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## The Uptake and Release of Label from Endocytosed 35S04-Labeled Proteins in Cultured Mouse Peritoneal Macrophages

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THE UPTAKE AND RELEASE OF LABEL FROM ENDOCYTOSED  
<sup>125</sup>I-LABELED PROTEINS IN CULTURED MOUSE  
PERITONEAL MACROPHAGES

By  
Susan Patterson Buktenica

A Dissertation Submitted to the Faculty of the  
Graduate School of Loyola University of  
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## VITA

The author, Susan Patterson Buktenica, is the daughter of Lester Benjamin Patterson and Edythe Friedman Patterson. She was born December 29, 1950 in Chicago, Illinois.

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This Dissertation is Dedicated to  
My Father

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## LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBZ	Benzyloxycarbonyl
cpm	Counts per minute
EDTA	Ethylenediaminetetraacetate
EI	Endocytic index
FCS	Fetal calf serum
GH	Growth hormone
Hb	Hemoglobin
HSA	Human serum albumin
LH	Luteinizing hormone
MIT	Monoiodotyrosine
NAD	Nicotinamide adenine dinucleotide
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulfate
TAME	Tosyl-arginine-methyl ester
TCA	Trichloroacetic acid
TEMED	N,N,N',N' -Tetramethylethylene diamine
TSH	Thyroid stimulating hormone

## CHAPTER I

### INTRODUCTION

The purpose of this dissertation is to characterize the process by which extracellular proteins are degraded by cultured macrophages. The degradation of extracellular protein occurs within the lysosome. It has been proposed that the lysosome may also be the site of intracellular protein degradation under some conditions. The study of extracellular protein degradation may thus be a model for some steps in intracellular protein turnover. A study of the degradation of extracellular proteins may therefore contribute to an understanding of intracellular protein turnover and may permit choices to be made between several models proposed for the latter process.

I will begin this introduction by reviewing the current literature on intracellular protein degradation, as of 1981, and will present models that have been proposed to explain this process. I will then discuss the degradation of extracellular proteins and how this process may be related to intracellular protein degradation.

### A. Intracellular Protein Degradation

Intracellular protein degradation is believed to serve several important functions in the cell. It can enable the cell to adapt to changes in its environment by facilitating rapid changes in the concentrations of enzymes within the cell. Thus, the more rapidly an enzyme is degraded, the faster its intracellular concentration can respond to changes in its environment (1, 2). In agreement is the observation in liver that enzymes which catalyze either the first or the rate-limiting steps in metabolic pathways have the shortest apparent half-lives (3).

In nutritionally poor environments, protein degradation could enhance cell survival by providing a continued supply of amino acids for protein synthesis and/or to serve as metabolic fuels for ATP formation. During starvation, protein degradation has been found to increase in bacteria (4-11), yeast (12, 13), and certain mammalian cells in culture (14, 15). During fasting by mammals, amino acids derived from cellular proteins in muscle and liver can be oxidized directly or their carbon skeletons can be converted into glucose by the liver and kidney (16).

Another function of protein turnover is probably the removal of abnormal proteins within the cell that might

arise by mutations, errors in gene expression, denaturation, or chemical modification. Mutant proteins, those with a single amino acid substitution, were found to be degraded at a faster rate than normal proteins in cultured mouse L-cells (17). Abnormal proteins created by growing cells in media containing amino acid analogs were degraded more rapidly than normal proteins in bacteria (18, 19), fibroblasts (20), and hepatoma cells (21, 22).

The degradative rates of different enzymes within bacterial and animal cells can vary widely. Heterogeneous rates have been found for membrane and organelle proteins (1, 23) as well as soluble proteins (2). In addition, these rates can vary under different physiological conditions. In most cases, degradation appears to be a first order process indicating that newly synthesized protein is as likely to be degraded as old protein. (2, 24, 25, 26). This suggests that for each individual protein, molecules are selected at random for degradation.

There appears to be a correlation between degradative rates and some physical properties of proteins. In rat liver, soluble and membrane proteins composed of larger molecular weight subunits turned over more rapidly than those with smaller subunits (27-29). This relationship did not hold for serum proteins (30).

However, in the latter case, the proteins must be cleared from the circulation before degradation can occur so that their turnover rate may be controlled by their rate of uptake into tissues. For both serum and tissue proteins a correlation may exist between their isoelectric points and half-lives (30-32), with the more acid proteins being degraded more rapidly. The relationships between degradation rate, subunit molecular weight, and isoelectric point were also found in the mouse blastocyst (33). A relationship may also exist between the hydrophobicity of a protein and its rate of turnover (34, 35) with the more hydrophobic molecules being degraded at a faster rate. It was suggested that this could be due to an increased association of hydrophobic proteins with cellular membranes, leading to a preferential uptake into lysosomes.

Conformation also appears to affect the rate of protein turnover. Thus, it has been suggested that the increased rates of degradation which have been observed for mutant or modified proteins (18-22, 32) may be a consequence of their abnormal and possibly less stable conformations. In bacterial mutants, incomplete chains of  $\beta$ -galactosidase which differ in conformation, as determined by their ability to cross-react with antibody, were degraded at different rates (36). Although a small correlation

existed between chain length and half-life, a much stronger correlation existed between conformation and half-life. Chains with conformations similar to that of the native protein, as determined by antibody cross-reactivity, were degraded more slowly. Additional evidence for the importance of protein conformation on degradative rates was shown by studies of the effects of bound ligands on protein half-lives. Tryptophan pyrrolase (37), serum albumin (38), aspartate transcarbamylase (39), staphylococcal nuclease (40), and ribonuclease (41) were observed to be degraded more slowly upon the binding of specific ligands in vivo. This effect was attributed to changes in the conformation and/or stabilization of the conformation of the proteins caused by ligand binding (42). Similarly, both hemoglobin and glycogen phosphorylase were shown to be degraded more rapidly in vitro on the binding of oxygen (43) and glucose (44) respectively. Thus, the concentration of various ligands within the cell, such as substrates, cofactors, inorganic ions, etc., could regulate the rate of degradation of individual proteins.

One study, however, appears to dispute the effect of conformation on turnover rate (45). Various tyrosine-amino-transferases containing different amino acid analogs were found to be less heat stable and only 2/3 as

catalytically active when compared to the native protein but their turnover rates in rat liver were found to be similar.

The mechanisms by which hormones and metabolites regulate the rates of protein turnover have been investigated in cells in culture, in tissue slices, and in perfused organs. Isolated hepatocytes labeled with  $^3\text{H}$ -amino acids degrade about 4.5% of their cellular protein per hour (46). This degradation was decreased by inhibitors of ATP formation and by the addition of insulin and several amino acids to the medium or perfusate.

The control of protein turnover by insulin, however, may be an involved process since it has been found to increase protein synthesis as well as decrease degradation in tissues (47, 48) and in cultured fibroblasts (49). Insulin appears to antagonize the adenylyl cyclase system, as do oxytocin and prostaglandins, while hormones such as epinephrine, glucagon, ACTH, TSH, LH, secretin, and GH stimulate it (50-57). As will be explained later, cAMP appears to be involved in endocytosis and, in the thyroid, TSH has been found to increase endocytosis (58). In contrast to insulin, glucagon appears to stimulate protein degradation in the liver (59). This effect appears to be

secondary to a glucagon induced depletion in the cellular levels of several free amino acids, notably glutamate (60).

Intracellular protein degradation also appears to be regulated by the availability of ATP. Murakami, et al., (61) looked at the degradation of proteins with abnormal conformations using cell-free extracts from E. Coli and found it to be an energy dependent process. Incomplete fragments of  $\beta$ -galactosidase generated through premature termination of protein synthesis were degraded in this system by a process that depended on a continued availability of ATP. Cell free extracts from E. Coli also degraded  $^{14}\text{C}$ -methylapohemoglobin in an ATP dependent fashion. Other nucleotide triphosphates and ADP were less stimulatory than ATP while AMP, NAD, NADH, guanosine tetraphosphate and  $\alpha$ ,  $\beta$ , and  $\gamma$ -methylene ATP did not afford significant stimulation. The requirement for ATP could not be explained by a requirement for protein synthesis as the stimulation by ATP was not affected by chloramphenicol (62). The pH optimum for this ATP dependent proteolytic activity was 8.0. The authors indicated the need for further studies to explain this ATP requirement.

Kowit and Goldberg (63), in studying the degradation



of a specific mutant  $\beta$ -galactosidase (X-90) in E. Coli, were able to demonstrate that high energy phosphate was required both for the initial proteolytic cleavage as well as for the subsequent hydrolysis of the degradative intermediates.

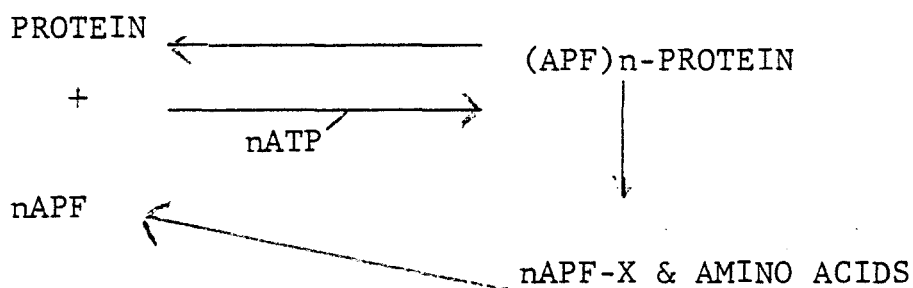
Extracts of E. Coli were purified and this ATP stimulating protease was found to have a molecular weight of 115,000. Reticulocytes also appear to have an ATP dependent protease activity and it appears to be a distinct cellular enzyme of non-lysosomal origins (62). It has an optimal pH of 7.8 and remains in the supernatant even after centrifugation at 100,000g for several hours. In contrast, fractions of cell homogenates enriched in lysosomes had little proteolytic activity at pH 7.8 and showed no stimulation by ATP. The ATP dependent proteolytic activity was not sensitive to the protease inhibitors pepstatin, leupeptin, or chymostatin which will inhibit the lysosomal cysteine proteases.

ATP could stimulate protein degradation by participating directly in the catalytic process. This could involve an ATP dependent transformation of the substrate, thereby increasing its susceptibility to proteolysis. Alternatively, ATP could act to modify the protease

directly by inducing a change in protein conformation, by participating in the reversible chemical modification of the protease, or by participating in the inactivation of an endogenous inhibitor.

In order to determine the chemical basis of the ATP requirement in intracellular protein turnover, Hershko and his associates have recently partially purified and characterized an ATP dependent proteinase system from rabbit reticulocytes (64). They found this system to be composed of several components. One component is a heat-stable polypeptide (APF-1) which has subsequently been identified as ubiquitin (65), a protein discovered earlier in many tissues but whose function was not known. During protein degradation, this polypeptide appears to become covalently linked to the protein substrate in an ATP dependent fashion forming a high molecular weight conjugate. This conjugation reaction is inhibited by N-ethylmaleimide. UTP and GTP could not substitute for ATP in this system. These properties of conjugate formation are similar to those of overall ATP dependent protein breakdown in the same system suggesting that the formation of a substrate-ubiquitin conjugate could be obligatory intermediates in the degradative process. A

mechanism for this process was proposed (66). The scheme is as follows:



Conjugation involves an enzymically catalyzed synthesis of a covalent bond between APF-1 and the  $\epsilon$ -NH<sub>2</sub> group of lysine in polypeptides and proteins. The portion of the APF-1 molecule that is involved in conjugation appears to be the COOH-terminal glycine (153). This enzymic activity, "APF-1-protein amide synthetase," is either processive or has a high preference for protein previously conjugated with APF-1, as derivatives with more than one APF-1 molecule will appear under conditions of excess free substrate protein. ATP is specifically required for this reaction. A reverse reaction, the generation of APF-1 from APF-1 protein, is catalyzed by an amidase. The amidase does not require ATP. (66).

(APF)<sub>n</sub>-Protein is cleaved by peptidases that act on (APF-1)<sub>n</sub> derivatives to yield nAPF-X where X is lysine or a small peptide. The Apf-1 is regenerated by an amidase (66).

It remains to be demonstrated whether such a system operates in eukaryotic cells other than reticulocytes. In addition, studies of the purified ATP dependent proteolytic system from E. Coli suggests that it may be very different from the rabbit reticulocyte system (154, 155).

Poole and Wibo (67) studied protein turnover in rat fibroblasts in culture and found that many cellular proteins could be divided into two categories; those with short half-lives (approximately 1-10 hours) and those with long half-lives. They observed that fluoride, iodoacetate, and chloroquine affected the rates of degradation of both types of proteins to similar extents (68). These compounds all inhibit proteolysis within the lysosome. In contrast, variations in the composition of the culture medium affected the degradation of proteins with long half-lives only. Their degradation was inhibited by increasing levels of serum in the culture media and enhanced by conditioned media. Addition of amino acids to the medium also inhibited the degradation of the proteins with long half-lives.

The requirement for ATP for the degradation of the two classes of proteins is not clear. Fluoride and iodoacetate decrease cellular ATP levels but the inhibition

of protein degradation observed did not parallel their effects on the cellular ATP levels. In general, these results could be interpreted to indicate that different pathways exist for the degradation of proteins with slow and fast turnover rates but that these pathways might share one or more steps in common (68). It was further suggested that the degradation of the more stable proteins involves a step not necessary for the degradation of more labile proteins. In another study (69), it was found that media deficient in serum, amino acids, phosphate, or glucose caused a twofold increase in the rate of degradation of long-lived cellular protein but had no effect on the rate of degradation of proteins with short half-lives. Insulin, dexamethasone, and fibroblast growth factor acted synergistically to inhibit the enhanced protein degradation caused by serum deprivation and to stimulate thymidine incorporation to levels similar to that seen with complete media containing serum. The enhanced proteolysis caused by serum-deficient media (long-lived protein) was found to be inhibited by vinblastine, a microtubule inhibitor, but vinblastine had no effect on basal proteolysis (70). Therefore, only enhanced proteolysis seemed to involve microtubules.

R. B. Hendil (71) found that the rates of degradation of protein with long and short half-lives were increased in fibroblasts maintained at a high cell density. In contrast, protein degradation in SV 40 transformed cells was affected by serum deprivation but had lost its response to high cell density.

The participation of the lysosomes in the degradation of long and short-lived proteins in isolated rat hepatocytes was investigated using reagents that inhibit the lysosomal digestion of protein (72). Ammonia inhibited by 70% the degradation of long half-life proteins but only inhibited the degradation of short half-life proteins by 45%. The results for chloroquine and methylamine, both of which are lysosomotropic reagents that concentrate in the lysosomes, were the same as those for ammonia. In addition, the inhibitions produced by these reagents were not additive. On this basis, it was concluded that there may be more than one pathway for the degradation of cellular proteins and these pathways may make different contributions to the degradation of long and short-lived proteins.

In a second study, leupeptin, an inhibitor of lysosomal cysteine proteinases, was also found to be unable

to completely inhibit protein turnover. In addition, the inhibition it produced was not additive with that produced by ammonia. From these results it was hypothesized that a non-lysosomal pathway may also contribute to cellular protein turnover. In addition, it was hypothesized that short-lived proteins were degraded mainly by a non-lysosomal pathway since the lysosomal protease inhibitors did not significantly affect the degradation of most of these proteins. The majority of the stable proteins appeared to be degraded by a lysosomal pathway as lysosomal protease inhibitors decreased their rate of breakdown by 70% (72).

Neff et al. (73) also studied the degradation of long and short-lived proteins in rat hepatocytes. They found that the protease inhibitors leupeptin, chymostatin, and antipain inhibited the degradation of long-lived proteins by 20-30%. A similar inhibition of degradation of the extracellular protein <sup>125</sup>I-asialofetuin was also observed. In contrast, leupeptin, chymostatin, and antipain did not significantly inhibit the degradation of proteins with short half-lives or abnormal proteins containing an amino acid analog. These results are also consistent with the existence of multiple pathways of

cellular protein breakdown which make different contributions to the degradation of proteins with long and short half-lives.

In summary, these studies suggest that normal cellular proteins fall into at least two populations: those which turn over slowly and those which turn over rapidly. It also appears that the pathways for their degradation may be different, although they may share one or more steps in common. Long-lived proteins may be degraded in lysosomes, as are extracellular proteins, while short-lived proteins may undergo initial degradation in the cytosol. The pathway for the degradation of short-lived proteins may resemble that of abnormal proteins which could arise from errors in translation, premature chain termination, or incorporation of an amino acid analog since abnormal proteins are also rapidly degraded (74). Normal proteins which turn over rapidly may be enzymes which play a regulatory role in metabolism (75).

The mechanism by which abnormal proteins are degraded has been investigated in E. Coli and in cultured mammalian cells (17, 18, 20-22). With E. Coli, abnormal proteins were degraded more rapidly than normal proteins by a process which is energy dependent. In addition,



while the degradation rates for normal proteins differed in growing and non-growing cells, the rates of degradation of abnormal proteins were similar under both conditions. The abnormal proteins were caused by errors in translation, by growth in the presence of amino acid analogs, and by puromycin which causes premature release of nascent proteins from the ribosome. Similar phenomena were also obtained in mammalian cells in culture (17, 18, 20-22). In addition, aging in cultured fibroblasts, which has been hypothesized to cause an increased accumulation of abnormal proteins, also causes an increased rate of proteolysis of cellular proteins (20).

Although some facts are known about intracellular protein degradation and the effects of various factors on its rate, the exact cellular mechanism is in doubt. Where does the degradation take place? Studies with reticulocytes and prokaryotes suggest that degradation might occur outside the lysosome (32, 76). Prokaryotes, which are capable of degrading intracellular proteins, lack lysosomes. The protease activity of reticulocytes against abnormal globin was found to be due to a soluble extra lysosomal system stimulated by ATP and having a basic pH optimum.

There is, however, strong evidence for the involvement of lysosomes in eukaryotic intracellular protein turnover. Liver perfusion studies showed the inhibition of protein degradation by pepstatin, a specific inhibitor of cathepsin D (77). This result is supported by additional perfusion studies which demonstrated that conditions which affect lysosomal proteolysis also affect cellular protein turnover in a similar manner. (78, 79). In addition, lysosomes isolated from liver perfused under conditions of increased protein turnover show differences in their densities and fragilities from those isolated under conditions of basal turnover (156). Measurements of the size of the lysosomal pool and volume of the lysosomal compartment in the perfused liver indicate that these increase under conditions of enhanced cellular protein degradation (157, 158). Calculations of the rate of lysosomal protein degradation, based on the size of the lysosomal substrate pool under various conditions, has been cited as evidence for the sole participation of lysosomes in cellular protein turnover. In vitro studies also suggest the involvement of lysosomal enzymes in intracellular protein degradation since the **relative rates** of degradation of various proteins by

lysosomal enzymes in vitro were similar to their relative in vivo degradation rates. In vivo studies with lysosomal protease inhibitors (46, 85-87) and agents that cause lysosomal dysfunction, such as chloroquine, (46, 85, 88-90) also support the involvement of lysosomes in protein degradation in the cell.

The importance of lysosomes in protein degradation, in the diseased state, was shown by the delayed degeneration of dystrophic muscle in cell cultures in the presence of pepstatin, leupeptin, and antipain (91) and in vivo in the presence of pepstatin and leupeptin (92). However, in all cases, lysosomal protease inhibitors and lysosomotropic reagents, either singly or in combination, are incapable of completely abolishing intracellular protein degradation. This perhaps implies the contribution of an extralysosomal pathway.

If eukaryotic protein degradation occurs in the lysosome, how are proteins transported into the lysosome from other cellular compartments? One possibility involves a kind of internal phagocytosis which has been called autophagy. The ATP requirement of protein turnover may thus be due to an energy dependence for autophagy. One problem with this hypothesis is that it has difficulty

accounting for the observation that the rates of turnover of proteins are quite variable. Even many of the proteins of a given cellular organelle show varied turnover rates indicating that the organelle is not degraded as a unit. To accommodate these observations, it has been proposed that proteins which can bind to the lysosomal membrane may be internalized more rapidly and thus are more rapidly degraded. It has been shown that proteins with short half-lives accumulate preferentially at water-organic solvent interphases when aqueous liver homogenates are incubated with an immiscible organic solvent (93) and tend to associate preferentially with lysosomal pellets (94). In agreement, proteins with more rapid turnover rates appear superficially more hydrophobic and bind preferentially to hydrophobic chromatography resins (34, 35). In addition, hormones that decrease protein degradation, such as insulin, have been shown to decrease cAMP, which is involved in endocytosis. However, it has also been reported that observation, which causes increased protein turnover, largely abolishes the differences in the degradation rates of various cellular proteins. Since starvation induced proteolysis is believed to be

autophagic and to involve lysosomes, this finding has been interpreted to mean that basal protein turnover occurs by a second pathway which is more sensitive to the physical properties of the cellular protein substrate (94b).

