially unaffected by temperature. As cellular protein leakage and intracellular vesicle formation (substrate sequestration step in Model 1) and vesicle fusion may all involve similar kinds of transient discontinuities in membrane structure, it may not be surprising that they show similar temperature dependencies.

Among the most convincing evidence for the participation of lysosomes in intracellular protein turnover is the inhibition caused by lipophilic weak bases such as chloroquine, methylamine, and ammonium chloride. These lysosomotropic agents are in fact acidotrophes, accumulating in acidic cellular compartments such as lysosomes and endosomes in which they act to raise the intravacuolar pH (Ohkuma & Poole, 1978). Buktenica et al. (1987) observed chloroquine at a concentration of 150 µM caused an 88%
inhibition of degradation and a stimulation of regurgitation of endocytosed bovine serum albumin in stimulated mouse peritoneal macrophages. Chloroquine also affects molecular sorting and recycling in endosomes (Tietze et al., 1980). Acidotropic agents and proton ionophores also exert effects at the trans-most cisterna of the Golgi stack, causing dilation of the Golgi cisterna and Golgi-associated vesicles (Millman et al., 1986). As a consequence of these effects, proteolytic processing of lysosomal enzymes is inhibited and their delivery to lysosomes blocked (Nishimura et al., 1988). In mouse 3T3 fibroblasts, latent precursor forms of cathepsins B and L accumulate intracellularly, and are secreted in large quantities into the culture medium (Achkar et al., 1990). Prolonged incubation of fibroblasts with chloroquine produces an increased vacuolization of the cell (possibly through vacuolar dilation) and profound deficiencies in the levels of lysosomal hydrolases. Removal of chloroquine results in cellular recovery of lysosome function (Wiesmann et al., 1975).

Despite the profound lysosomal dysfunction likely to be induced by chloroquine and other weak bases, these agents were incapable of completely abolishing intracellular protein turnover. Inhibitions of cellular protein degradation of 40 to 70% have also been reported by others, depending on the tissue or cell and its metabolic state (Knowles & Ballard, 1976; Dean, 1979; Hapgood et al., 1977; Seglin et al., 1979; Amenta et al., 1979; Poole & Wibo, 1973). The incomplete inhibition of intracellular protein turnover afforded by weak bases has often been cited as evidence for the participation of extralysosomal proteolytic enzyme systems in intracellular protein turnover (Seglen et
In this present study, chloroquine and ammonium chloride produced, respectively, a 20% and a 24% decrease in $k_{obs}$ for the appearance of TCA/PTA-soluble and -insoluble radioactivity. However, the decrease in $k_{obs}$ was partially masked by a modest increase in the rate of leakage of proteins from the cells, as reflected in a substantial decrease in $R_T$ from the control value of about 0.7 to 0.45 and 0.57 in the presence of chloroquine and ammonium chloride. Recalling that $k_{obs} = k_T$ (or $k_{D'}$ for Model 3) + $k_L$, it is possible to calculate from the experimental value of $(P_3)_{sol}/(P_3)_{total}$ that chloroquine and ammonium chloride produced a 50% and 36% inhibition of $k_T$ (Model 1) or $k_{D'}$ (Model 3) and a 48 and 8% increase in leakage ($k_L$), respectively. From this result, it seems reasonable to conclude that a lysosomal degradative pathway contributes 35 to 50% to the turnover of cellular proteins in fibroblasts in the fed state, as it seems less likely that chloroquine and ammonium chloride would affect extralysosomal protein degradation occurring in a pH-neutral compartment.

The lack of effect on cellular protein turnover of lysosomal proteinase inhibitors and the incomplete inhibition caused by chloroquine prompted an examination of cellular protein degradation in the absence of serum and an essential amino acid. It has been well established that protein/amino acid deprivation causes a marked increase in cellular protein turnover and in the size of the lysosomal substrate pool in perfused rodent liver (Schworer et al., 1980; Mortimore & Ward, 1981). Serum and amino acid deprivation has also been reported to result in an increase in intracellular protein degradation in
cultured rat fibroblasts (Poole et al., 1978; Poole, 1975; Poole & Wibo, 1973) and in cultured hepatoma cells (Epstein et al., 1975; McIlhinney & Hogan, 1974). Furthermore, it has been suggested that lysosomes are responsible for the enhanced rate of cellular protein turnover under conditions of nutritional deprivation (Mortimore & Ward, 1981; Dean, 1975). Consequently, I had expected to see an increased sensitivity of protein degradation to lysosomal proteinase inhibitors and chloroquine in the absence of serum and amino acids.