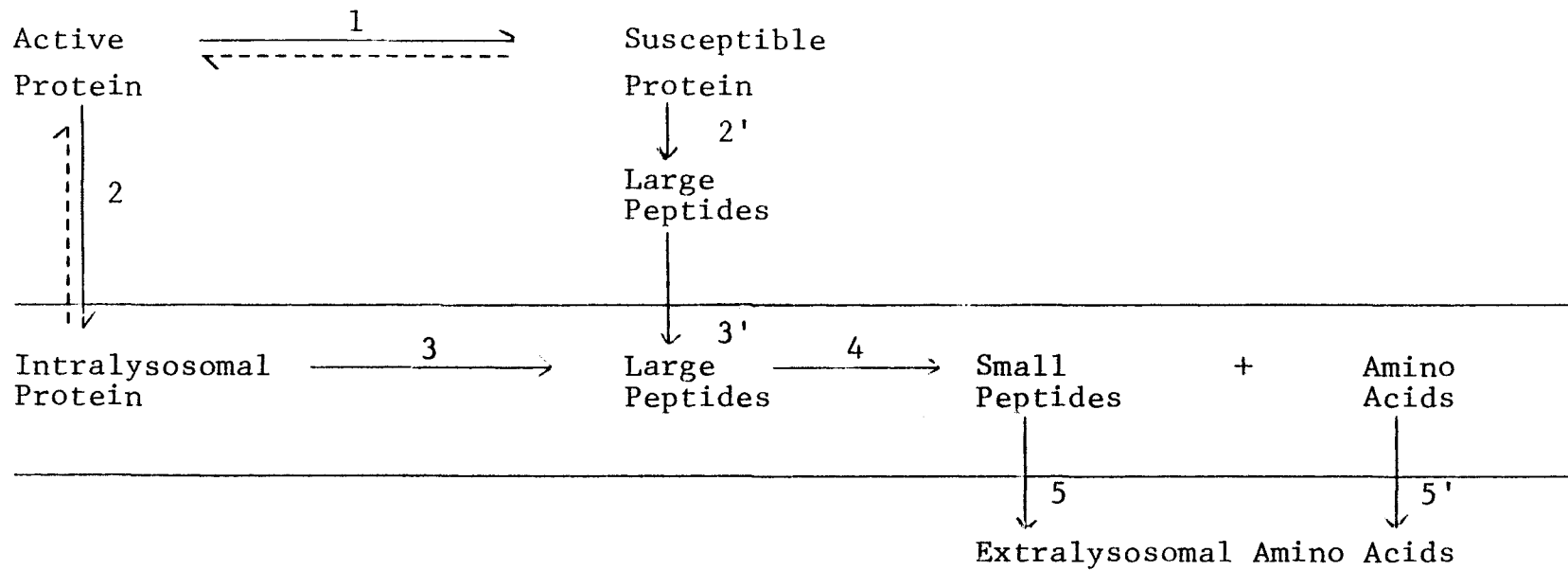
An alternative model involving lysosomes has been proposed to account for differences in the rate of degradation of cellular protein. According to this model, a protein's natural susceptibility to degradation by lysosomal proteases may determine its biological half-life. Many studies with lysosomal extracts and intact cells offer some support for this hypothesis (87, 95-98). It was found that the half-lives of proteins in vivo correlated roughly with their rates of degradation in vitro. There was also found to be some correlation between the activity of lysosomal proteases and the rate of intracellular protein degradation. It was postulated that the rate-limiting steps of protein turnover occur in the lysosome and that differences in degradative rates between various proteins reflect inherent differences in their susceptibilities to lysosomal proteases. One problem with this hypothesis is that it requires that a

protein which escapes digestion can exit the lysosome. According to this hypothesis, proteins which are degraded more slowly by lysosomal enzymes would have a greater probability of escaping the organelle intact. Segal et al. (98) demonstrated that yeast invertase could escape intact from the lysosomes and proposed a model for intracellular protein degradation (Figure 1). Step number 3, the rate of degradation to large peptides by lysosomal proteases, is hypothesized to be rate limiting.

In this model, a protein can leave as well as enter the lysosome intact (Step 2). The protein would then be degraded to large peptides (Step 3). Step 3, the rate of degradation by lysosomal proteases, is hypothesized to be rate limiting under some circumstances. The protein can also be altered in the cytoplasm (Step 1) to become susceptible to possible extra-lysosomal degradation (Step 2). The large peptides produced in Step 2 would then enter the lysosome where they would be degraded further. The final product of degradation would be the amino acids that are released from the lysosome.

Haider and Segal (99) demonstrated that substances that affect in vivo turnover of proteins were also capable

Figure 1. Model for intracellular protein degradation. Native protein can enter the lysosome directly (Step 2) or after modification in the cytoplasm (Step 1). The modified proteins either enter the lysosome or are partially degraded (Steps 1, 2', and 3'). The intact proteins in the lysosome are degraded to large peptides (Step 3). The large peptides are further degraded in the lysosome. The resulting amino acids are then released from the lysosomes. The active, native protein is able to escape degradation by exiting the lysosome before (Step 3) has occurred.





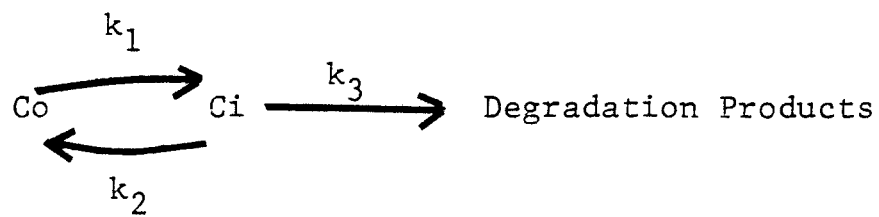
of modulating degradation of protein in isolated lysosomes as well and they formulated equations to explain the rates they observed (Figure 2). In their model, there is non-differentiated engulfment followed by enzymic inactivation. In Figure 2,  $C_o$  equals the concentration of protein,  $C_i$  equals its concentration inside the lysosome, and  $k_1$  is the rate constant describing the transport of the protein into the lysosome. The protein can also escape the lysosome with a rate constant of  $k_2$  or it can be degraded by the lysosomal proteases with a rate constant of  $k_3$ . The specific removal rate ( $k_{sp}$ ) of a protein from a cell is therefore dependent on the rates of these three processes and is given by

$$k_{sp} = \frac{k_1 k_3}{k_2 + k_3}$$

under steady state conditions. When  $k_3 \gg k_2$ , rate of degradation much greater than rate of exit from the lysosomes, the specific removal rate will equal  $k_1$ , the rate of entry into the lysosome. Substances that affect  $k_3$  will have only small effects on the turnover of protein.

Figure 2. Proposed equations describing intracellular protein turnover.  $C_o$  represents the concentration of a protein in the cytoplasm while  $C_i$  represents its concentration within the lysosome. The rate constants  $k_1$  and  $k_2$  refer to the rate of entry and exit of the protein from the lysosome. The rate of intralysosomal degradation of the protein is given as  $k_3$ . The removal of a protein from the cell is therefore described by  $k_{sp}$ , the specific removal rate, and is equal to  $k_1 k_3$

$$\frac{k_1 k_3}{k_2 + k_3}$$



$$\text{ksp (Specific Removal Rate)} = \frac{k_1 k_3}{k_2 + k_3}$$

If  $k_2 \gg k_3$ ,  $k_{sp}$  is approximately equal to  $(k_1/k_2) k_3$  and substances that affect  $k_2$  and  $k_3$  will now also affect the turnover rate as well.

One result that supports the escape of peptides from the lysosome is the finding that lysosomal extracts from liver are incapable of completely digesting protein substrates (100). Further degradation occurred upon the addition of the high speed supernatant at pH 8. It was postulated that small peptides were transported or diffused across the lysosomal membrane to be further degraded in the cytoplasm. However, in experiments with isolated lysosomes (101) and lysates from macrophages (102), it was found that there was a limit to the molecular size of solute molecules which can cross the lysosomal membrane. Polypeptides would not be able to escape. Only small molecules could cross the lysosomal membrane.

Thus, it appears that the results of Segal et al. (98) which demonstrated the transfer of invertase from the lysosome to the cytosol may represent an exception. At present, there is no evidence for an appreciable transfer of intact proteins from the lysosome to the

cytosol as a generalized phenomenon.

It has also been argued that protein denaturation may be the rate determining step in protein turnover (103). On incubating proteins with lysosomal extracts in vitro, it was found that denaturation was required for extensive digestion of this mixture by lysosomal hydrolases. The rate of denaturation of a protein in vivo may therefore determine its rate of degradation. Although this can account for variable turnover rates for proteins, it does not explain how denatured proteins might be preferentially taken up into lysosomes. However, it is possible that denaturation could expose the hydrophobic regions of a protein which could interact with the lysosomal membrane causing increased uptake into the lysosomes.

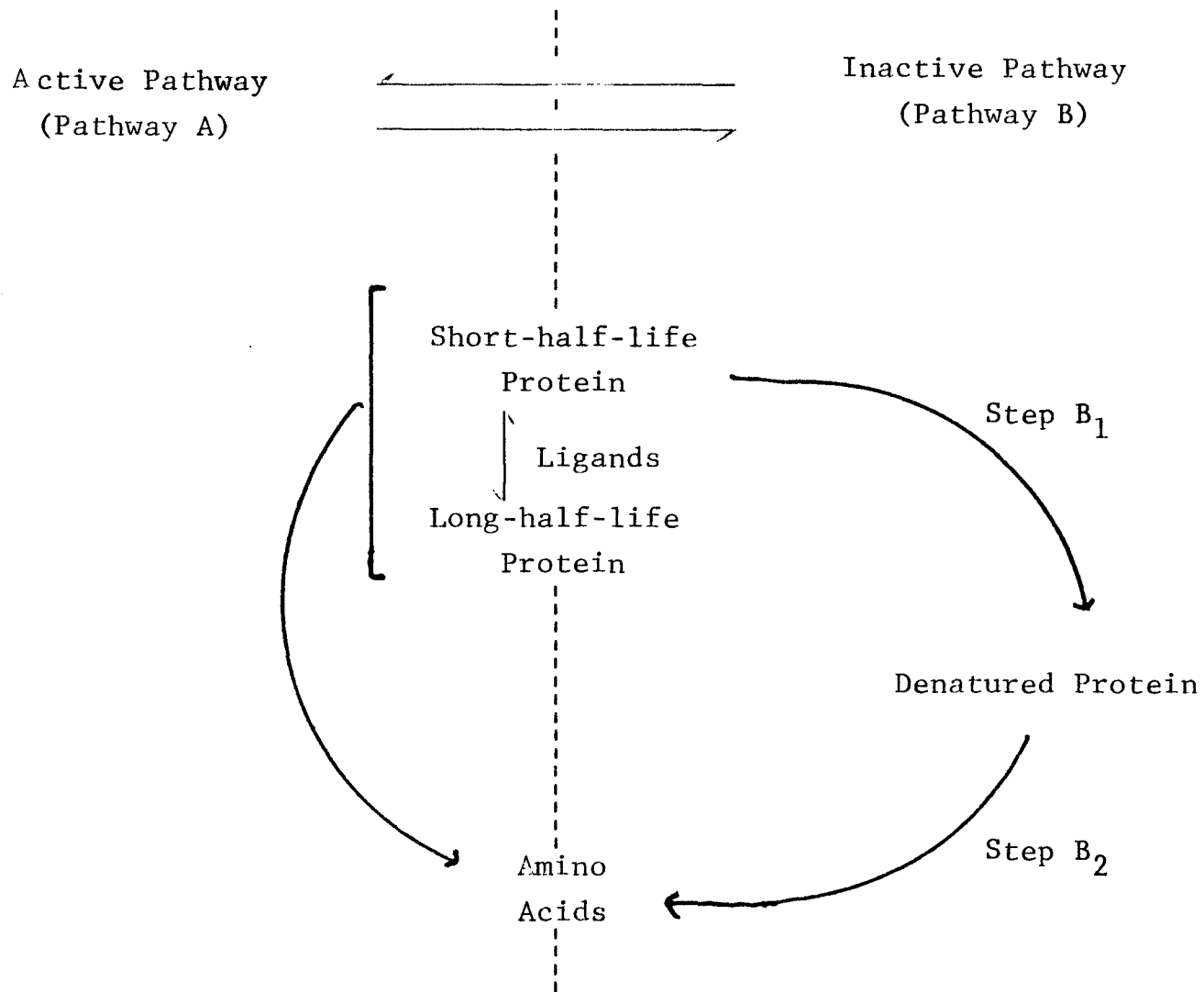
The model proposed in Figure I proposes that while early steps in protein degradation may occur in the cytosol, the latter stages occur primarily in the lysosome. In contradiction, some evidence exists for an independent non-lysosomal pathway for protein degradation. This includes: the finding that lysosomal protease inhibitors

can not completely block protein degradation; the observations that there exists two classes of proteins whose degradation rates are differentially affected by hormones and which show differential susceptibilities to the action of lysosomal protease inhibitors; the finding that inhibitors of ATP formation block basal protein turnover but not the augmented protein turnover observed in nutritionally deprived cells; the observation that starvation abolishes differences in the degradation rate of cellular protein; and the discovery of a non-lysosomal proteolytic activity in the extracts of reticulocytes and possibly liver.

A model for the degradation of cellular protein by a dual pathway has recently been proposed by Knowles and Ballard (22). In studies of the degradation of normal proteins and aberrant proteins containing an amino acid analog, 1-canavanine, in hepatoma cells, some evidence was obtained for the model shown in Figure 3. It was found that normal proteins were degraded more rapidly than native proteins. Furthermore, in contrast with normal proteins the degradation of abnormal proteins

Figure 3. Dual pathway model for intracellular protein degradation. The active pathway (Pathway A) involves protein uptake into and degradation by lysosomes. This pathway degrades short and long lived proteins at similar relative rates which are determined by the rate of autophagy. The inactive pathway (Pathway B) would degrade various cellular proteins at different rates which would depend on their rates of denaturation and thus on their inherent stabilities.

This stability could be modulated by ligands. The first and rate determining step in this pathway,  $B_1$ , involves denaturation of the protein. The denatured protein is then degraded in Step  $B_2$ . Pathway B is suggested to be extralysosomal. The active pathway, pathway A, becomes activated on nutritional step-down as a consequence of increased autophagy.





was not inhibited by lysosomal protease inhibitors, fetal calf serum, insulin, or cyclohexamide. To account for these observations, it was proposed that all proteins could be degraded by an active pathway involving autophagy (Pathway A). This pathway could be inhibited by lysosomal protease inhibitors and lysosomotropic reagents.

It was proposed that the lysosomal pathway degrades the different classes of cellular proteins at similar relative rates. Therefore, to account for the observation that various proteins are degraded at different rates, it was proposed that a second pathway, termed the inactive pathway, is also present. This pathway, which was hypothesized to degrade abnormal or inactive proteins by a process which did not involve lysosomes, was presumed to be regulated by the intrinsic stability of each protein. Accordingly, protein breakdown is initiated by denaturation (Step B<sub>1</sub>). The rate of denaturation would reflect the inherent stability of each protein molecule and would vary, thereby accounting for differences in degradation rates between proteins. In addition, the stability of various proteins could be modulated by ligands. According

to this hypothesis, short-lived proteins are those which are rapidly denatured or inactivated in vivo. Step B<sub>2</sub> depicts the degradation of denatured proteins to free amino acids. It is suggested that the inactive pathway, Pathway B, does not involve lysosomes because the rates of degradation of short-lived molecules are not significantly affected by the lysosomal protease inhibitors.

This proposal is attractive because it provides a means by which the cell may remove abnormal proteins which is free from the regulatory constraints associated with the physiological degradation of normal proteins.

In summary, the process of intracellular protein degradation is not completely understood; there is disagreement as to exactly where in the cell degradation occurs, whether one or more pathways participate, and on the nature of the steps involved. At least some steps may occur in lysosomes. However, mechanisms in which the lysosome is the exclusive site of cellular protein degradation now seem untenable.

Essential components of any explanation of protein **degradation must** account for differences in the turnover

rates for various cellular proteins. It has been suggested that the rate of turnover of an individual protein is determined by its intrinsic sensitivity toward endogenous proteases (80-84). However, why different proteins show different susceptibilities toward digestion is unknown. In addition, the sequence of events in protein degradation within the lysosome is also only poorly described. Information about lysosomal protein degradation may be obtained by studying the degradation of extracellular proteins since these appear to be taken up and degraded exclusively within these organelle. This approach also permits one to determine possible correlations between known structural features of proteins and their intrinsic sensitivity toward digestion in lysosomes and to study a process which may be a model for one or more steps in intracellular protein turnover.

#### B. Extracellular Protein Degradation

Extracellular proteins are taken up by cells in culture by endocytosis. Ehrenreich and Cohn (104) demonstrated that the conditions for uptake of  $^{125}\text{I}$ -HSA by macrophages correlated well with the conditions favorable

for pinocytosis; fetal calf serum (50%) and dextran sulfate enhanced uptake while parafluorophenylalanine, which inhibits pinosome formation, had an inhibitory effect. Uptake of non-digestible material as well as denatured albumin by macrophages demonstrated that uptake was linear with time for 7 hours (105). Similar results were also obtained with sarcoma cells (106). Experiments utilizing rat yolk-sac showed that extracellular protein enters in the liquid phase. Denaturation of the protein increased its uptake by permitting its binding to cell surface components (107), a result that was also duplicated in other experiments with yolk sacs (108). It was suggested that the digestive capacity of the lysosomes exceeded the capacity of cells to take up proteins, and therefore, pinocytosis was the rate determining step in the processing of exogenous proteins.

Factors influencing the rate of uptake of extracellular protein, other than protein structure, were also investigated. Inhibitors of glycolysis and respiration reduced pinocytosis in macrophages. Protein synthesis is also required for pinocytosis since it was inhibited by

puromycin and P-fluorophenylalanine (109). A reduction in pinocytosis was also found as the temperature was lowered. Pinocytosis is stimulated by high concentrations of calf serum (110, 111) as well as by anionic molecules (112). It is also stimulated by cAMP, ADP, and ATP (113). Catecholamines and prostaglandins  $E_1$ ,  $PGA_1$ , and  $F_2$ , which elevate cAMP levels, also stimulate pinocytosis (114). A similar effect was also observed for theophylline and papaverine, inhibitors of cyclic nucleophosphodiesterase. Other hormones which affect cellular cAMP levels (47-58) would therefore affect pinocytosis.

Endocytosis of digestible material causes an increase in the activity of the lysosomal enzymes. This appears to involve de novo protein synthesis since this induction is inhibited by puromycin (115). The induction is also inhibited by colchicine (116) and stimulated by chloroquine (117). This induction of digestive enzymes does not occur after endocytosis of non-digestible material (115).

After uptake, endocytosed material is localized within the lysosome (118-122) where the endocytosed protein is then digested by lysosomal proteases. In vitro

experiments demonstrated the ability of lysosomal protease to digest proteins. Studies using lysosomal protease inhibitors showed the importance of cathepsins B<sub>1</sub>, C, and D for protein degradation (123). The importance of cathepsin D was also shown by studies utilizing an antiserum to the enzyme that inhibited protein degradation in cultured cells (124).

Degradation of extracellular proteins pre-loaded in macrophages was found not to require ongoing pinocytosis, oxidative phosphorylation, or protein synthesis but was found to be temperature dependent (104). In contrast, in liver slices it was found that degradation of extracellular protein in lysosomes does require ATP, as inhibitors of energy formation were also able to partially inhibit protein degradation (125). These liver slices were collected 30 minutes after intravenous injection of Formaldehyde treated <sup>125</sup>I-BSA into mice. The degradation was studied for 60 minutes. However, there was only 60% cell viability after 60 minute incubation. Studies with isolated lysosomes (101) and cultured macrophages (102), as discussed previously, suggested that digestion of

protein within the lysosome is complete as small peptides are too large to escape from the lysosome. Proof of this comes from the analysis, by paper and column chromatography, of the products released when cultured cells (104, 126-128) and isolated phagosomes, vesicles that appear as the result of **phagocytosis** (129), degraded exogenous  $^{125}\text{I}$ -labeled protein. In each case, the only labeled products released into the medium are free  $^{125}\text{I}$ , mono-iodotyrosine, diiodotyrosine, and glycyliodotyrosine; no intermediate molecular weight products were observed. In the isolated phagosomes, the degradation was inhibited by sucrose and salts which is similar to the effect that these two substances have on the degradation of pinocytosed proteins in vivo.

It has frequently been observed that a significant quantity of material endocytosed by cultured cells may subsequently be released into the media. Approximately 20% of the total colloidal  $^{128}\text{Au}$ -gold taken up by macrophages is released into the media within the first hour, and thereafter, very little further release is seen in subsequent hours (105). A similar result was obtained for

<sup>125</sup>I-PVP. The cells therefore appear to regurgitate some undigested material in an initial period following uptake. A qualitatively similar result has been obtained on feeding <sup>192</sup>Ir to macrophages. However, in this case, 50% of the label was released from the cells in the first day following uptake but no more was released for the next 21 days (130).

Degradation of extracellular protein by cultured cells has been examined by two methods. Steinman and Cohn (131) fed soluble horseradish peroxidase to mouse peritoneal macrophages and studied the loss of peroxidase activity from macrophages with time. The half-life obtained by this method was approximately 8 hours. In contrast, when <sup>125</sup>I-labeled peroxidase was used, the half-life based on the loss of radioactivity from the cells was 20 to 30 hours. The discrepancy in rates could be due to the larger number of peptide bond cleavages required before the label can exit the cell compared with the number of peptide bond cleavages required for inactivation. Studies utilizing <sup>125</sup>I-albumin (104, 126) found that the loss of



label from the cells was biphasic, a fast phase followed by a slower one. The rate of loss was increased when the concentration of serum in the media was raised. After 24 hours, 75% of the labeled albumin originally present in the cells was released.

To make sure that the release of  $^{125}\text{I}$  activity into the media was due to protein degradation, Ehrenreich and Cohn (127) studied the degeneration of  $^{125}\text{I}$ -Hb and leucine- $^3\text{H}$ -Hb by macrophages and found the results to be identical. The degradation of Hb was found to be slower than that of albumin as only 50% of the label was released from the cells after 24 hours. The isotope lost by the cells could be accounted for as TCA - soluble isotope appearing in the medium.

### C. Purpose

The purpose of this study was two-fold. First we wanted to characterize the process by which extracellular proteins, incorporated into macrophages by endocytosis, are degraded. This process takes place in lysosomes, which are also possible sites of intracellular protein

degradation. In the model for intracellular degradation illustrated in Figure 2, the degradation of long-lived proteins is suggested to occur primarily in the lysosome. In addition, it has been suggested that the rate of turnover of many intracellular proteins may be determined by their rate of degradation in the lysosome. Therefore, it was hoped that studies of protein degradation following pinocytosis might contribute to an eventual understanding of the mechanism of intracellular protein turnover. In addition, it was felt that these studies could contribute to an understanding of the structural features of proteins which control their rates of proteolytic degradation. However, in order to carry out studies on the cellular degradation of extracellular proteins, it was first necessary to develop a methodology that required a minimum amount of cells, media, and labeled protein substrates and which yielded reliable and reproducible results.

## CHAPTER II

### EXPERIMENTAL

#### A. Materials

##### 1. Substrates for Cellular Uptake and Degradation

Dextran, human serum albumin, cytochrome c, phosphorylase b, hemoglobin, arginase, ribonuclease,  $\beta$ -galactosidase, thyroglobulin, catalase, and enolase were obtained from Sigma Chemical Company. Ovalbumin was a product of Nutritional Biochemical Corporation. Dextran-sulfate (molecular weight  $2 \times 10^6$ ) was from Pharmacia Fine Chemicals and  $^{125}\text{I}$ -bovine serum albumin was obtained from New England Nuclear.

##### 2. Labeling Reagents

$\text{Na } ^{125}\text{I}$ ,  $^{14}\text{C}$ -glycine,  $^3\text{H}$ -glycine and  $^{14}\text{C}$ -acetic anhydride were obtained from Amersham-Searle. Cyanogen bromide was purchased from Aldrich Chemical Company. Chloramine-T and lactoperoxidase were products of Sigma Chemical Company and the enzymobead kit for radioiodination was obtained from Bio-Rad Laboratories.

### 3. Inhibitors

Antipain, leupeptin, and pepstatin were products of the Protein Research Foundation. Soybean trypsin inhibitor (Kunitz) was from Worthington. Chloroquine was obtained from Sigma Chemical Company.

### 4. Cell Culture Material

Lux polystyrene tissues culture petri dishes (35 x 10 mm) and multiplates were obtained from Microbiological Associates. Media 199 (1 x, with Earles salts, 2.2 g/l sodium bicarbonate, and l-glutamine), Earles balanced salt solution, virus screened fetal calf serum, penicillin-streptomycin sulfate, and l-glutamine were from Grand Island Biological Company.

Media was supplemented with fetal calf serum (1-50%) penicillin (1,000 units /ml), streptomycin sulfate (1,000 µg/ml), and l-glutamine (2mM) before use. The media used in the uptake of radioactively labeled substrates contained 50% fetal calf serum. The media used in measuring rates of degradation of pinocytosed proteins contained varying amounts of fetal calf serum although

experiments were most commonly performed with media containing 1% fetal calf serum. All cells were maintained in a Forma Scientifica CO<sub>2</sub> incubator in an atmosphere of 5% CO<sub>2</sub> and at 37°C.

#### 5. Gel Chromatography

Sephadex G-25, G-50, and G-75 were from Pharmacia Fine Chemicals. Affigel-Blue was obtained from Bio-Rad Laboratories.

#### 6. Electrophoresis

Electrophoresis on agarose film was done with the Corning cassette electrophoresis system. For polyacrylamide disc gel electrophoresis, acrylamide, methyl-bis-acrylamide, and TEMED were obtained from Eastman Kodak Company and sodium doedecyl sulfate was a product of Sigma Chemical Company.

#### 7. Miscellaneous

Eight-week-old male Swiss mice were obtained from Scientific Small Company. Fibroblasts, low passage baby hamster kidney cells (BHK-21/Clone 13), were a gift from Dr. Gene Majors. Freund's complete adjuvant was obtained

from Grand Island Biological Company. Acrodisc filters (0.2 $\mu$ m) were from Gelman and dialysis tubing was obtained from Fisher Scientific Company. Trypsin was obtained from Sigma Chemical Company.

## B. Methods

### 1. Synthesis of Labeled Substrates

#### a. Labeled Dextran

<sup>14</sup>C-dextran and <sup>14</sup>C-dextran-sulfate were synthesized by the method of Marshall and Rabinowitz (132). Fifty  $\mu$ Ci of <sup>14</sup>C-glycine was incubated for 24 hours with 550 mg CNBr-activated dextran, yielding a label product with a specific activity of 50 cpm/ $\mu$ g. Dextran-sulfate was labeled in a similar manner by adding 250  $\mu$ Ci <sup>14</sup>C-glycine to 600 mg of the CNBr-activated dextran and stirring in the cold for 23 hours. The resultant <sup>14</sup>C-labeled dextran-sulfate had a specific activity of 125 cpm/ $\mu$ g.

<sup>3</sup>H-dextran was synthesized by the same procedure using <sup>3</sup>H-glycine. Two hundred and fifty  $\mu$ Ci of tritiated glycine was added to 500 mg activated dextran and the reaction was allowed to proceed for one week. The resultant labeled dextran had a specific activity of 190 cpm/ $\mu$ g.

In preparing the various labeled dextrans, cold glycine was added at the end of each reaction to modify any of the free CNBr-activated sites which had not previously reacted with the label. The solutions were then dialyzed against distilled water in the cold to remove any unbound label. The samples were then lyophilized and refrigerated.

Occasionally, the extent of labeling was monitored during the course of the reaction by chromatographing samples of the reaction mixture on a column of Sephadex G-75. The extent of the reaction could be determined from the amount of label which co-chromatographed with the high molecular weight dextran.

b. Iodination of Proteins

Proteins were iodinated by three different techniques. Hemoglobin and ovalbumin were iodinated by the method of Hunter and Greenwood (133) using chloramine-T and Na <sup>125</sup>I. The labeled proteins obtained had specific activities of less than 0.1  $\mu\text{Ci}/\mu\text{g}$  after purification on a Sephadex G-75 column.

Human serum albumin was iodinated with lactoperoxidase by the method of J. Marchalonis (134) and purified by chromatography on G-75. It had a specific activity of 1.3  $\mu\text{Ci}/\mu\text{g}$ .

All of the proteins, with the exception of HSA, were also labeled utilizing the Bio-Rad enzymobead kit. The reaction was stopped by filtering the reaction mixture through an acrodisc to remove the bead-bound enzyme. Unreacted  $^{125}\text{I}$  was subsequently removed by dialysis. The specific activities obtained varied from 0.1 to 21.  $\mu\text{Ci}/\mu\text{g}$  depending on the protein. Ovalbumin and hemoglobin labeled by this and the chloramine-T method gave similar results in all experiments.

The labeled proteins were refrigerated after purification and generally used within 3 days. Some were also frozen for later experiments. Media was added to the labeled protein solution just before use. The radio-activity of protein samples stored for any period of time was corrected for the known decay rate of  $^{125}\text{I}$ .



## 2. Collection of Macrophages

Eight-to-twelve-week-old male mice were injected intraperitoneally with 0.75 ml 4% thioglycollate media through a sterile filter to elicit stimulated macrophages. The cells were collected three days later by flushing the peritoneum with sterile Earls balanced salt solution through a syringe and collecting the lavage solution in a second syringe. Flushing of the peritoneum was repeated twice. The washings were combined in sterile centrifuge tubes and the cells collected by low speed centrifugation. The salt solution was then aspirated off and the cells resuspended in media. The pooled cells collected from 3 to 8 mice were counted in a hemocytometer and aliquots containing  $4 - 9 \times 10^6$  cells were plated on glass slides or plastic petri dishes. These numbers of cells were found to pinocytose sufficient labeled material to yield significant radioactivity when the cells were counted. Cell preparations were examined by phase contrast microscopy during initial studies and routinely with a cell culture microscope.

Studies with  $^{14}\text{C}$ -dextran and  $^{14}\text{C}$ -dextran-sulfate were performed on glass coverslips. However, it was found that  $^{125}\text{I}$ -proteins incubated with glass coverslips adhered to the glass giving rise to high background counts. Plastic plates were used to minimize this non-specific binding. Since fetal calf serum was found to compete with labeled proteins for the non-specific binding sites on the surface of the plastic plates, the uptake media routinely contained 50% fetal calf serum. This level of fetal calf serum was also found to stimulate pinocytosis (110, 111).

After the cells were dispersed onto glass coverslips or plastic tissue culture dishes, they were incubated at  $37^{\circ}\text{C}$  for one to three hours. During this time, the macrophages adhered to the surface of the plates. The plates were then washed with sterile saline to remove any non-adherent cells and fresh media added. Cells used for an overnight labeling experiment were then exposed to media containing the labeled material for 12 to 20 hours at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Alternatively, in some experiments

unlabeled media was added at this point and the cells subsequently treated with label the next day.

In some experiments, activated macrophages were elicited by injecting 0.5 ml Freund's complete adjuvant instead of thioglycollate media into the peritoneal cavity. The cells were collected and treated in the same manner as described for stimulated macrophages (those elicited by thioglycollate media).

### 3. Uptake of Labeled Material by Cells

Media containing the radioactively labeled material was added to the cell cultures and uptake of the label was allowed to proceed for various periods of time. All cell cultures used in a given experiment were plated at the same time and with the same known number of macrophages. Fibroblast cultures were confluent monolayers when used. At the end of the uptake period, the labeled media was removed by aspiration and the cells washed five times with sterile saline. They were then incubated in media for 20 minutes and washed once more with saline. This procedure was adopted to insure removal of any non-specific label adsorbed to the surface of the plates.

The efficacy of this procedure was tested with blank plates (plates without cells).

Cells cultured on coverslips were placed directly into test tubes or scintillation vials for counting. Cells cultured on plates were collected by sonifying them in 1 ml distilled water for 10 minutes to disrupt the cells. The disrupted cells were transferred to test tubes. The plates were then washed with an additional 0.5 ml water and the washings added to the disrupted cells. The combined 1.5 mls obtained were then counted. examination of the plates with a tissue culture microscope verified that no cells were left on the plate. This method was found to maximize the collection of cell-associated radio-activity while minimizing the release of any labeled protein that might be bound non-specifically to the plate. This was confirmed by comparing various collection procedures with both blank culture dishes and culture dishes containing cells.

Blank plates or slides, those without cells, were run concurrently with those containing cells in order to determine what percentage of the label adsorbed to the surface of the support. Background counts, those from

non-specifically bound label as well as noise from the counting instruments, were subtracted from the data. In most experiments, the blank represented less than 5% of the label collected.

#### 4. Degradation and Release of Pinocytosed Labeled Material by Cells

The cell cultures were treated as described for the uptake experiments except that the cells were not collected after washing. Instead, fresh unlabeled media containing varying amounts of fetal calf serum was added to each plate and the rate of release of radioactivity into the fresh media was measured. Aliquots of media were removed at various times. These aliquots were then treated with an equal volume of 20% trichloroacetic acid - 1% phosphotungstic acid and the mixture incubated in an ice water bath for 30 minutes. The precipitated protein was removed by centrifugation. Both the supernatant and precipitate were collected and counted in a gamma counter. Media aliquots from experiments with the labeled dextrans were placed directly into scintillation vials without precipitation. Rates of protein release were also measured in a

second way. In these experiments, all the media was removed at each time point and replaced with fresh media, or only a small aliquot was removed and replaced with an equal volume of fresh media. This was done in order to determine if nutrient depletion from the media or accumulation of metabolic end products in the media might have affected the degradation and release of labeled protein. These experiments also provide some information about whether any of the TCA-soluble radioactivity formed could have been due to the degradation of intact labeled protein in the media by any extracellular proteases which might be present or released by the cells. The possibility of extracellular proteolysis was also checked by examining the effects of conditioned media on the labeled substrates. In these experiments, no evidence was obtained that the appearance of TCA-soluble radioactivity could be due to the action of extracellular proteases on intact proteins which might have been regurgitated from the cell or adsorbed to the surface of the culture dish.

At the end of the release experiment, the cells

were collected in the manner described for the uptake experiments and counted.

This method was developed so that all the data points from one experiment came from a single plate followed over time and therefore the need to normalize the data from different plates is obviated. This is important since estimates of cell density based on protein content are not sufficiently accurate when dealing with small numbers of cells, can occasionally lead to anomalously high values due to contamination of cells with even low levels of serum proteins normally present in the culture media, and can vary with cellular activity. One problem with this approach, however, is that the cell density in a given plate may change with time as a consequence of cell detachment and/or cell death. It was found that the amount of labeled protein collected from one plate before release agreed within the variability observed in duplicate uptake experiments ( $\pm 15\%$ ) with the total amount of label associated with the cells undergoing release. In the cells undergoing release, the total amount of label is expressed

as the sum of the TCA-soluble radioactivity released, the TCA-insoluble radioactivity released, and the radioactivity remaining in the cell at the end of the release experiment. Blanks, plates without cells, were also run in an identical manner.

#### 5. Analysis of Released Label

Labeled material released into the media by cells was analyzed by gel chromatography utilizing Sephadex G-25 or G-50. The gels were poured into a column and equilibrated with at least three column volumes of buffer before use.

The Sephadex G-25 column (1.6 x 10.5 cm) was eluted with water:phenol:acetic acid (1:1:1) at 20 ml/hr. A 0.7 ml sample of TCA-soluble label was applied to the column and 16 drop fractions collected to be counted in a gamma counter. Monoiodotyrosine (MIT) and Na <sup>125</sup>I were also run on the column in an identical manner. The MIT was detected in the fractions by the ninhydrin method of Moore & Stein (136).

Media samples, as well as TCA-soluble material,



were chromatographed on a 1.6 x 60 cm Sephadex G-50 column with 0.05M phosphate buffer, pH 7.3, containing 0.15 M NaCl at 30 ml/hour. The sample size for this column was 1.5 ml and 32 drop fractions were collected and counted.

Uptake media, release media, and sonified cell samples were also analyzed by SDS gel electrophoresis according to the procedure of Weber and Osborn (137).

Samples were run for 23 hours on an 18% cross-linked acrylamide gel. The gels were then sliced and each section counted. All samples contained bromphenol blue as a marker and the media samples also had phenol red. The solutions used in preparing the gels were as follows:

Gel Buffer: 0.2 M Phosphate, pH 7.2

0.2% SDS

Sample Buffer: 0.01 M Phosphate, pH 7.2

1% SDS

1% 2-Mercaptoethanol

Acrylamide B: 22.2 g Acrylamide/75 ml

0.6 g Methyl Bisacrylamide

Ammonium Persulfate: 15 mg/ml

Running Gel: 15 ml Acrylamide B

7.5 ml Gel Buffer

1.0 ml Ammonium Persulfate

0.045 ml TEMED

Reservoir Buffer: 1 part Gel Buffer to 1 part  
distilled water

The tubes were run at room temperature at a  
constant voltage of 4 mA/tube

## 6. Miscellaneous

### a. Analysis of Proteins Used

The proteins used for iodination were analyzed using the Corning cassette electrophoresis system with agarose film. The buffer was 0.05 M sodium barbital, pH 8.6, with 0.035% EDTA. Samples, 0.002 ml of a 4 mg/ml solution, were

run for 40 minutes and the film stained for 20 minutes in an amido black solution followed by washing with acetic acid: methanol: water (1:7:2). The film was optically scanned in a Gelman scanner.

The proteins were also analyzed by SDS disc gel electrophoresis utilizing the method of Weber and Osborn (137) as described previously, except that the samples were run on 7% gels. After electrophoresis, the proteins were fixed with acetic acid, stained with amido black, and washed with acetic acid: methanol: water (1:7:2).

b. Analysis of Labeled Material

Radioactively labeled dextran, dextran-sulfate, and protein were analyzed by column chromatography. After labeling,  $^{14}\text{C}$ -Dextran was applied, in a 0.4 ml sample in acetate buffer, to a 15 ml Sephadex G-75 column and fractions collected. Aliquots of each fraction were added to 10 ml Bray's solution and counted. The hexose content of each fraction was determined with phenol-sulfuric acid (138).  $^{14}\text{C}$ -Dextran-sulfate was analyzed in a similar manner.

Iodinated hemoglobin was also analyzed by chromatography. In this procedure, Sephadex G-75 equilibrated in acetate buffer was used. The fractions obtained were

analyzed for both radioactivity and protein. Twenty-five percent of the label was found associated with the protein peak.

The  $^{125}\text{I}$ -BSA obtained commercially was subjected to affinity chromatography on an Affi-gel blue Sepharose column which specifically binds native albumin.  $^{125}\text{I}$ -BSA was added to the column in 0.02 M phosphate buffer, pH 7.1, and the column washed with this buffer until no more radioactivity eluted. The bound  $^{125}\text{I}$ -BSA was then eluted from the gel with phosphate buffer containing 1.4 M NaCl. Samples of affinity purified  $^{125}\text{I}$ -BSA were then dialyzed before use against water to remove salts.

Other proteins used in cellular uptake and degradation experiments were also analyzed by SDS gel electrophoresis on 7% gels essentially as described previously.

c. Denaturation of Protein

$^{125}\text{I}$ -BSA was denatured by immersing a solution containing 0.2 mg/ml in a boiling water bath for 10 minutes. Media was added after the solution cooled and this was filtered before use. The labeled heat-treated protein remained soluble at these concentrations. The denaturation of the labeled BSA was verified by chromatography

on an Affi-gel blue affinity column.

$\beta$ -Galactosidase was denatured by freezing and thawing after labeling with  $^{125}\text{I}$ . The degree of denaturation was tested by assaying for activity (139).

d. Protein Degradation by Cell Lysates

Stimulated macrophages were plated and allowed to incubate overnight before use. Cell lysates were collected from the plates by sonification for 10 minutes in 1 ml 0.1 M acetate buffer, pH 5.

Degradation of labeled protein by these cell lysates was studied by two techniques. In the first, labeled protein was added to the cell lysate to give a final concentration of  $9 \times 10^5$  cpm/ml. In the second method,  $10^7$  cpm/ml  $^{125}\text{I}$ -protein was added to the cell cultures for the overnight incubation and the cells allowed to pinocytose the label. The cells were washed as described previously and the lysates prepared. For both techniques, aliquots of the lysates were removed at various times, treated with an equal volume of 20% TCA-1% phosphotungstic acid, and analyzed as described previously for TCA-soluble and TCA-insoluble radioactivity.

e. Partial Purification of Cathepsin B<sub>1</sub>

Cathepsin B<sub>1</sub> was partially purified from mouse liver by the method of Bajkowski and Frankfater (140). The liver was homogenized and stirred in 1% NaCl, 2% N-butanol,  $10^{-3}$ M EDTA, 0.2% Triton X-100, pH 6.1, for one hour and centrifuged to remove the precipitate. The pH of the supernatant was adjusted to 4.4 with HAc and spun again at 17,000 rpm. The supernatant was mixed with 1.3 volumes acetone and again centrifuged as before. The precipitate was dissolved in 0.025M NaAc, pH5.1, with 0.1 M NaCl and  $10^{-3}$ M EDTA and dialyzed overnight against this same buffer. The sample is then further purified by chromatography on Sephadex G-150.

f. Enzyme Assays

1. Cathepsin B

Cathepsin B activity was assayed by the method of Bajkowski and Frankfater (140) using CBZ-glycine-nitrophenyl ester as the substrate. The enzyme is incubated for 15 minutes with dithiothreitol in 0.025M NaAc, pH5.1 with  $10^{-3}$ M EDTA before the addition of substrate.

## 2. Papain

Papain was assayed by the same method as Cathepsin

B.

## 3. Trypsin

Trypsin was assayed utilizing tosyl-arginine-methyl ester (TAME) as a substrate (160). The buffer used was 0.5M Tris-HCl, pH 8.1.