In contrast to expectations, the combined effects of amino acid deprivation and protein starvation caused a 55-60% decrease in $k_{obs}$ and large decreases in $(P_3)_{solv}/(P_3)_{total}$ (Table 14, part B and Table 15, part B). $k_T$ (Model 1) or $k_D'$ (Model 3) was inhibited 85-90% (Table 20). The reason for the differences between my results and those of Poole et al. (1978), who reported that degradation of long-lived endogenously-labeled proteins in rat lung fibroblasts more than doubled in the absence of serum, is presently unclear. The results may reflect differences in short-term (Poole et al., 1978) and long-term (my results) responses to nutritional deprivation. Differences between my results and those of Mortimore and Ward (1981), who observed increased protein catabolism in rat liver, could be explained in terms of the role of liver in amino acid homeostasis. Thus, amino acid and protein deprivation could be expected to increase liver protein degradation to mobilize amino acids for release into the blood. Fibroblasts, which are not known to function in the same manner as liver in this regard, might be expected to respond to long-term amino acid and serum deprivation by reducing
protein turnover in order to conserve cellular proteins. Interestingly, my results show that the addition of 1% serum to the media is not sufficient to restore $k_{obs}$ to the control values in the fed state. However, when the medium was supplemented with 1 mM leucine in addition to 1% serum, $k_{obs}$ for the slow phase of protein turnover was restored to the value seen in the fed state. Of significant interest is the finding that both nutritional deprivation and cycloheximide cause a significant inhibition of cellular protein turnover in fibroblasts. As amino acid and serum deprivation, like cycloheximide, is expected to inhibit protein synthesis, these results imply that protein synthesis and protein degradation in fibroblasts are reciprocally and coordinately regulated.

Inhibition of protein degradation by inhibitors of protein synthesis has been reported previously in perfused liver (Pastan et al., 1975), liver slices (Simpson, 1953), and hepatoma cells (Epstein et al., 1975) maintained in vitro in medium lacking insulin or serum. Under such conditions, protein degradation is accelerated above basal levels, and inhibitors of protein synthesis act to reduce protein degradation rates to basal levels. Thus, it appears that the behavior of human fibroblasts is fundamentally different from that of liver. In liver, nutritional deprivation produces enhanced proteolysis which is inhibitable by inhibitors of protein synthesis, while under basal conditions liver protein degradation is insensitive to cycloheximide and puromycin. In fibroblasts, however, basal proteolysis is sensitive to inhibition by cycloheximide (49-69%), and nutritional deprivation brings about a decrease in intracellular protein turnover (84-85%). The decrease in the rate of cellular protein turnover caused by serum and amino acid
deprivation was probably not due to a decrease in lysosomal proteases, as cathepsin B levels were only slightly (18%) decreased (Table 28).

From Table 20 it is also apparent that amino acid and serum deprivation produced a 30% increase in the rate of leakage or cellular disruption. This was accompanied by an increase in the amount of radioactivity in the 14,000 x g and 109,000 x g pellet following centrifugation of media samples. The proportion of radioactivity in these pellets was much greater during day 3 than during day 1, indicating that leakage and/or disruption became more severe the longer the fibroblasts were exposed to serum and amino acid deprived conditions. As noted earlier, these results could not be explained by Model 2.

Antipain and pepstatin A in the fasted state produced increases in k_{obs} for the slow phase (Table 15, Part B). These increases were principally due to increases in cell leakage or death (Table 20). However, k_T (Model 1) or k_D' (Model 3) were largely unaffected (Table 20).