## CHAPTER III

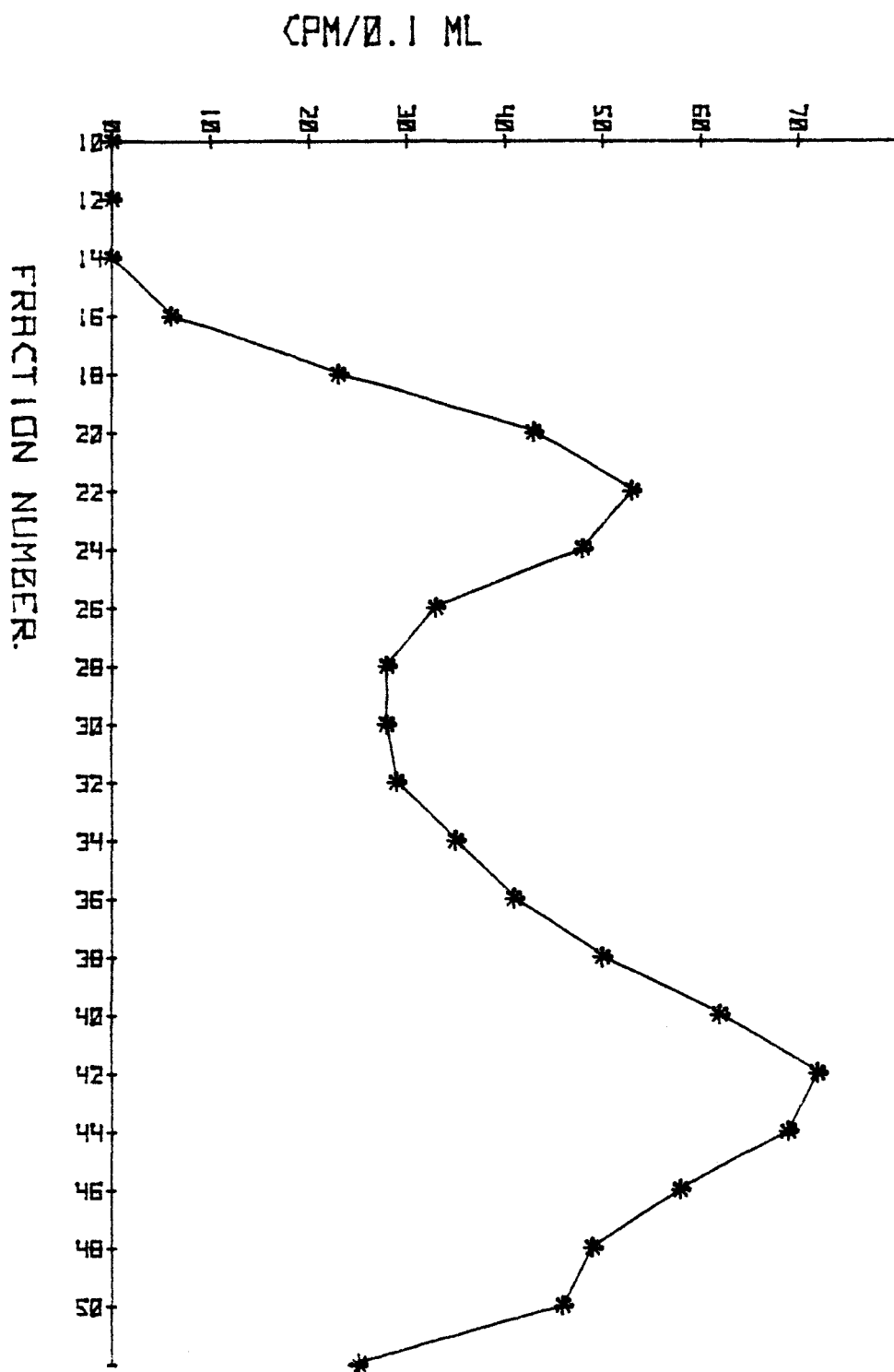
### RESULTS

#### A. Characterization of Labeled Substrates

Reaction mixtures containing CNBr activated dextran or dextran-sulfate and radioactively labeled glycine were analyzed by Sephadex G-25 chromatography at varying times during the reaction to monitor the extent of the coupling. Figure 4 shows the elution profile of the labeling solution of dextran-sulfate after 24 hours demonstrating that  $^{14}\text{C}$  activity is associated with both high and low molecular weight species. Similar analysis of aliquots of the labeling solution after 1, 2, and 13.5 hours demonstrated a progressive increase in the percentage of  $^{14}\text{C}$  activity associated with the high molecular weight peak. The percent of label in the high molecular weight fraction increased from 15% after 1 hour to 27% and 85% after 2 hours and 13.5 hours respectively. Similar results were also obtained for labeling experiments with  $^{14}\text{C}$ -glycine or  $^3\text{H}$ -glycine and dextran. Proteins used in the preparation of  $^{125}\text{I}$ -labeled substrates were analyzed prior to labeling by electrophoresis with the Corning cassette electrophoresis system to determine their purity. Most



Figure 4. Chromatography of the  $^{14}\text{C}$ -Dextran-sulfate labeling solution on Sephadex G25 in 0.5M acetate buffer, pH 5.0. Aliquots (0.1 ml) of each 0.3 ml fraction were added to 10 mls Bray's solution to be counted in the scintillation counter. The reaction was allowed to proceed 24 hours before analysis.



proteins yielded a single major band. Ovalbumin and catalase preparations appeared to contain minor impurities which represented no more than 20% of the total protein. Enolase showed two major bands whose proportions varied with protein concentration and which probably represented a monomer-dimer equilibrium. Similar results were obtained for these proteins by SDS disc gel electrophoresis. Each lot of protein obtained was analyzed before use.

After labeling with  $^{125}\text{I}$ , the protein substrates were further analyzed. Figure 5 is an elution profile for  $^{125}\text{I}$  labeled hemoglobin obtained with a Sephadex G-75 column (1.6 x 30 cm). It shows about 10% of the radioactivity associated with the hemoglobin peak. From the areas under the peak and knowledge of the total protein, the specific activity of the labeled hemoglobin could be estimated.

Other proteins, after labeling, were precipitated with 10% TCA on ice and the amount of label associated with the TCA-insoluble fraction was measured. These results were then used to estimate the specific activity of these proteins. The results summarized in Table 1 list the proteins labeled, the technique used, and the

Figure 5. Chromatography of the  $^{125}\text{I}$ -hemoglobin labeling solution on Sephadex G75 in 0.5 M acetate buffer, pH 5.0. Aliquots of each 1.8 ml fraction were removed for counting.

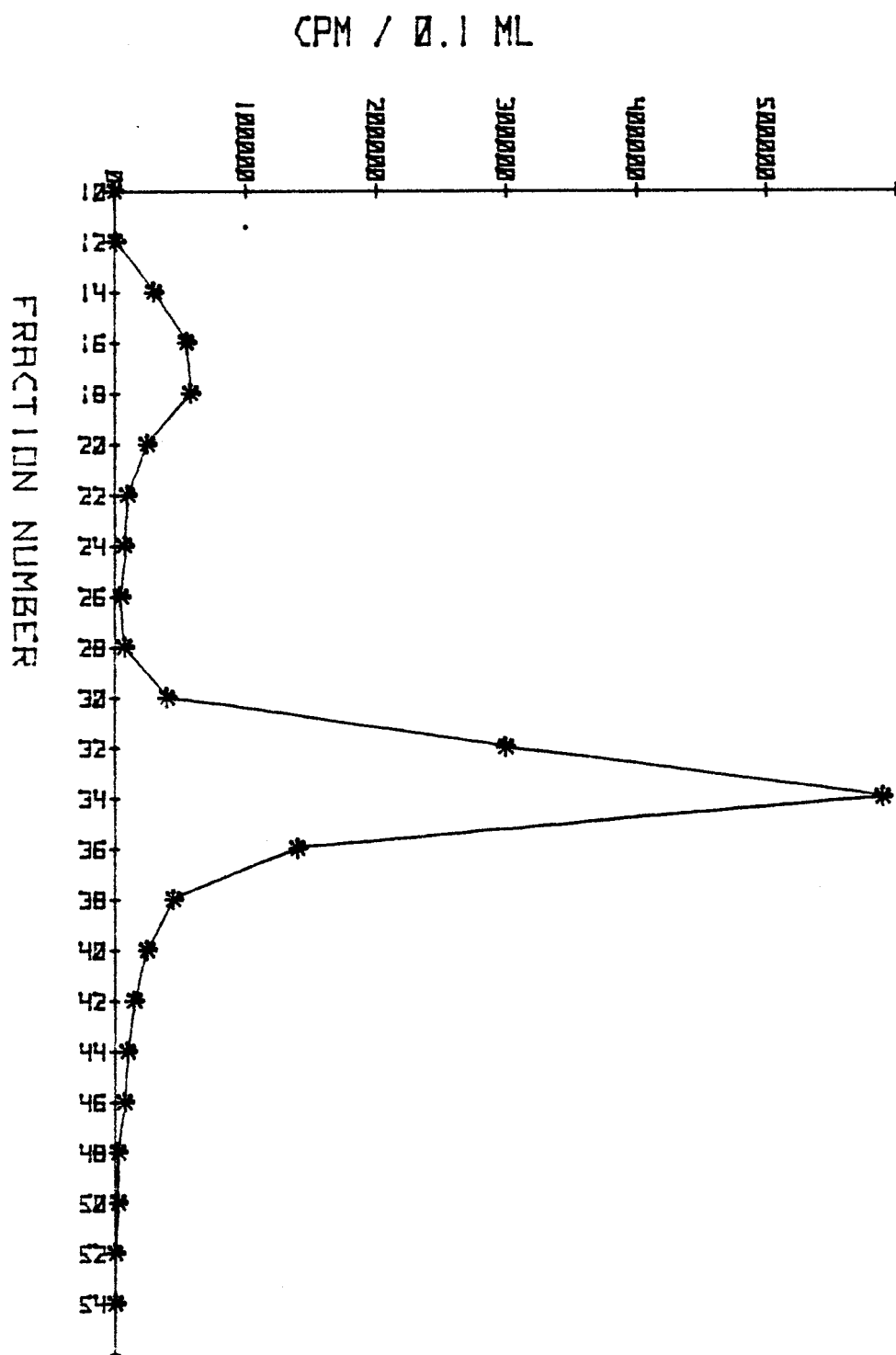


TABLE 1

## LABELED PROTEINS USED

<u>Protein</u>	<u>Labeling Technique</u>	<u><math>\mu\text{Ci}/\mu\text{g}</math></u>
Arginase (Prep. 1)	Enzymobead	0.72
Arginase (Prep. 2)	Enzymobead	0.56
Catalase	Enzymobead	0.17
Cytochrome c (Prep. 1)	Enzymobead	1.17
Cytochrome c (Prep. 2)	Enzymobead	1.83
Enolase	Enzymobead	0.30
$\beta$ -Galactosidase	Enzymobead	4.30
Hemoglobin	Enzymobead	1.63
HSA (Prep. 1)	Lactoperoxidase	1.30
HSA (Prep. 2)	Lactoperoxidase	0.73
Ovalbumin (Prep. 1)	Chloramine T	<0.001
Ovalbumin (Prep. 2)	Enzymobead	0.43
Phosphorylase b (Prep. 1)	Enzymobead	1.11
Phosphorylase b (Prep. 2)	Enzymobead	2.10
Ribonuclease (Prep. 1)	Enzymobead	1.90
Ribonuclease (Prep. 2)	Enzymobead	0.04
Thyroglobulin	Enzymobead	1.27

specific activities obtained.

<sup>125</sup>I-BSA obtained commercially was characterized by affinity chromatography on an Affi-gel blue column. In this chromatographic support, Cibacron Blue F3GA, a reactive blue dye, is covalently linked to an insoluble agarose matrix. The resulting gel will bind albumin and other proteins which contain an organic anion binding site. With one batch of albumin, 3 separate determinations yielded 81%, 79%, and 83% of the total radioactivity bound to the affinity column. A similar analysis of a second batch indicated 80% of the total <sup>125</sup>I activity bound to the affinity column. The bound fractions were eluted as described previously.

#### B. Uptake of Nondigestible Materials

The uptake of nondigestible material by cultured macrophages was first investigated. Cells were allowed to pinocytose <sup>3</sup>H- or <sup>14</sup>C-dextran or <sup>14</sup>C-dextran-sulfate for varying periods of time. The cells were then washed and cell samples collected. In order to normalize the data for differences in the amounts and specific activities of the labeled materials and differences in the quantities of cells used in the various experiments, the results are expressed as microliters of medium ingested per 10<sup>6</sup> cells

plated (141).

This value was calculated by the formula:

$$\mu\text{l}/10^6 \text{ Cells} = \frac{\text{Amount of Cell Associated Radioactivity} / \text{Concentration of Radioactivity in Medium}}{\text{Number of Cells Plated}/10^6}$$

The uptake of  $^{14}\text{C}$ -dextran-sulfate, which is known to cause increased pinocytotic vesicle formation (142), is shown in Figure 6. Our data for the uptake of labeled dextran is similar except that it occurs at only about 1/20 the rate of uptake of dextran-sulfate.

#### C. Uptake of Digestible Substrates

$^{125}\text{I}$ -BSA uptake by cells was also studied (Figure 7). In this case, the accumulation was less than that for nondigestible materials; possibly due to the ongoing degradation of  $^{125}\text{I}$ -BSA and release of free label into the media. The rate of accumulation of  $^{125}\text{I}$ -BSA by the cells was similar in the presence of dextran and markedly increased in the presence of dextran-sulfate, which causes increased pinocytotic vesicle formation (142).

The effect of different concentrations of labeled BSA in the media on the amount taken up over a 12 hour labeling period was also investigated (Table 2). These experiments were performed with two different batches of



Figure 6. Uptake of <sup>14</sup>C-dextran-sulfate by macrophages. Uptake was performed in 50% FCS with 5 mg/ml dextran-sulfate (160,000 cpm/ml) for varying time periods. Each time point represents a separate plate with  $3 \times 10^6$  macrophages.

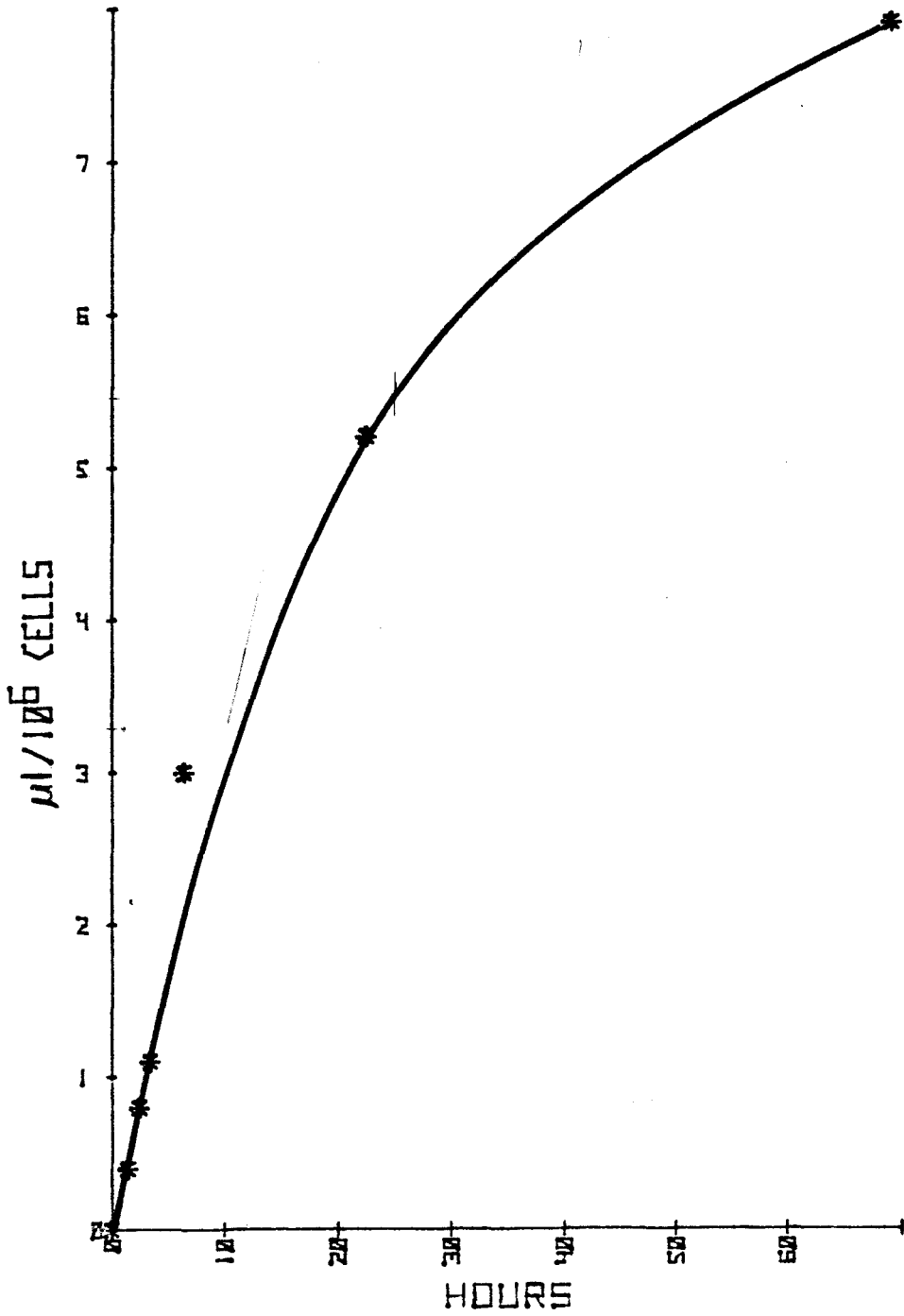


Figure 7. Uptake of  $^{125}$ I-BSA by macrophages. Uptake was performed in media with 50% FCS with  $1 \times 10^7$  cpm/ml in the absence (\*) and the presence of 1 mg/ml dextran-sulfate (□). Each point represents a single plate with  $2.6 \times 10^6$  cells.

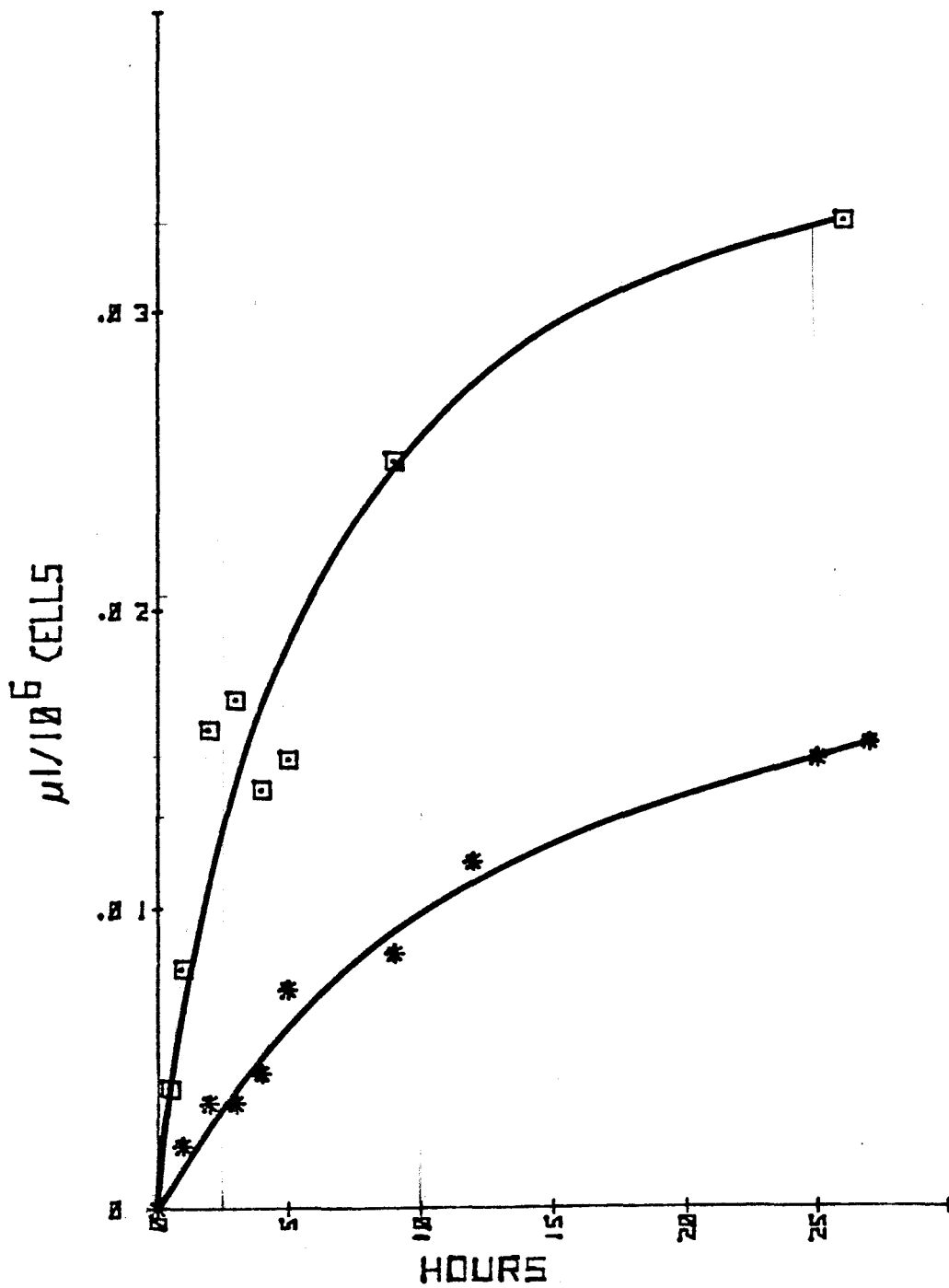


TABLE 2

<sup>125</sup>I-BSA Uptake With Different Concentrations of <sup>125</sup>I-BSA in the Uptake Media<sup>a</sup>

<u>cpm/ml x 10<sup>6</sup> <sup>125</sup>I-BSA</u>	<u>Macrophage Preparation</u>	<u>Cell Associated Radioactivity (cpm)</u>	<u>μl/10<sup>6</sup> Cells Plated<sup>b</sup></u>
1.7	1 <sup>c</sup>	516,545	0.04
6.7	1 <sup>c</sup>	2964,1924	0.05
15.0	1 <sup>c</sup>	5755,4851	0.04
4.0	2 <sup>d</sup>	557,622	0.03
6.0	2 <sup>d</sup>	783,948	0.03
12.0	2 <sup>d</sup>	1419,1737	0.03

<sup>a</sup> Uptake was performed in media with 50% FCS for 12 hours.