Chloroquine caused a more substantial increase (100%) in k_{obs} for the slow phase release of products in the absence of leucine and serum (Table 15, Part B). This was principally due to an increase in the release of TCA-insoluble radioactivity. According to Models 1 and 3, this was principally due to a 2-fold increase in the leakage rate constants k_L, which is easily explained by the cytotoxic nature of this amine. However, k_T (Model 1) or k_D' (Model 3) was, if anything, slightly increased. Thus, protein turnover in the absence of serum and amino acids is completely insensitive to inhibition by acidotropic agents, suggesting that lysosomes are not the site of cellular protein degradation under
such conditions.

The serum and amino acid deprived cells tolerated the 26°C incubation better than the serum-supplemented cells. It is proposed that the deprived state may induce changes in cellular physiology which render the cells more resistant to lower temperatures. For Models 1 and 3, the 27% decrease in $k_{obs}$ seen at 26°C in the fasted state was due almost entirely to a decrease in leakage, $k_L$ (Table 20). Decreased leakage was also noted in serum-supplemented conditions at 26°C (Table 19). What differs between the results obtained in the presence and absence of serum is that serum-supplemented cells show marked decreases in cellular protein turnover ($k_T$ or $k_D'$) at 26°C, whereas in the absence of serum and amino acids $k_T$ (Model 1) or $k_D'$ (Model 3) are, if anything, increased at 26°C from serum and amino acid deprived controls. Thus, the slow phase of cellular protein degradation was not inhibited when fibroblasts were transferred from 37°C to 26°C under starvation conditions, in contrast to results obtained in the fed state.

It is proposed that there are 2 proteolytic systems which participate in the turnover of long-lived proteins (slow phase) within fibroblasts; one system is inhibitable by decreasing temperatures between 37 and 26°C, while the other is largely unaffected by this transition. In the deprived state, only the system unaffected by decreasing temperature is operable. The observation that chloroquine did not inhibit degradation in deprived conditions indicates that the temperature-insensitive system is non-lysosomal. In contrast, the chloroquine sensitivity of degradation in the fed state suggests that the
temperature dependent pathway is lysosomal.

The results obtained with serum-supplemented cells suggest that Model 1 may account for 30 to 50% of the degradation of long-lived proteins. Model 3 is less consistent because it does not account for the decrease in degradation effected by the lysosomotropic amines. When examining the results collected from serum and amino acid deprived cells, however, Model 3 is more consistent. Chloroquine did not decrease degradation during the slow phase; in fact, there was a small increase in $k_T$ (Model 1) or $k_D'$ (Model 3). Also, at 26° C, there was no concomitant decrease in $k_T$ or $k_D'$ as compared with serum-supplemented conditions (Table 20). Therefore, it appears that there are at least two degradative pathways responsible for the slow phase of protein turnover in fibroblasts:

1. A system which functions in a pH neutral compartment (non-lysosomal), is temperature-insensitive between 26-37° C, and predominates in serum and amino acid deprived conditions.

2. A lysosomal system which increases in rate between 26-37° C and which contributes 50% or more to the slow phase of protein degradation in the fed state.

Thus, the proposed model for protein turnover under normal culture conditions is a combination of Models 1 and 3 (Figure 39). In the fed state, both models are probably operative, with the lysosomal pathway making a significant contribution to protein degradation (53% based on chloroquine sensitivity, 37% based on temperature dependency, and 82% based on sensitivity to serum and amino acid deprivation) such that
Figure 39. Illustration for the proposed model of the metabolism of cellular proteins in fibroblasts. P₁, P₂, kD and kS are explained in Figure 18. In the fed state, proteins in P₃ may be transferred (kT) to the lysosomes (P₃''), where they are degraded (kD''), degraded by a non-lysosomal degradative system (kD'''), or leaked from the cell (kL). Under conditions of serum and amino acid deprivation, kT approaches 0.
kobs for the slow phase is equal to $k_T + k_D' + k_L$. Under deprived conditions, the lysosomal pathway is non-operative, such that $k_T$ approaches 0 and $k_{obs} = k_D' + k_L$ (Model 3).