<sup>b</sup> Average of 2 determinations

<sup>c</sup> Number of cells plated was  $7.9 \times 10^6$

<sup>d</sup> Number of cells plated was  $5.3 \times 10^6$

macrophages as indicated. The rate of uptake was seen to vary somewhat between the two preparations of cells. In general, results of many such experiments show a one to two fold variation in uptake rates between different batches of macrophages. From the results in Table 2, it can be seen that the cpm of  $^{125}\text{I}$ -BSA accumulated by the cells was dependent on the initial cpm/ml in the media. When the data is normalized for the different cpm/ml media and cell numbers by expressing the results as  $\mu\text{l}$ s of media ingested per  $10^6$  cells, it can be seen that the amount of labeled media ingested is relatively constant. If the uptake were receptor mediated, one would expect that the calculated volume of media taken up per  $10^6$  cells would appear to decrease with increasing concentration of labeled protein in the media. Therefore, if receptors are involved in BSA uptake, they could not have been saturated with BSA under these conditions. This might be expected in view of the large quantities of extraneous proteins present in the uptake media arising from the presence of fetal calf serum (50). Consistent with this finding is the observation that excess cold BSA does not inhibit the uptake of labeled BSA (Table 3). These results also eliminate the possibility

TABLE 3

<sup>125</sup>I-BSA Uptake in the Presence of Varying Amounts of Cold BSA<sup>a</sup>

Cold BSA Added (mg/ml)	Cell Associated Radioactivity (cpm)	$\mu\text{l}/10^6$ Cells <sup>b</sup>
0	15,420;16,542	0.023
0.1	12,314;17,686	0.021
1.0	11,699;19,888	0.023

<sup>a</sup> Uptake was performed in media containing no FCS for 18 hours with  $1.4 \times 10^7$  cpm/ml <sup>125</sup>I-BSA (0.01mg/ml).

<sup>b</sup> Average of 2 determinations

that the cell associated radioactivity represents protein non-specifically bound to the surface of cells or the culture dishes.

Similar results have been obtained in uptake studies of labeled albumin macrophages by others (104). Results obtained for uptake of other proteins in this work are generally similar.

Uptake experiments were also performed with  $^{125}\text{I}$ -BSA before and after dialysis in order to verify that uptake was not due to the accumulation of free  $^{125}\text{I}$  by the cells or the binding of free  $^{125}\text{I}$  to the culture plates. The results for the dialyzed  $^{125}\text{I}$ -BSA were similar to those for  $^{125}\text{I}$ -BSA before dialysis (Table 4).

Blank plates, those with no cells added, treated in the same manner as the plates with cells, took up less than 10% of the amount of label as did plates containing cells, further indicating that the apparent accumulation of radioactivity by the cells was not due to nonspecific binding of label to the plates.

The simultaneous addition of lysosomal protease inhibitors with the  $^{125}\text{I}$ -BSA caused an increased overnight accumulation of radioactive label (Table 5). This could



TABLE 4

<sup>125</sup>I-BSA Uptake With Nondialyzed and Dialyzed <sup>125</sup>I-BSA<sup>a</sup>

<u>Sample</u>	<u>% TCA Soluble</u>	<u>μl/10<sup>6</sup> Cells<sup>b</sup></u>
Nondialyzed	7.5	0.018 <sup>c</sup> ± 0.004
Dialyzed	0.3	0.016 <sup>d</sup> ± 0.001

<sup>a</sup>Uptake was performed in media with 50% FCS for 16 hours.

<sup>b</sup>Results expressed as mean ± standard deviation

<sup>c</sup>Average of 5 determinations

<sup>d</sup>Average of 4 determinations

TABLE 5

<sup>125</sup>I-BSA Uptake in the Presence of Inhibitors<sup>a</sup>

Inhibitor	Inhibitor Concentration in Media (mg/ml)	Volume of Media in Cells $\mu\text{l}/10^6$ Cells
None	-	0.013 $\pm$ .006 <sup>b</sup>
Antipain	0.29	0.050 $\pm$ .017 <sup>b, c</sup>
Leupeptin	0.29	0.073 $\pm$ .012 <sup>b, d</sup>
Pepstatin	0.06	0.040 $\pm$ .010 <sup>b, c</sup>
Chloroquine	0.05	0.060 $\pm$ .014 <sup>e</sup>

<sup>a</sup>Uptake was performed in media with 50% FCS for 18 hours.

<sup>b</sup> 3 Determinations. Mean  $\pm$  standard deviation

<sup>c</sup>Difference from control statistically significant,  
p < .05.

<sup>d</sup>Difference from control statistically significant,  
p < .01.

<sup>e</sup>Average of two determinations

$$\pm \sqrt{\frac{\sum x^2}{n - 1}} \quad .p < .1.$$

be due to decreased degradation of the pinocytosed labeled protein by the cells.

The effect of alterations in protein structure on the overnight accumulation of labeled BSA was also investigated. Binding and nonbinding fractions of Affi-gel blue purified BSA were found to be accumulated by macrophages at comparable rates (Table 6). In contrast,

<sup>125</sup>I-BSA denatured by boiling, which demonstrated a decreased binding to the Affi-gel blue column, showed an increased accumulation uptake after an overnight incubation with cells.

The uptake of other iodinated proteins by macrophages following overnight incubation is shown in Table 7. The value reported for ovalbumin was obtained both at ovalbumin concentrations of 1.1  $\mu\text{g/ml}$  and 45  $\mu\text{g/ml}$ . The observation that the calculated volume of media is independent of the concentration of label in the media is evidence that uptake of this protein was also non-receptor mediated. The uptake of  $\beta$ -galactosidase denatured by freezing was less than that of the native form after an overnight incubation with cells. This is in contrast to the results obtained with native and denatured BSA. However, it will subsequently be shown that the denatured

TABLE 6

Effect of Protein Conformation on the Uptake of  $^{125}\text{I}$ -BSA<sup>a</sup>

Affi-Gel Blue Column Samples

Sample	Radioactivity in Uptake Media (cpm/ml x 10 <sup>6</sup> )	Calculated Volume of Media Taken Up $\mu\text{l}/10^6$ Cells <sup>b</sup>
Before column	3.9	0.020 $\pm$ .006 <sup>c</sup>
Binding fraction	3.5	0.023 <sup>d</sup>
Nonbinding fraction	3.5	0.018 <sup>d</sup>

Boiled  $^{125}\text{I}$ -BSA Samples

Sample	cpm/ml x 10 <sup>6</sup> Uptake Media	$\mu\text{l}/10^6$ Cells <sup>b</sup>
Before boiling	6.3	0.018 $\pm$ 0.002 <sup>c</sup>
After boiling	1.5	0.087 $\pm$ 0.012 <sup>e</sup>

<sup>a</sup> Uptake was performed in media with 50% FCS for 24 hours.

<sup>b</sup> Values given as the mean  $\pm$  standard deviation

<sup>c</sup> Average of 3 determinations

<sup>d</sup> Value represents a single determination

<sup>e</sup> Average of 5 determinations. Difference statistically significant ( $p < .0005$ ).

TABLE 7

Uptake of  $^{125}\text{I}$ -Labeled Proteins<sup>a</sup>

<u>Protein</u>	<u><math>\mu\text{l}/10^6</math> Cells<sup>b</sup></u>
Ribonuclease	$0.043 \pm 0.015^c$
Arginase	$0.034 \pm 0.015^d$
Enolase	$0.078 (0.05, 0.10)^e$
Ovalbumin	$0.263 \pm 0.010^f$
Catalase	$0.049 \pm 0.016^f$
BSA	$0.030 \pm 0.012^g$
Phosphorylase b	$0.220 (0.340, 0.100)^e$
$\beta$ -Galactosidase	$0.090 (0.091, 0.089)^e$
Thyroglobulin	$0.396 \pm 0.110^f$

<sup>a</sup>Uptake was performed in media with 50% FCS for 18 to 24 hours.

<sup>b</sup>Values expressed as the mean  $\pm$  standard deviation

<sup>c</sup>Average of 4 determinations

<sup>d</sup>Average of 5 determinations

<sup>e</sup>Average of 2 determinations. Individual values in parentheses

<sup>f</sup>Average of 3 determinations

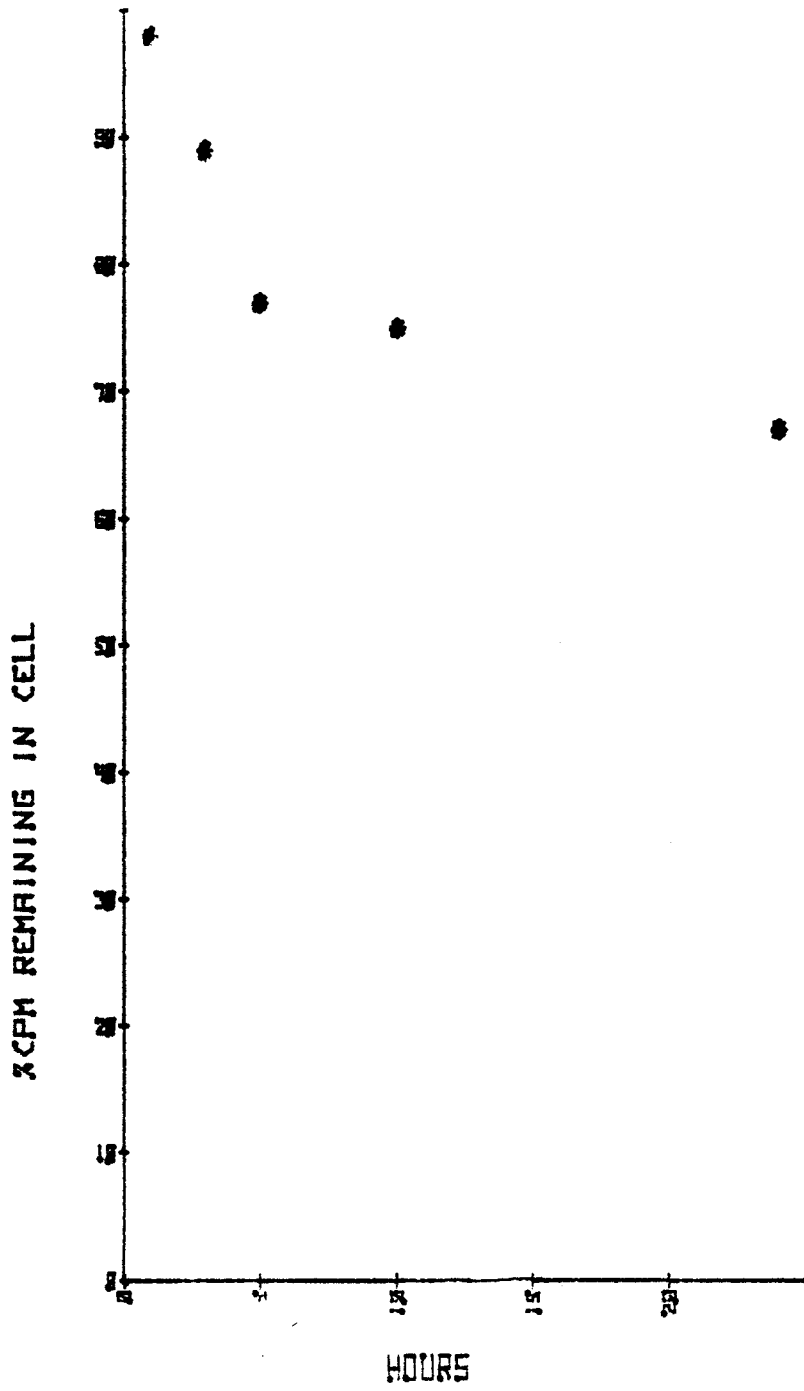
<sup>g</sup>Average of 17 determinations

form of  $\beta$ -galactosidase is degraded at a very much greater rate than the native form. Since the amount accumulated probably represents a steady state between uptake and degradation, one would expect less of the denatured form to accumulate.

#### D. Release of Nondigestible Materials

The release of labeled dextran-sulfate into the media from cultured macrophages was studied in order to determine if nondigestible macromolecules could escape from the cells and, if so, to quantitate the extent of this release (Figure 8). This experiment was performed by counting both the cells and the media at various times during the release. It can be seen that there appears to be a rapid release of radioactivity within the first 5 hours, followed by a very slow loss of label from the cells (less than 0.5% per hour). Approximately 20% of the label was lost in the initial phase. Similar results have been obtained in studies of the uptake and release of  $^{125}\text{I}$ -PVP and insoluble  $^{128}\text{Au}$  labeled gold salts (105). This has been attributed to regurgitation of the contents of some of the pinocytic and phagocytic vesicles by the macrophages rather than to cell detachment and/or cell death.

Figure 8. Release of <sup>14</sup>C-activity from macrophages that had pinocytosed 1 mg/ml dextran-sulfate (32,000 cpm/ml) for 24 hours. The samples were then washed and fresh media added. Uptake and release media contained 30% FCS. Each point represents a single plate that was washed, the cells scraped off the plates in 0.2 mls water, and Bray's solution added to the cell suspension for scintillation counting.



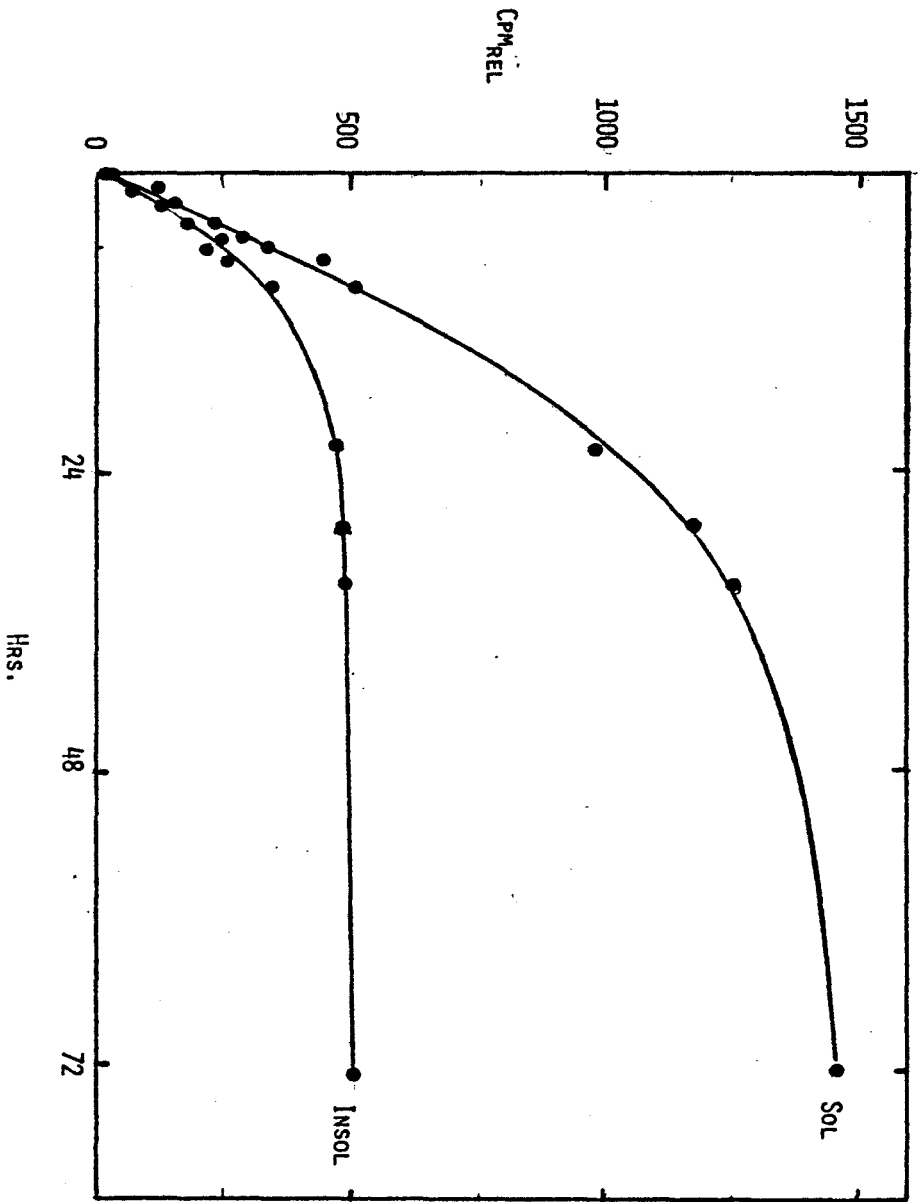


### E. Degradation of Protein Substrates

The release of label from cells containing digestible  $^{125}\text{I}$ -labeled protein was also studied. Figure 9 illustrates the time course of the appearance of radioactivity in the media after uptake of  $^{125}\text{I}$ -BSA for 15 hours. As with dextran-sulfate, the release of TCA insoluble radioactivity is not linear. Approximately 70% of the released TCA insoluble radioactivity appears in the initial rapid phase. During the slow phase, less than 0.1% per hour of the total label within the cell is released as TCA insoluble radioactivity. The similarity between this result and that obtained with labeled dextran-sulfate suggests that the release of TCA insoluble radioactivity represents regurgitation of intact protein. The release of TCA soluble radioactivity also appears not to be linear. In this case, however, the release of TCA soluble label continues well after the release of insoluble label has reached a plateau. Similar results were obtained with the other proteins studied.

One object of the experiments, whose results are shown in Figure 9, is to measure the rate of degradation of pinocytosed labeled proteins. One method is to plot the log of the percent of radioactively labeled protein

Figure 9. Release of  $^{125}\text{I}$ -activity in the media from macrophages incubated with  $1 \times 10^7$  cpm/ml  $^{125}\text{I}$ -BSA in 50% FCS for 15 hours. Media samples (1% FCS) were removed from a single plate at various time points and precipitated with an equal volume of 20% TCA - 1% phosphotungstic acid before counting. The cells were counted at the end of the experiment.



remaining in the cell vs time. The percent cpm remaining could be obtained with the help of the following relationship:

$$\% \text{cpm remaining} = \frac{\text{Total cpm taken up} - \text{cpm released}}{\text{Total cpm taken up}} \times 100$$

However, if the percent cpm remaining is plotted vs time it yields estimates for the rate of protein degradation which are too large because this equation fails to account for label which is released through regurgitation of intact or partially degraded protein. Alternatively, we could express percent cpm remaining as being equal to:

$$\frac{\text{Total cpm taken up} - \text{TCA soluble cpm released}}{\text{Total cpm taken up}} \times 100.$$

However, this underestimates rates of protein degradation by overestimating the amount of label remaining in the cell since the loss of TCA insoluble radioactivity through regurgitation is not counted. Clearly, therefore, some method is needed to correct for the loss of TCA insoluble label from the cells with time. An exact and rigorous correction is not possible because the mechanism by which the TCA insoluble radioactivity is released is unknown.