It is probable that the compartmentalization of cellular proteolysis is tissue-specific. In rat liver, the lysosomal substrate pool is largest during starvation and smallest during the fed state (Mortimore & Ward, 1981). Our results in human fibroblasts indicate that lysosomal degradation is minimal during prolonged starvation. However, fibroblasts and hepatocytes have different functions within the intact organism; therefore, it should not be surprising that they respond differently to a particular stimulus (starvation). In addition, when working with tissue culture, it must be remembered that results cannot be interpreted too generally. For example, results obtained with an intact organ (e.g., liver) may differ somewhat from those obtained with cultured cells (hepatocytes), because organs are generally composed of several cell types which interact, and because the culture medium may not replicate the extracellular fluids which bathe the intact organ.
CHAPTER V

SUMMARY

Large unilamellar liposomes (LUV) were successfully prepared from the calcium salt of phosphatidylserine (PS) as putative vehicles for the injection of labeled proteins into the cytosolic compartment of fibroblasts. Methods were developed to quantify liposome-cell fusion which did not rely on cellular biological responses. My studies concluded that PS-LUV do not fuse with cellular membranes, but instead adhere tightly to the cell surface.

Since our PS-LUV preparations were unsuitable for microinjection, intracellular protein degradation was investigated using proteins metabolically-labeled with \([4, 5-^3\text{H}]\)-L-leucine. Both TCA-soluble and -insoluble products were recovered from the culture medium, and their appearance was monitored over time. The release of both products occurred in 2 phases. Approximately 10% of the labeled protein products were released in the fast phase, which was largely completed within 5 hours. The remaining labeled proteins were released in a slow phase with a half-life of approximately 5.4 days.

TCA-insoluble radiolabeled products released into the cell culture medium were similar in composition to whole cell extracts, as revealed by electrophoretic analysis. This suggested that these products resulted from cellular leakage or cellular dissolution. Addition of inhibitors of protein synthesis did not affect the release of TCA-insoluble
products, thereby precluding the possibility that TCA-insoluble radioactivity resulted from the reincorporation of labeled amino acids into secretory proteins. In addition, centrifugation of the culture medium revealed that dead or detached cells accounted for only a small fraction of the TCA-insoluble products released.

Our results show that at least 2 pathways exist for cellular protein turnover in fibroblasts, one inhibited at 26° C and the second, a temperature-insensitive system. The temperature-sensitive pathway presumably involves lysosomes, is inhibited by lysosomotropic amines, is more prominent under control ("fed") conditions, and is responsive to changes in protein synthesis. The temperature-insensitive pathway is dominant under deprived conditions, and is presumably nonlysosomal because it is largely unaffected by acidotropic amines known to inhibit lysosomal protein degradation.
APPENDIX

DERIVATIONS OF THE RATE EQUATIONS FOR THREE MODELS OF CELLULAR PROTEIN METABOLISM

Definition of Rate Constants

\[ k_D = \text{degradation rate constant for proteins in pool 1} \]
\[ k_S = \text{exocytosis rate constant for proteins in pool 2} \]
\[ k_T = \text{rate constant for transfer of protein in pool 3 to lysosomes} \]
\[ k_D' = \text{degradation rate constant for proteins in pool 3} \]
\[ k_L = \text{rate constant for cellular leakage of proteins in pool 3} \]
\[ k_R = \text{rate constant for regurgitation of proteins in pool 3} \]

Model 1

In this model a fraction of newly synthesized proteins may undergo rapid degradation (pool 1) or secretion (pool 2). A third pool of cytosolic proteins (pool 3) may slowly leak from the cell or may be slowly transferred to lysosomes (pool 3') where they are degraded.

\[ P_1 = (P_1)_0 e^{-k_D t} \]
\[ P_2 = (P_2)_0 e^{-k_S t} \]
\[ P_3 = (P_3)_0 e^{-(k_T + k_L) t} \]

\[ \frac{dP_3'}{dt} = 0 = k_T P_3 - k_D' P_3' \]

Assuming
\[
\text{then } P_3' = \frac{k_T P_3}{k_D'}
\]

\[
\frac{d}{dt} (TCA)_{ins} = k_S P_2 + k_L P_3
\]

\[
\frac{d}{dt} (TCA)_{sol} = k_D P_1 + k_D' P_3' = k_D P_1 + k_T P_3
\]

\[
\frac{d}{dt} (TCA)_{ins} = k_S (P_2)_0 e^{-k_S t} + k_L (P_3)_0 e^{-(k_L + k_T) t}
\]