As a first approximation we have assumed that the TCA insoluble radioactivity released represents labeled substrate which is regurgitated prior to any significant exposure to the hydrolytic enzymes in the cell. Accordingly, we have subtracted the TCA insoluble radioactivity released from the total radioactivity taken up by the cell. The amount of labeled protein taken up which is available for degradation is then expressed as being equal to the sum of the TCA soluble radioactivity released and the radioactivity remaining in the cell at the end of the release experiment as shown below:

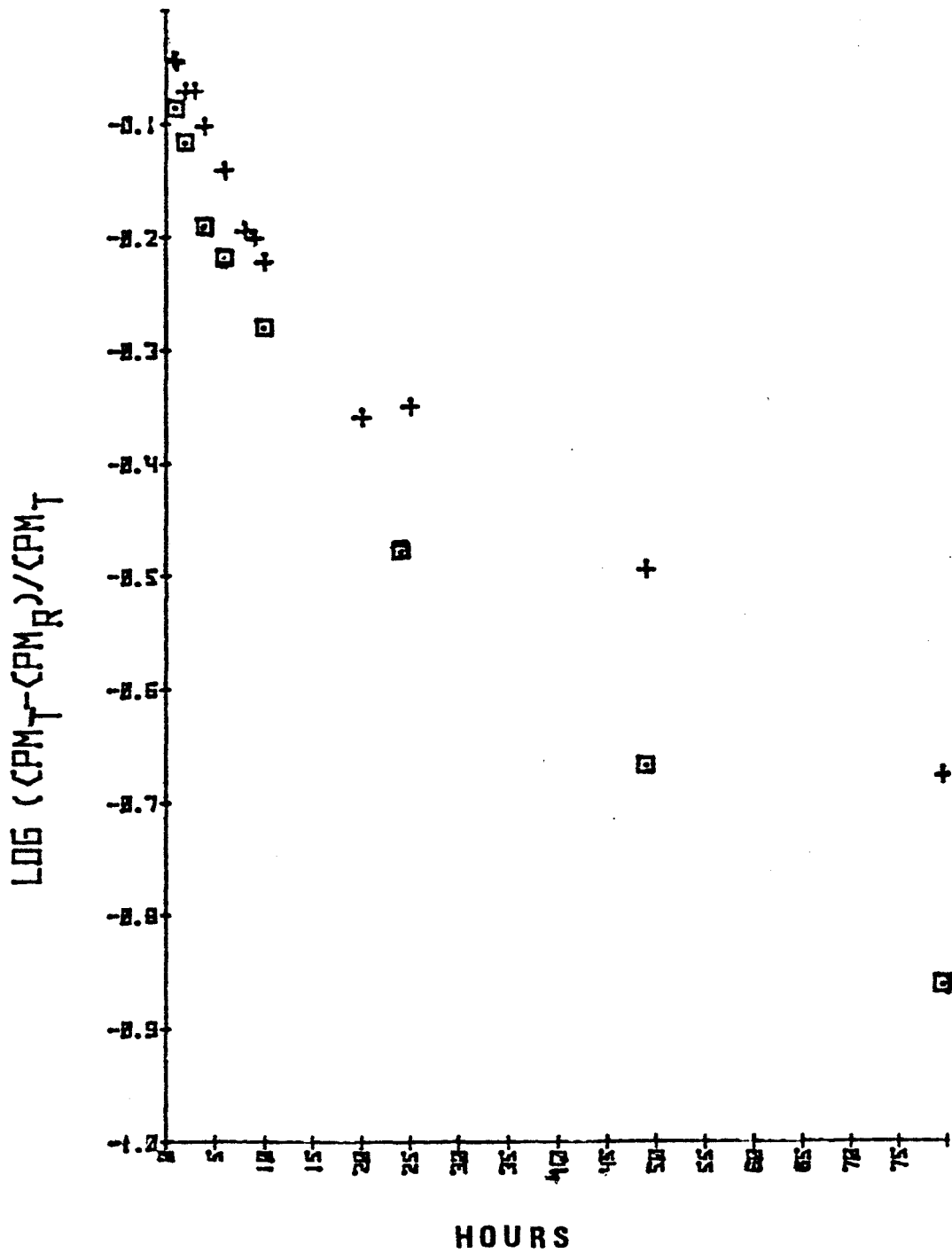
$$\begin{array}{l} \text{Total cpm avail-} \\ \text{able for hydrol-} \\ \text{ysis} \end{array} = \begin{array}{l} \text{Total TCA sol-} \\ \text{uble cpm re-} \\ \text{leased} \end{array} + \begin{array}{l} \text{TCA cpm remaining at} \\ \text{end of release} \\ \text{period} \end{array}$$

The percent radioactivity remaining in the cell at any time,  $t$ , is thus given by:

$$\begin{array}{l} \% \text{ cpm remain-} \\ \text{ing}_t \end{array} = \frac{\begin{array}{l} \text{Total cpm} \\ \text{available} \end{array} - \text{TCA soluble cpm released}_t}{\begin{array}{l} \text{Total cpm} \\ \text{available} \end{array}} \times 100$$

Figure 10 illustrates the results in Figure 9 replotted according to this equation (closed circles). The open circles show the same data uncorrected for the release of TCA insoluble label. In the latter case, the initial rate of release of label (initial slope) appears more

Figure 10. A replotting of Figure 9 using the equation  $(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t = \% \text{ cpm remaining at time } t$ . The log of this value is plotted versus the time in hours. The crosses represent the uncorrected data plotted in this fashion. The squares show the same data corrected for TCA insoluble label.



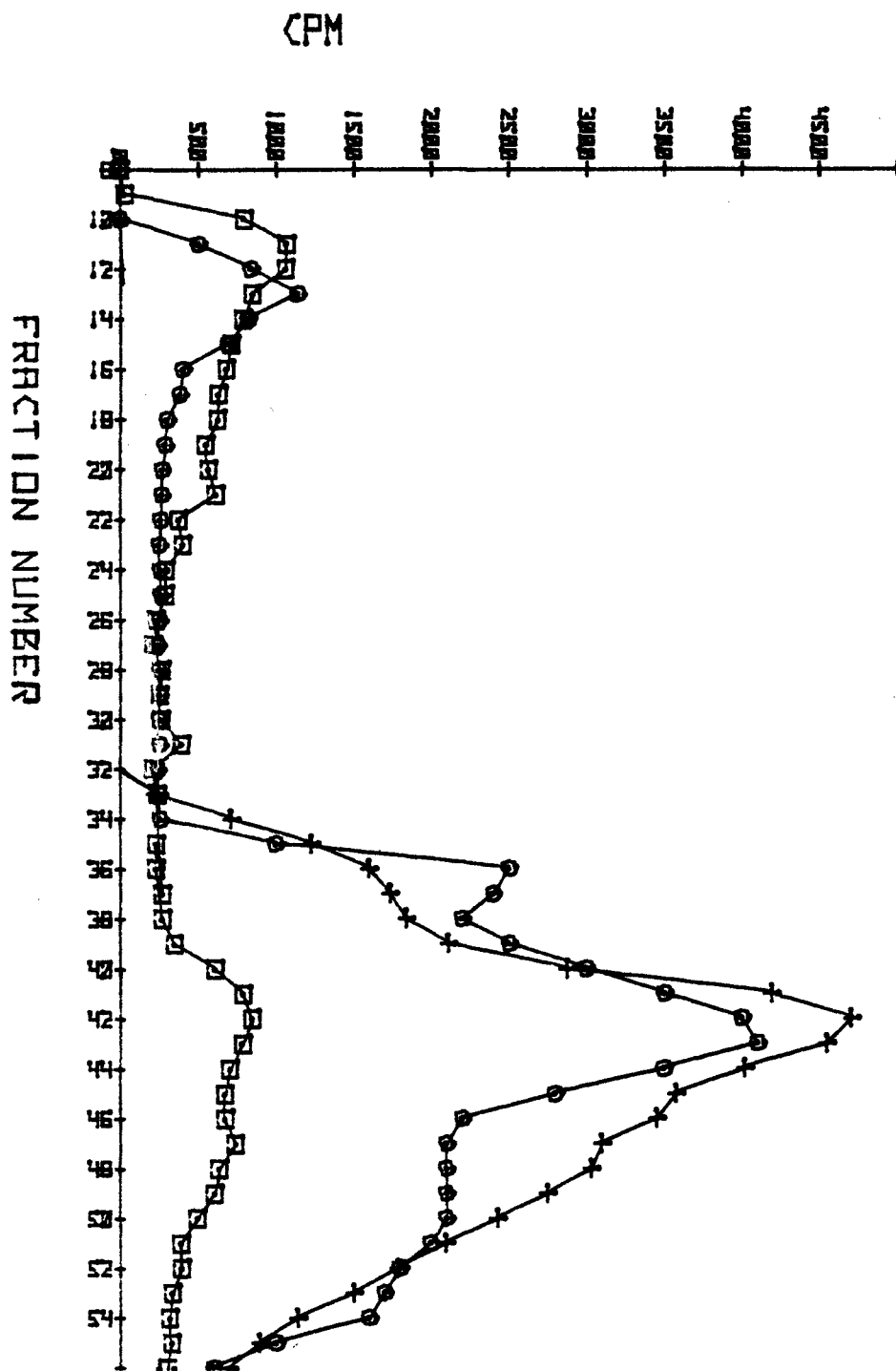
rapid because of regurgitation of TCA insoluble label from the cells. However, the final rates of release are more nearly similar for both curves since by 20 hours the rate of loss of label is due primarily to protein degradation in both cases.

In order to verify that the TCA insoluble radioactivity released by the cells into the media represented larger molecular weight species, and that TCA precipitation was effective in removing these, media samples were analyzed by Sephadex chromatography before and after precipitation with TCA. Figure 11 shows the Sephadex G-50 elution profiles for the release media obtained on incubating  $^{125}\text{I}$ -BSA laden fibroblasts in unlabeled media for 24 hours. Similar results were also obtained with release media from  $^{125}\text{I}$ -BSA laden macrophages. The elution profile for the media prior to TCA precipitation shows the presence of both large and small molecular weight species. The small molecular weight peak was made up of at least 3 components. This is in agreement with results obtained by others (104, 126-128) which demonstrated the release of labeled monoiodotyrosine, diiodotyrosine, glycyliodotyrosine and free  $^{125}\text{I}$  from cells but no species of intermediate molecular weight (300 to 10,000). TCA precipitation of the media removed the high molecular weight



Figure 11. Chromatography of media (O), TCA soluble media (+), and cell extracts (■) from a

<sup>125</sup>I-BSA release experiment where fibroblasts were allowed to take up label overnight. Each 1.5 ml fraction was counted directly in a gamma counter. The Sephadex G50 column was eluted with 0.05M phosphate, 0.15 M NaCl, pH 7.3 at 24 ml/hour. The column was previously calibrated and  $V_0$  corresponded to fraction 12 while  $V_i$  was fraction 42.



material. Similar elution profiles were obtained for media samples from macrophages fed  $^{125}\text{I}$ - $\beta$ -galactosidase and  $^{125}\text{I}$ -phosphorylase b. In each case, the high molecular weight component eluted in the void volume of the column indicating a molecular weight greater than 30,000. SDS electrophoresis of media yielded similar results; only large and small iodinated molecular weight species present in the release media. In addition, the TCA insoluble material was shown to be of the same size as the labeled protein before addition to the media.

The degradation and release of  $^{125}\text{I}$  activity by cells fed  $^{125}\text{I}$ -BSA appeared to be nonlinear when plotted on semilog paper (Figure 10). There are a number of possible explanations for this behavior. Some of these are:

- 1) The  $^{125}\text{I}$ -BSA preparations used are heterogeneous, containing impurities which are degraded at rates which differ from that of authentic  $^{125}\text{I}$ -BSA.
- 2) The  $^{125}\text{I}$ -BSA preparations contain denatured forms of BSA which are degraded at different rates than native BSA.

- 3) The <sup>125</sup>I-BSA preparations are relatively homogeneous, but the physical and chemical environments of the individual labeled amino acid residues in the protein are heterogeneous.
- 4) All the labeled amino acid residues in the protein are equally susceptible to release by cellular proteases but the cells themselves are heterogeneous with some having a greater hydrolytic capacity than others.
- 5) All the cells are homogeneous but each cell contains a heterogeneous population of lysosomes, which remain segregated during the release experiment, and which have different levels of various hydrolases.
- 6) The rate of release becomes progressively slower with time because of progressive changes which occur in the media during the course of release.
- 7) Some combination of the above explanations.

To distinguish between some of these possible explanations, release experiments were performed utilizing a variety of labeled protein substrates, different batches of macrophages, different cell types, and a different experimental procedure. The results of these experiments, which will be described below, tend to support explanation 3 for the biphasic appearance of the protein degradation curves.

To determine if the nonlinear curves for the release of label from  $^{125}\text{I}$ -BSA laden macrophages is due to the presence of labeled impurities or denatured forms of the protein, commercial preparations of  $^{125}\text{I}$ -BSA were purified by affinity chromatography on Affi-gel blue. This gel binds albumin through an anion binding site on the protein and denaturation of the protein would presumably lead to the destruction of this site. Both the binding and non-binding fractions of these labeled BSA preparations yielded nonlinear release curves which were similar to each other and to those obtained with commercial preparations before purification. Curves obtained with heat treated  $^{125}\text{I}$ -BSA show some initial enhancement in the rate of degradation of the pinocytosed protein substrate but are still remarkably similar to curves for

the native protein (Figure 12). These results argue against explanations 1 and 2.

To determine if the nonlinear curves were due to the presence of heterogeneity in the population of macrophages used in these studies, the experiments were repeated with a number of different batches of stimulated macrophages (thioglycolate media induced macrophages), with activated macrophages (Freunds adjuvant induced macrophages), and with baby hamster kidney fibroblasts. All preparations of stimulated macrophages gave similar time curves. In addition, while it was observed that activated macrophages, stimulated macrophages, and fibroblasts degraded albumin at slightly different rates, the shapes of the curves were similar suggesting that the curvature was a property of the protein and not the cell preparations. This conclusion was reinforced by results obtained for the degradation of other protein substrates. Figures 13 through 23 show the data obtained for protein substrated. The lines were generated by linear regression of the data points. In many cases, the early time points appeared to fall above the line while the intermediate time points appeared to be below the line. In addition, in almost all cases, the least squares line did not

Figure 12. Release of TCA soluble  $^{125}\text{I}$ -activity from macrophages incubated with  $6 \times 10^6$  cpm/ml heat treated  $^{125}\text{I}$ -BSA for 22 hours. Each point represents the mean  $\pm$  standard deviation. The least squares line is indicated. Correlation coefficient = 0.977.

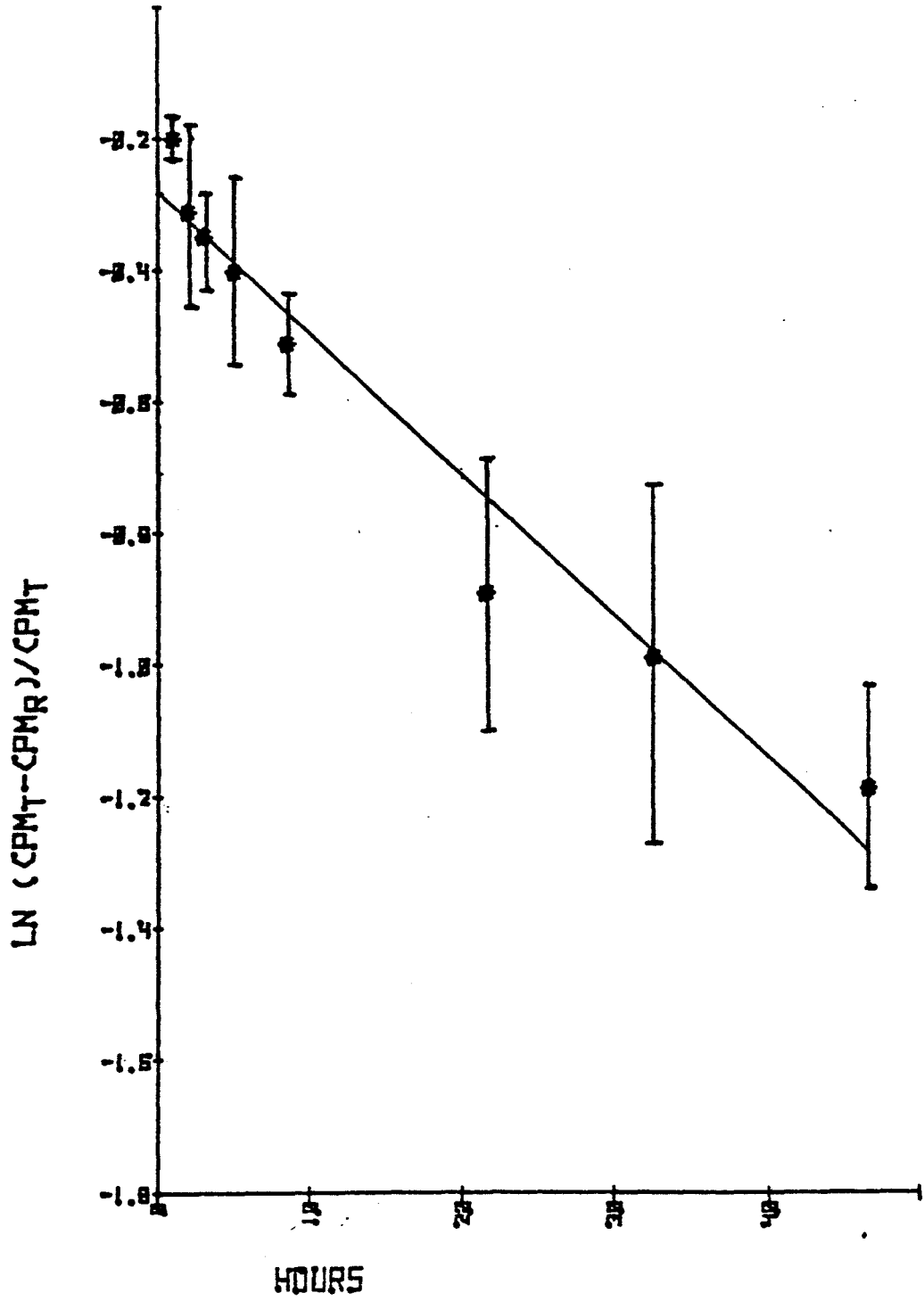




Figure 13. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}$ I-cytochrome c. The points indicate the mean  $\pm$  standard deviation for 6 experiments. Cell preparations used were numbers 7, 8, 10, and 11. The least squares line is indicated. Correlation coefficient = 0.838.

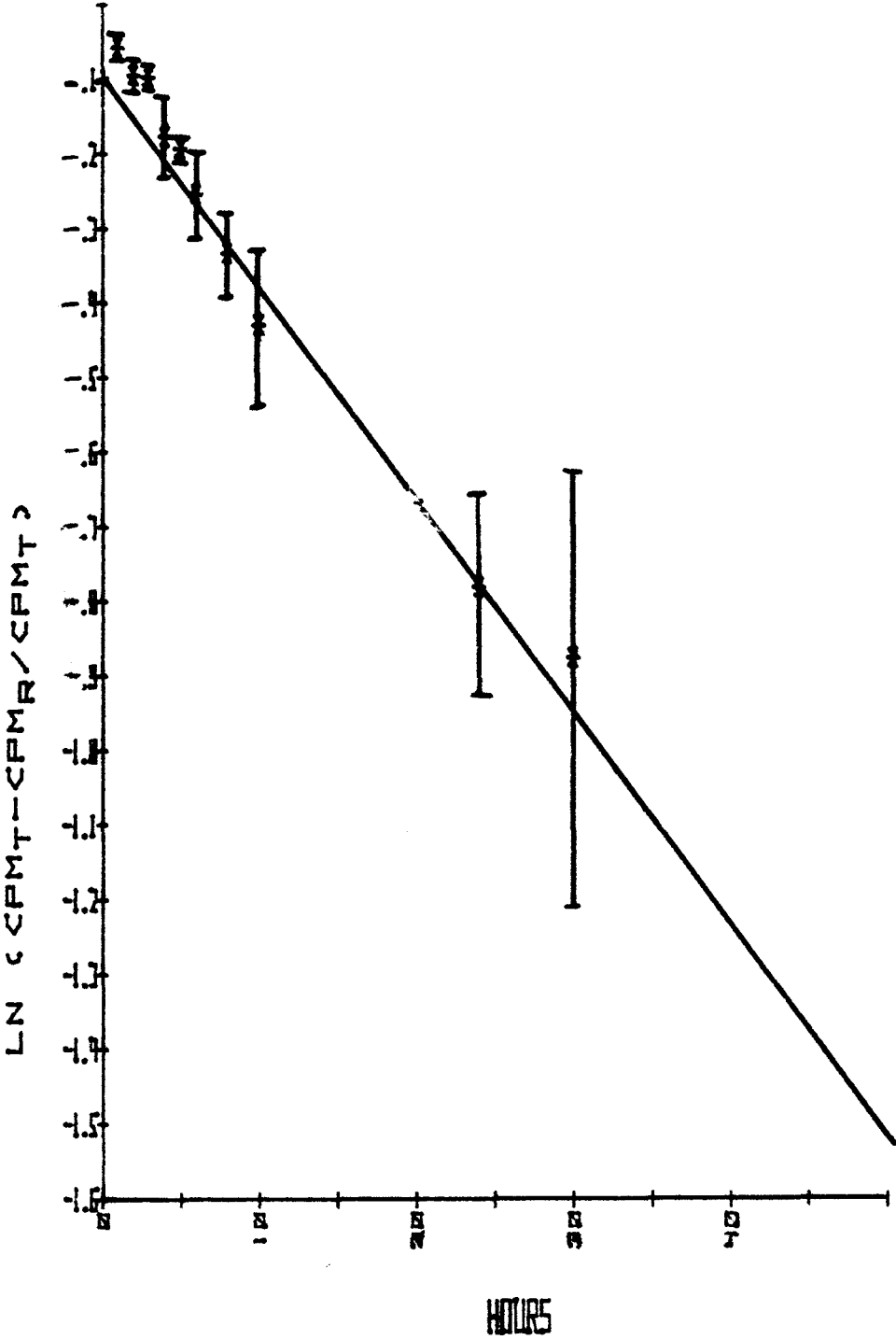


Figure 14. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -ribonuclease. The points indicate the mean  $\pm$  standard deviation for 3 experiments. Cell preparations used were numbers 12, 17, and 18. The least squares line is indicated. Correlation coefficient = 0.859.

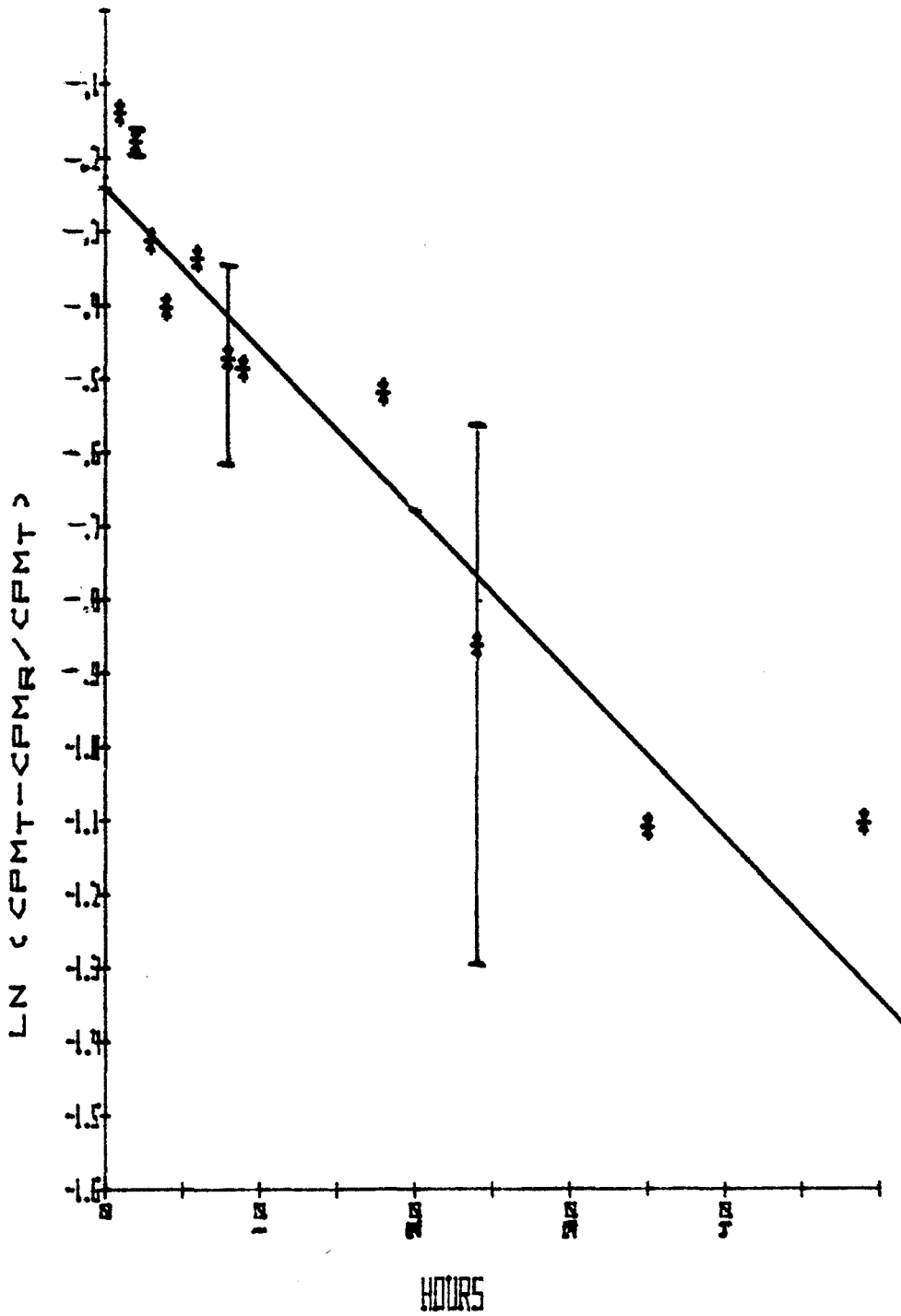


Figure 15. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -hemoglobin. The points indicate the mean  $\pm$  standard deviation for 6 experiments. Cell preparations used were numbers 9, 10, and 11. The least squares line is indicated. Correlation coefficient = 0.955.

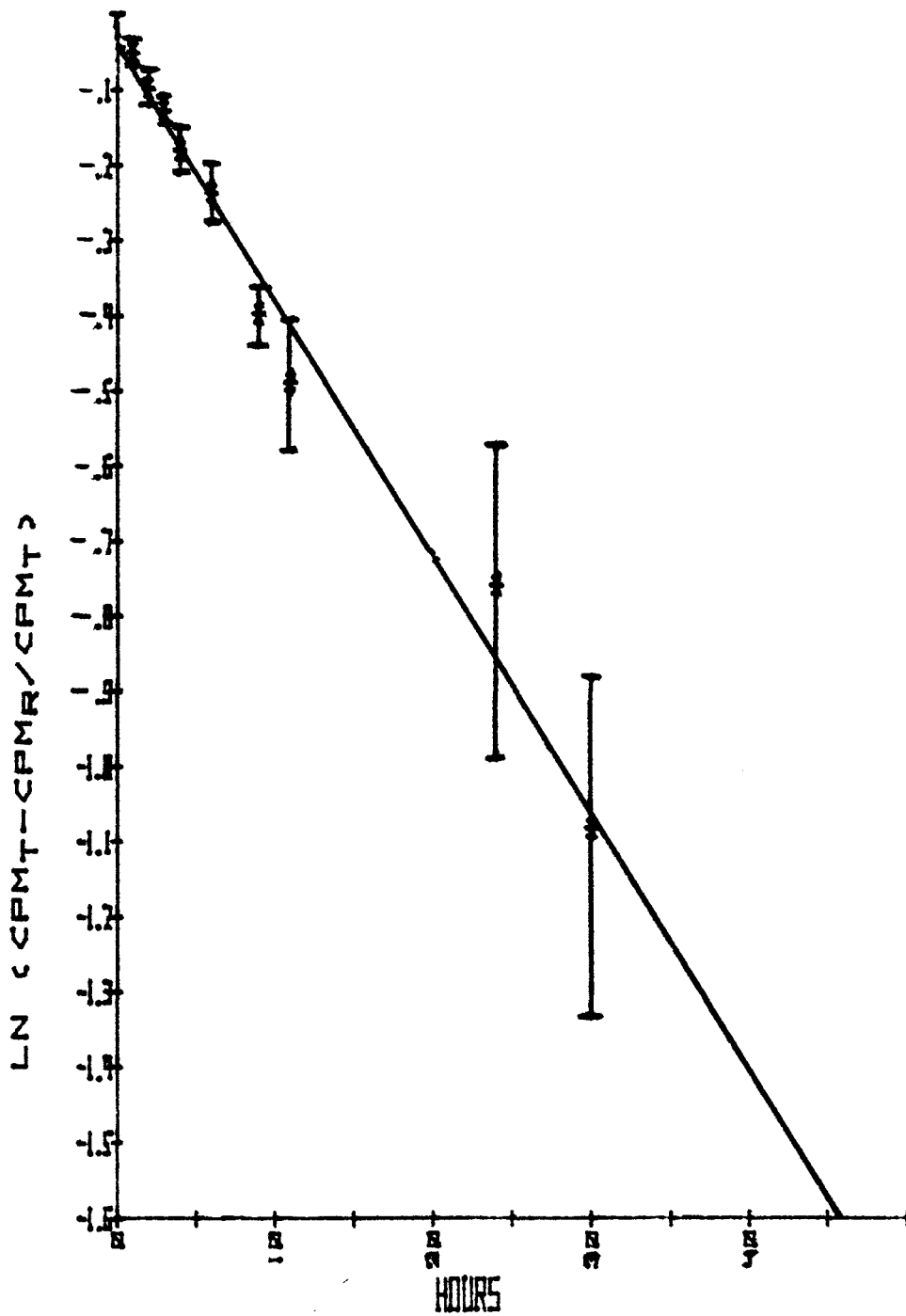


Figure 16. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -arginase.

The points indicate the mean  $\pm$  standard deviation for 7 experiments. Cell preparations used were numbers 7, 10, 11, 17, and 18. The least squares line is indicated. Correlation coefficient = 0.959.

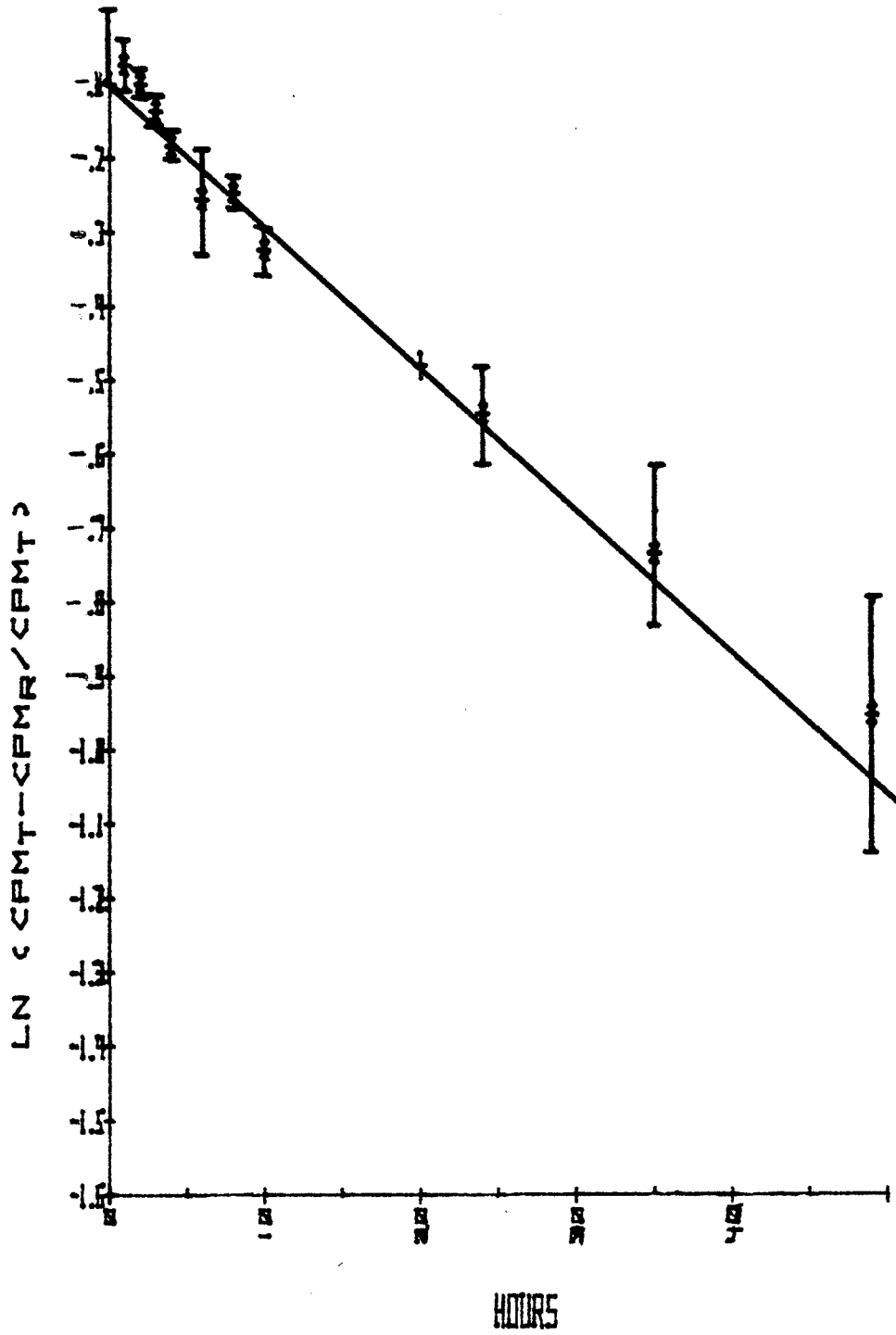




Figure 17. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -enolase. The points indicate the average  $\pm$  variation for 2 experiments. Cell preparations used were numbers 17 and 18. The least squares line is indicated. Correlation coefficient = 0.802.

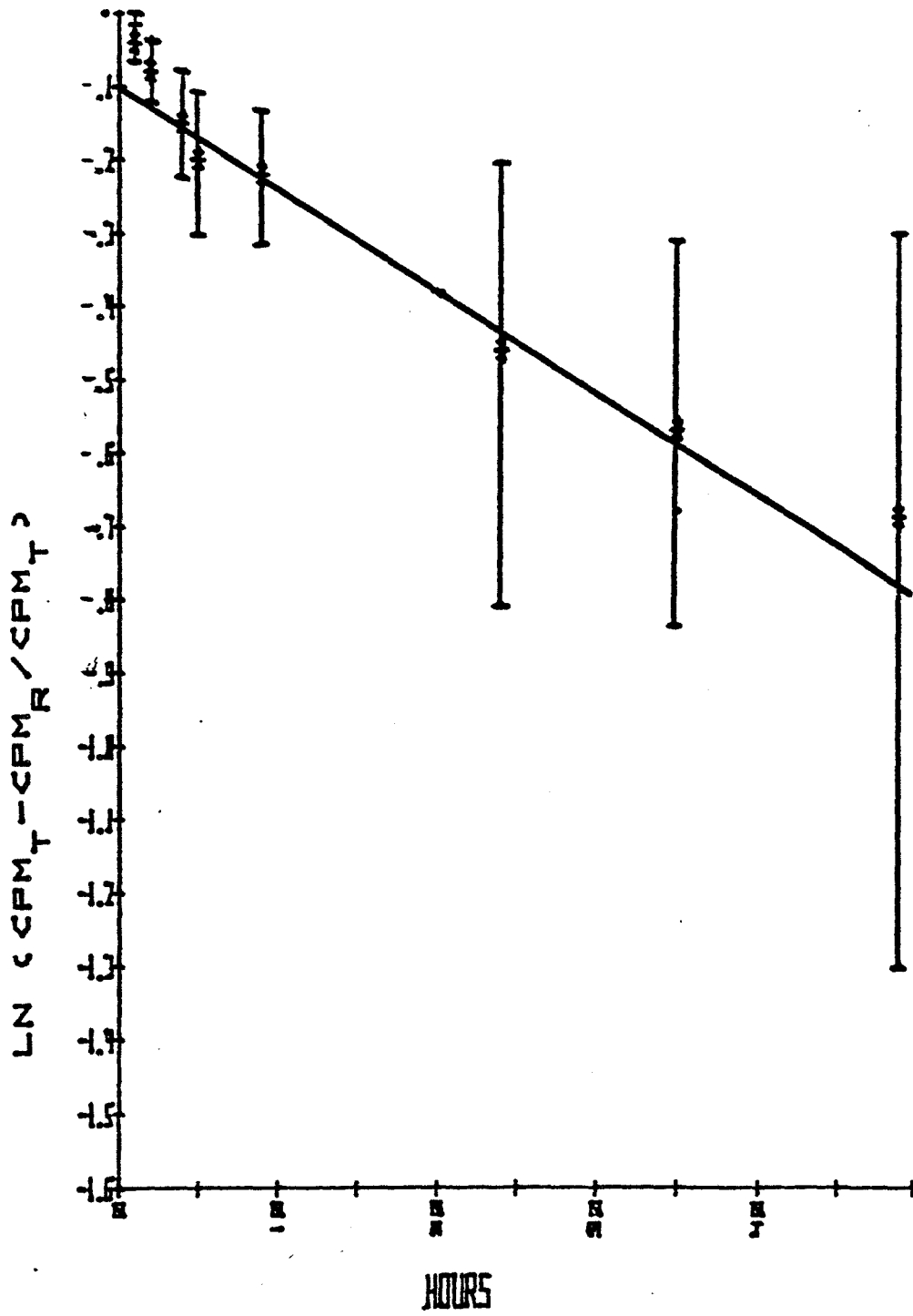


Figure 18. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}$ I-ovalbumin. The points indicate the mean  $\pm$  standard deviation for 4 experiments. Cell preparations used were numbers 5, 10, and 11. The least squares line is indicated. Correlation coefficient = 0.861.

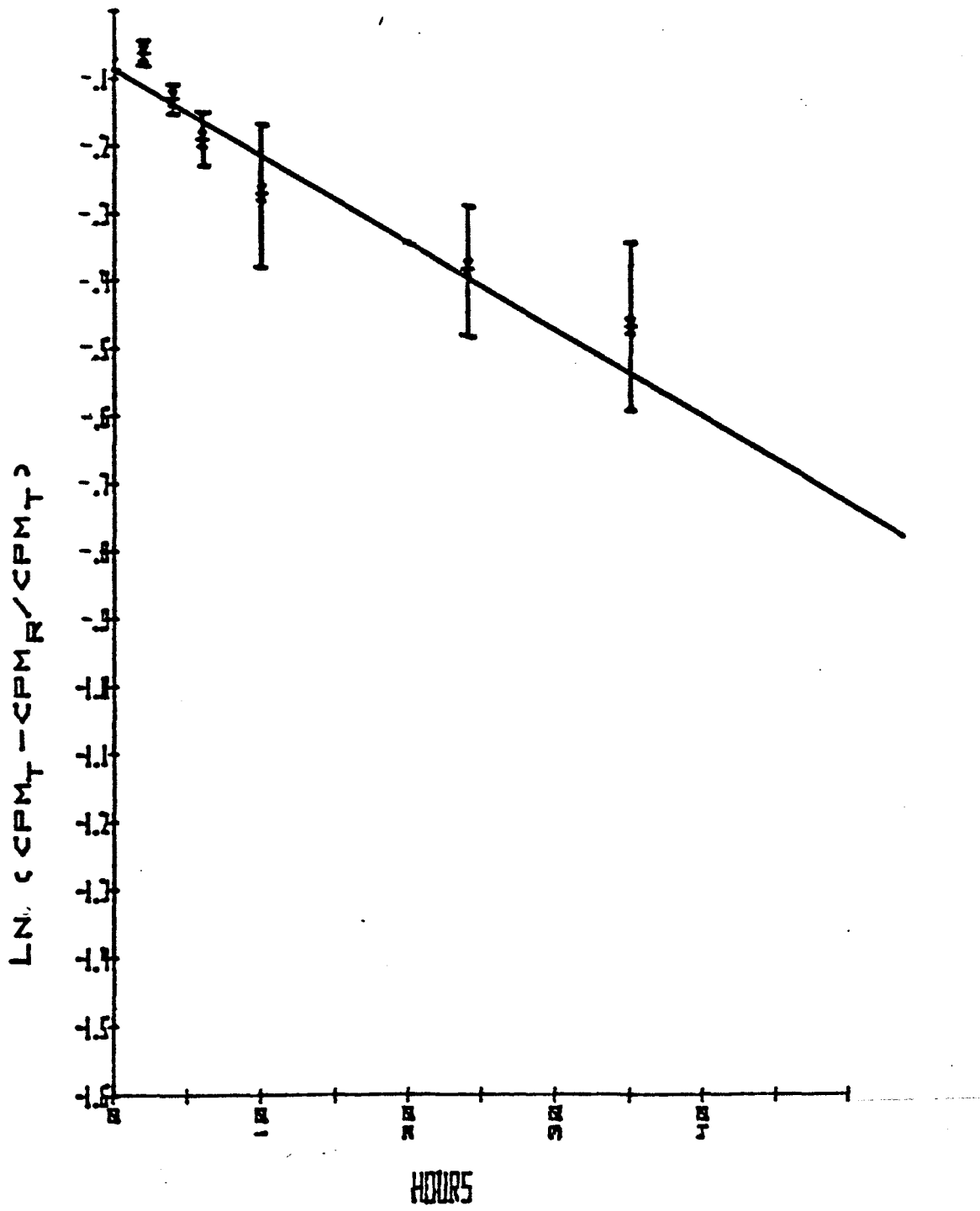


Figure 19. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -catalase. The points indicate the mean  $\pm$  standard deviation for 3 experiments. Cell preparation used was number 13. The least squares line is indicated. Correlation coefficient = 0.958.

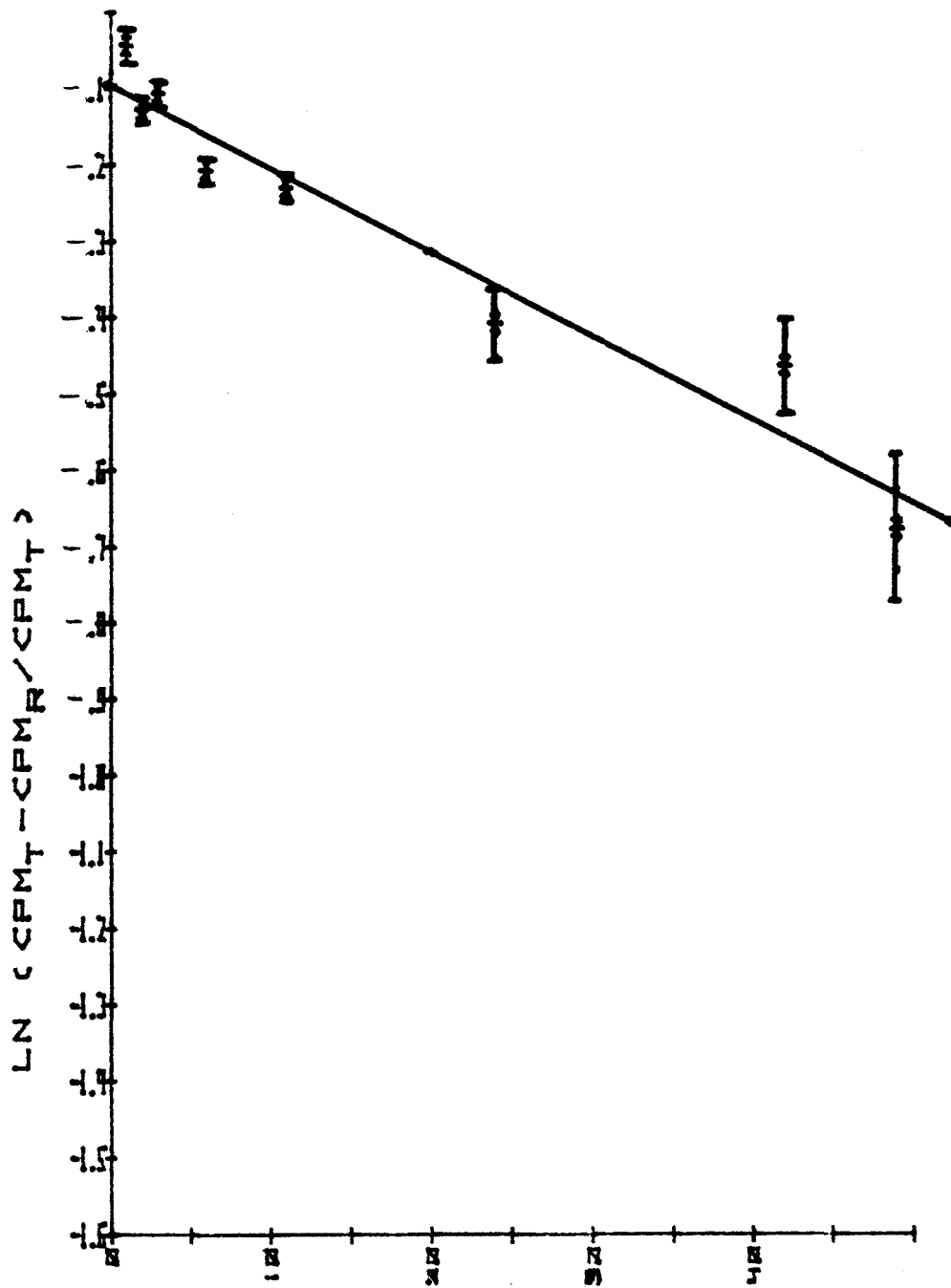


Figure 20. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -BSA. The points indicate the mean  $\pm$  standard deviation for 23 experiments. Cell preparations used were numbers 1, 2, 6, 12, 14, 15, 16, 18, 19, and 20. The least squares line is indicated. Correlation coefficient = 0.937.