Integrating yields:

\[
(TCA)_{ins} = -(P_2)_0 e^{-k_S t} - \frac{k_L}{k_L + k_T} (P_3)_0 e^{-(k_L + k_T) t} + C
\]

The constant can be evaluated at the limit of \(t=0\), where \(e^{-k_{obs} t} = 1\).

\[
\text{then } C = (P_2)_0 + \frac{k_L (P_3)_0}{k_L + k_T}
\]

Hence:

\[
[(TCA)_{ins}]_t = (P_2)_0 [1 - e^{-k_S t}] + \frac{k_L (P_3)_0}{k_L + k_T} \times [1 - e^{-(k_L + k_T) t}]
\]

In this equation, \(P_2)_0 + \frac{k_L (P_3)_0}{k_L + k_T}\) (the constant \(C\)) is equal to the total TCA-insoluble radioactivity released by the end of the reaction, \([(TCA)_{ins}]_\infty\).

\(P_2)_0\) and \(\frac{k_L (P_3)_0}{k_L + k_T}\) can be determined directly from a non-linear regression analysis of progress curves for the release of TCA-insoluble radioactivity obtained by plotting
\([\text{(TCA)}_{\text{ins}}]_\infty - [\text{(TCA)}_{\text{ins}}]_t \text{ vs. time}\)

This procedure also yields two observed rate constants, \(k_S\) and \((k_L + k_T)\).

In a similar fashion, it is possible to demonstrate that

\[
[(\text{TCA})_{\text{sol}}]_t = (P_1)_0 [1 - e^{-k_D t}] + \frac{k_T (P_3)_0}{k_L + k_T} \times [1 - e^{-(k_L + k_T) t}]
\]

again

\[
(P_1)_0 \text{ and } \frac{k_T (P_3)_0}{(k_L + k_T)}
\]
can be determined directly by a nonlinear regression analysis of a plot of \([\text{(TCA)}_{\text{sol}}]_\infty - [\text{(TCA)}_{\text{sol}}]_t \text{ vs. time}\).

This procedure also yields the two rate constants \(k_D\) and \((k_L + k_T)\). Note that the rate constant for the degradation of proteins in the lysosome, \(k_P^T\), is not obtainable from this analysis.

**Model 2**

In this model, cellular leakage is thought to be insignificant, and \((\text{TCA})_{\text{ins}}\) radioactivity arises solely by exocytosis from secretory vacuoles in steps \(k_S\) (pool 2) and regurgitation from the lysosomes in step \(k_R\) (pool 3').

\[
P_1 = (P_1)_0 e^{-k_D t}
\]

\[
P_2 = (P_2)_0 e^{-k_S t}
\]

\[
P_3 = (P_3)_0 e^{-k_T t}
\]

\[
P_3' = \frac{k_T (P_3)_0}{k_D' + k_R}
\]

\[
\frac{d (\text{TCA})_{\text{ins}}}{dt} = k_S P_2 + k_R P_3' = k_S P_2 + \frac{k_R k_T P_3}{k_D' + k_R}
\]

\[
\frac{d (\text{TCA})_{\text{sol}}}{dt} = k_D P_1 + k_D' P_3' = k_D P_1 + \frac{k_D k_T P_3}{k_D' + k_R}
\]
\[
\frac{d (TCA)_{\text{ins}}}{dt} = k_S(P_2)_0 e^{-k_S t} + \frac{k_R k_T}{k_D' + k_R} (P_3)_0 e^{-k_T t}
\]

Integrating yields

\[
[(TCA)_{\text{ins}}]_t = -(P_2)_0 e^{-k_S t} - \frac{k_R}{k_D' + k_R} (P_3)_0 e^{-k_T t} + C
\]

Solving for the constant of integration at \( t = 0 \), where \( e^{-k_{\text{obs}} t} = 1 \) gives

\[
[(TCA)_{\text{ins}}]_t = (P_2)_0 [1 - e^{-k_S t}] + \frac{k_R}{k_D' + k_R} (P_3)_0 [1 - e^{-k_T t}]
\]

Similarly

\[
[(TCA)_{\text{sol}}]_t = (P_1)_0 [1 - e^{-k_D t}] + \frac{k_D'}{k_D' + k_R} (P_3)_0 [1 - e^{-k_T t}]
\]

Again, \( (P_1)_0 \), \( (P_2)_0 \), \( k_D \), \( k_S \), and \( k_T \) can be obtained from the nonlinear regression analysis of plots of \([(TCA)_{\text{sol}}]_\infty - [(TCA)_{\text{sol}}]_t \) vs. time and \([(TCA)_{\text{ins}}]_\infty - [(TCA)_{\text{ins}}]_t \) vs. time.

Again, as for Model 1, the intrinsic degradation and exocytosis rate constants \( k_D' \) and \( k_R \) cannot be determined. However, in this case the ratio \( k_D'/k_R \) can be calculated.

**Model 3**

According to this model, there is no significant protein degradation in the lysosomes. Instead, protein degradation occurs within the cytosol and involves two substrate pools (and possibly two degradative systems). One protein pool \( (P_1) \) is degraded rapidly, while the other \( (P_3) \) is degraded slowly. The differences in degradation rates for these two populations may depend on the presence or absence of protein signals which are recognized by particular cytosolic degradative systems, or may be due to differences in the rates with which proteins in each pool undergo a modification which precedes degradation.
\[ P_1 = (P_1)_0 e^{-k_D t} \]
\[ P_2 = (P_2)_0 e^{-k_S t} \]
\[ P_3 = (P_3)_0 e^{-(k_D + k_D')t} \]
\[ \frac{d (TCA)_{ins}}{dt} = k_S P_2 + k_L P_3 \]
\[ \frac{d (TCA)_{ins}}{dt} = k_S (P_2)_0 e^{-k_S t} + k_L (P_3)_0 e^{-(k_L + k_D')t} \]

Similarly:
\[ \frac{d (TCA)_{sol}}{dt} = k_D P_1 + k_D' P_3 \]
\[ \frac{d (TCA)_{sol}}{dt} = k_D (P_1)_0 e^{-k_D t} + k_D' (P_3)_0 e^{-(k_L + k_D')t} \]

Integrating these equations yields
\[ [(TCA)_{ins}]_t = (P_2)_0 [1 - e^{-k_S t}] + \frac{k_L}{k_L + k_D'} (P_3)_0 x \left[ 1 - e^{-(k_L + k_D')t} \right] \]
\[ [(TCA)_{sol}]_t = (P_1)_0 [1 - e^{-k_D t}] + \frac{k_D'}{k_L + k_D'} (P_3)_0 x \left[ 1 - e^{-(k_L + k_D')t} \right] \]

Again, nonlinear regression analysis of plots of [(TCA)_{ins}]_t vs. time and [(TCA)_{sol}]_t vs. time yields values for
\[ \left( P_1)_0, (P_2)_0, \frac{k_L (P_3)_0}{(k_D' + k_L)}, \frac{k_D' (P_3)_0}{(k_D' + k_L)}, k_D, k_S \text{ and } (k_D' + k_L) \]

The kinetic consequences of Model 3 differ from Models 1 and 2 in that the intrinsic degradation rate constant, \( k_D' \), for the slow phase of release of (TCA)_{sol} radioactivity can now be calculated. However, \( k_D' \) may not correspond to a true degenerative step, but to a step in which a cytosolic protein is first recognized or modified.
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VITA

The author, Sarah Jones Olenick, was born on May 29, 1957, in McLeansboro, Illinois.

At Loyola University Sarah embarked on a program of studies leading to the M.D. and Ph.D. degrees. She entered the Graduate School in the Department of Molecular and Cellular Biochemistry in January, 1983, where she carried out her dissertation research under the direction of Dr. Allen Frankfater. She entered Loyola University Stritch School of Medicine in August, 1983. She received the Doctor of Medicine degree in June, 1989, and was awarded the Loyola University Faculty Award for Excellence in Basic Science. Since July, 1989, Sarah has been a resident in the Department of Pathology at the University of Illinois, and was Chief Resident from 1992 to 1993.

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The Dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Ph.D.

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