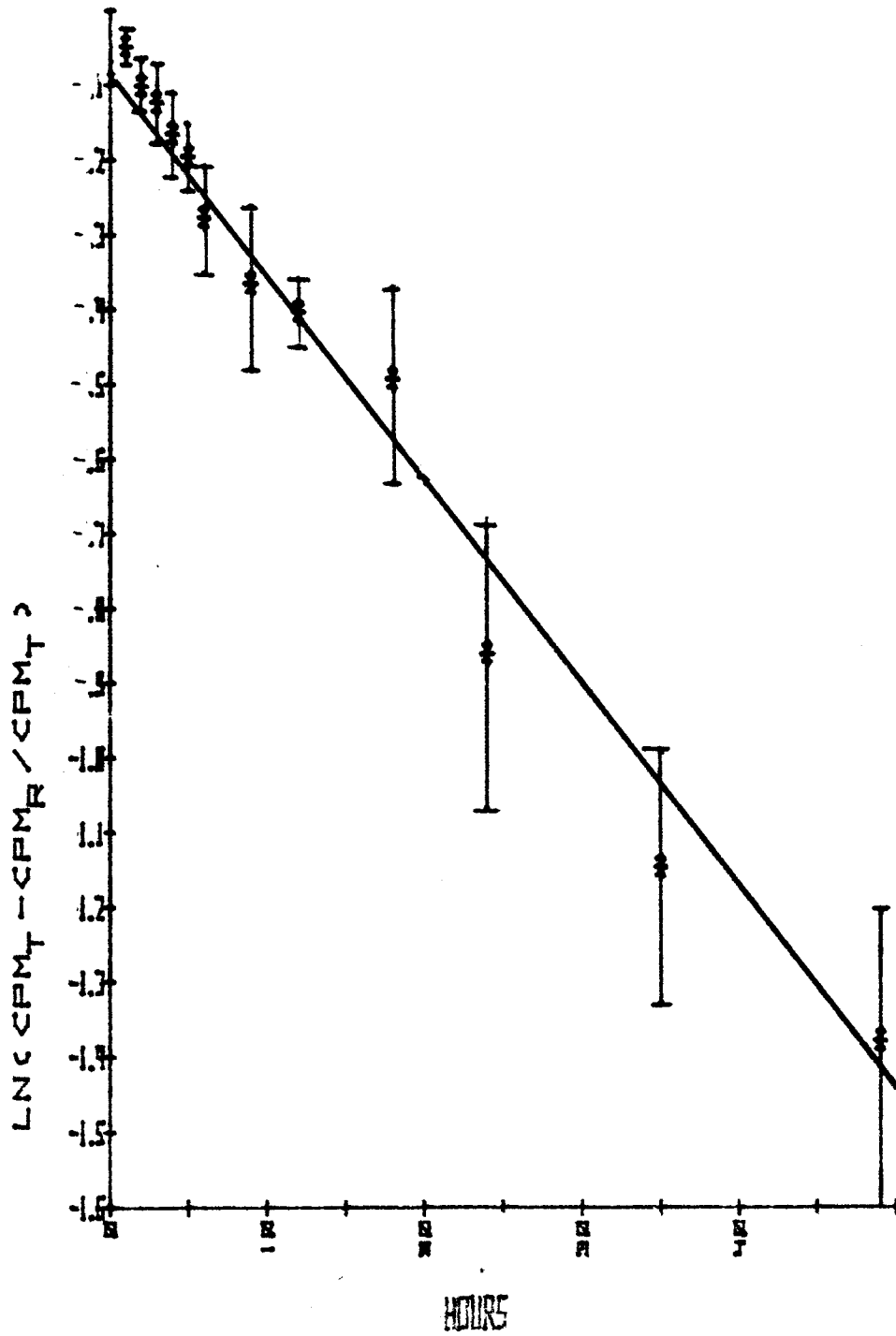




Figure 21. Release of TCA soluble radioactivity from macrophages incubated overnight with <sup>125</sup>I-phosphorylase b. The points indicate the mean  $\pm$  the standard deviation for 4 experiments. Cell preparations used were numbers 7 and 17. The least squares line is indicated. Correlation coefficient = 0.872.

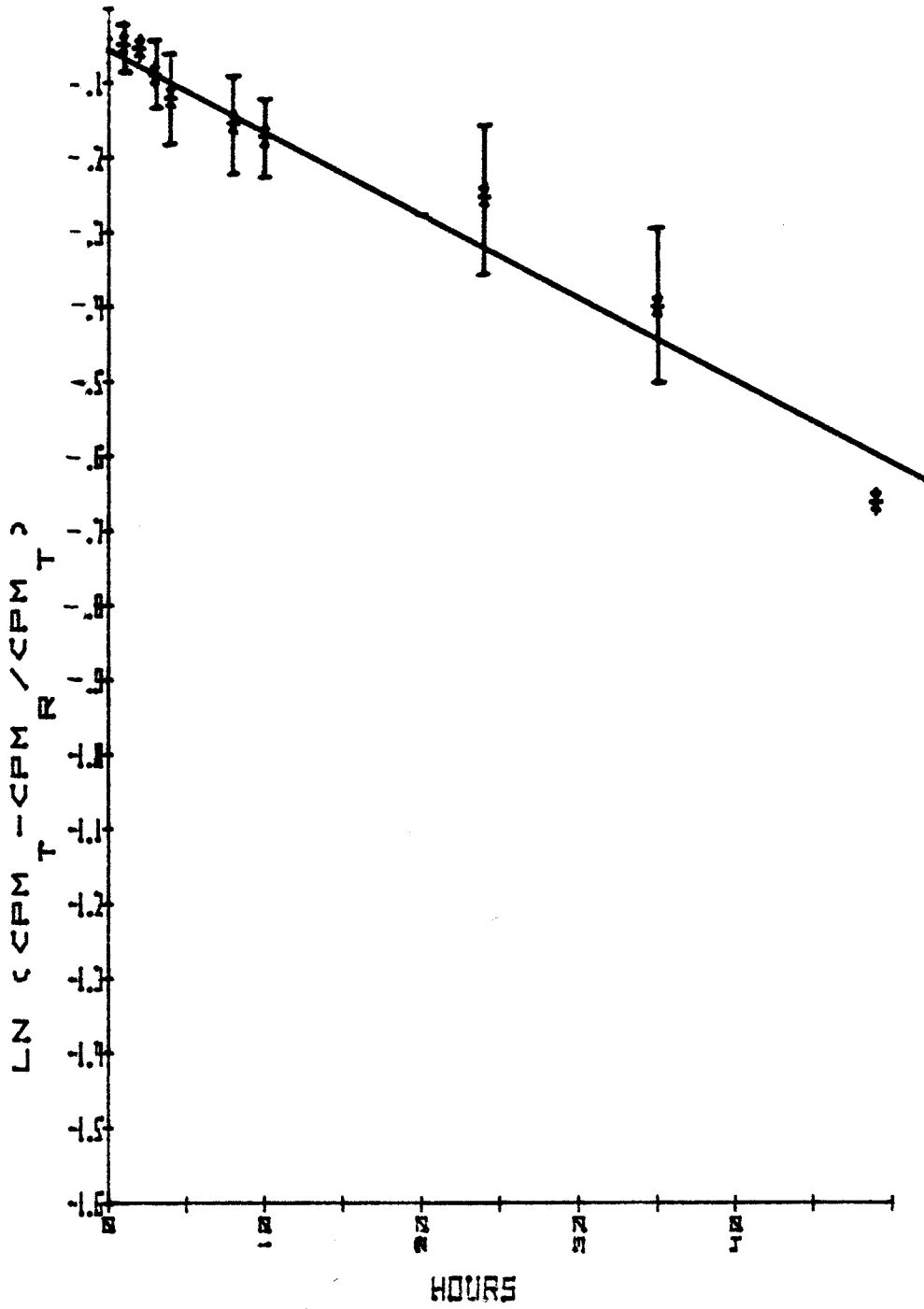


Figure 22. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ - $\beta$ -galactosidase. The points indicate the average  $\pm$  variation for 2 samples. Cell preparation used was number 13. The least squares line is indicated. Correlation coefficient = 0.937.

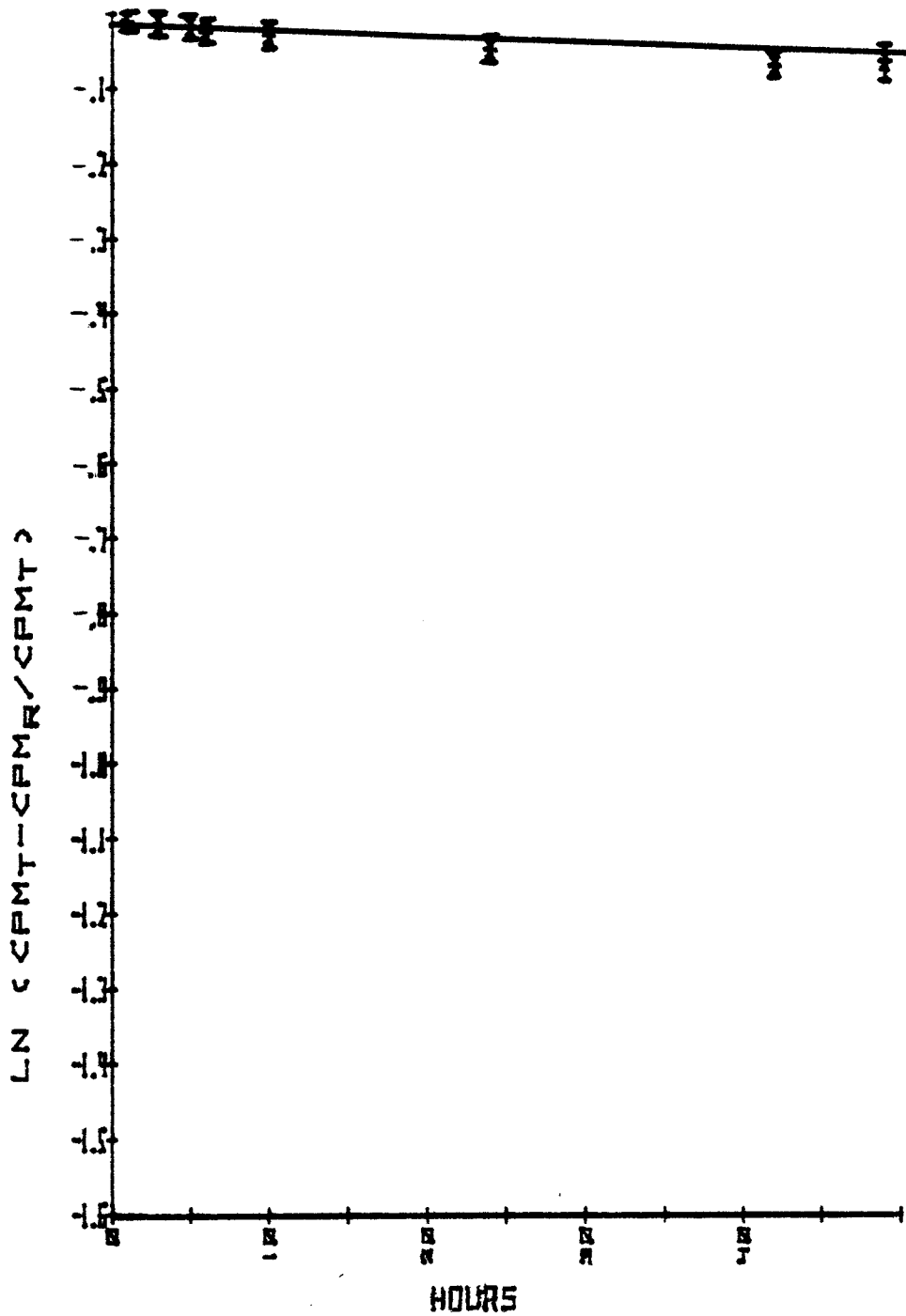
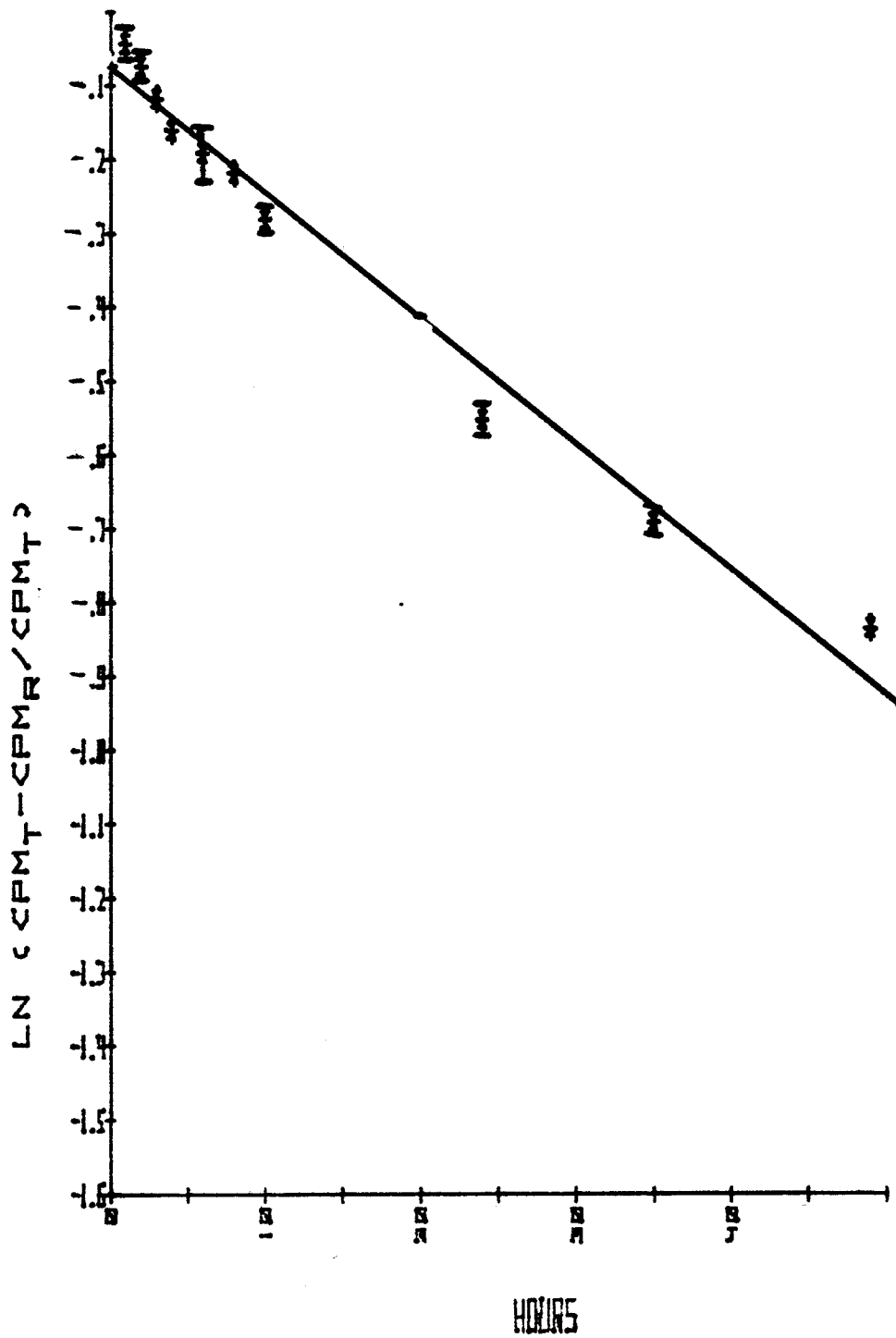


Figure 23. Release of TCA soluble radioactivity from macrophages incubated overnight with  $^{125}\text{I}$ -thyroglobulin. The points indicate the average  $\pm$  variability for 2 experiments. Cell preparations used were numbers 12 and 18. The least squares line is indicated. Correlation coefficient = 0.985.



extrapolate to zero on the Y axis. The results of the linear regression calculations are shown in Table 8. For each protein, the degradation rate constant,  $k$  ( $\text{hr}^{-1}$ ), was determined from the slope of the log plots of cpm remaining vs time. In an effort to judge the contribution of the slower phase of the curves to the total release of soluble label, least squares lines drawn through later time points ( $> 8$  hours) were extrapolated to time zero. Table 8 also contains the percent of total label released during the latter stage of the degradation reactions for the proteins studied. With the apparent exception of BSA, and thyroglobulin, the proportion of label released at the slower rate seems to increase with the increasing size of the protein.

If the fast and slow phases of the release curves were due to the contributions of two distinct populations of macrophages, one would have to postulate that the low molecular weight protein substrate was preferentially taken up by cells with a high hydrolytic activity, whereas high molecular weight substrates preferentially accumulated in cells with low levels of hydrolases. No obvious mechanism is known at present whereby this could

TABLE 8

## Calculated Rate Constants and Y - Intercepts

Protein	Apparent First Order <sup>a</sup> Rate Constant (X 10 <sup>3</sup> )	Y-Intercept <sup>b</sup>
Cytochrome c	28.3 ± 2.8	- .183 (83)
Ribonuclease	22.3 ± 3.7	- .333 (72)
Hemoglobin	33.5 ± 1.5	- .134 (87)
Arginase	18.5 ± 0.8	- .136 (87)
Enolase	14.3 ± 2.7	- .141 (87)
Ovalbumin	12.6 ± 1.6	- .185 (83)
Catalase	11.0 ± 0.8	- .110 (90)
BSA	27.3 ± 0.8	- .236 (79)
Phosphorylase b	11.0 ± 1.2	- .029 (97)
β-Galactosidase	1.2 ± 0.1	- .039 (96)
Thyroglobulin	17.5 ± 0.9	- .135 (87)

<sup>a</sup>Calculated from all data points. Value given is mean ± standard deviation. Units are h<sup>-1</sup>.

<sup>b</sup>Calculated from all data points after 8 hours.

The value in the parentheses is the percentage of label released from the cells in the slower phase.



occur. These results obtained with different protein substrates also tend to eliminate explanation 5 since no mechanism has been reported which could account for segregation of large and small pinocytosed proteins into different populations of lysosomes. Finally, the following experiment was performed to determine if the nonlinear protein degradation curves were due to accumulation of inhibitory metabolites in the media, as macrophages are known to secrete inhibitors (159), or depletion of some essential factor or nutrient from the media. Cells loaded with  $^{125}$ I-BSA as described previously were allowed to release label as before, except at each time point all the media was removed and replaced with fresh media at 37° C. The results obtained did not differ significantly from those of previous experiments. This experimental result also argued against the possibility that some of the TCA soluble radioactivity resulted from the action of extracellular proteases on any intact  $^{125}$ I-BSA present in the media. In order to more definitively eliminate this possibility, macrophages at a density of  $4 \times 10^6$  cells added per plate were incubated for 12 hours in media. At the end of this incubation, the conditioned media was transferred to a cell free culture

plate and  $^{125}\text{I}$ -BSA ( $10^6$  cpm/ml) added. There was no observable increase in the TCA soluble radioactivity even after 18 hours of incubation at  $37^\circ\text{C}$ .

#### F. Effect of Protein Size on Degradation Rate

The results of the protein degradation experiments described in Figures 13 - 23 were performed with iodinated protein substrates of varying molecular weight. In general, it was found that proteins with smaller subunit molecular weights were degraded more rapidly than those with larger subunits. This is shown graphically in Figure 24 where cpm remaining/cpm total at 35 hours is plotted against the log (subunit molecular weight).

The data was analyzed by computer and linear regressions were calculated from all the data points. The results of those calculations are shown in Table 8. For each protein, the degradation rate constant,  $k$  ( $\text{hr}^{-1}$ ), was determined from the slope of the log plots of cpm remaining vs time (Figures 13 - 23). In general, the rate constant,  $k$ , decreases with increasing molecular weight. The results are shown graphically in Figure 25.

#### G. Effect of Various Factors on Protein Degradation

The observation that the degradation of radio-labeled proteins by macrophages was reproducible and

Figure 24. Plot of  $(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t$  versus the log of the subunit molecular weight of the protein.

- a) Least squares line for all data points but BSA and Thyroglobulin. Correlation coefficient equals 0.952.
  
- b) Least squares line for all data points but BSA, thyroglobulin, and  $\beta$ -Galactosidase. Correlation coefficient equals 0.962.

## LOG SUBUNIT MOLECULAR WEIGHT

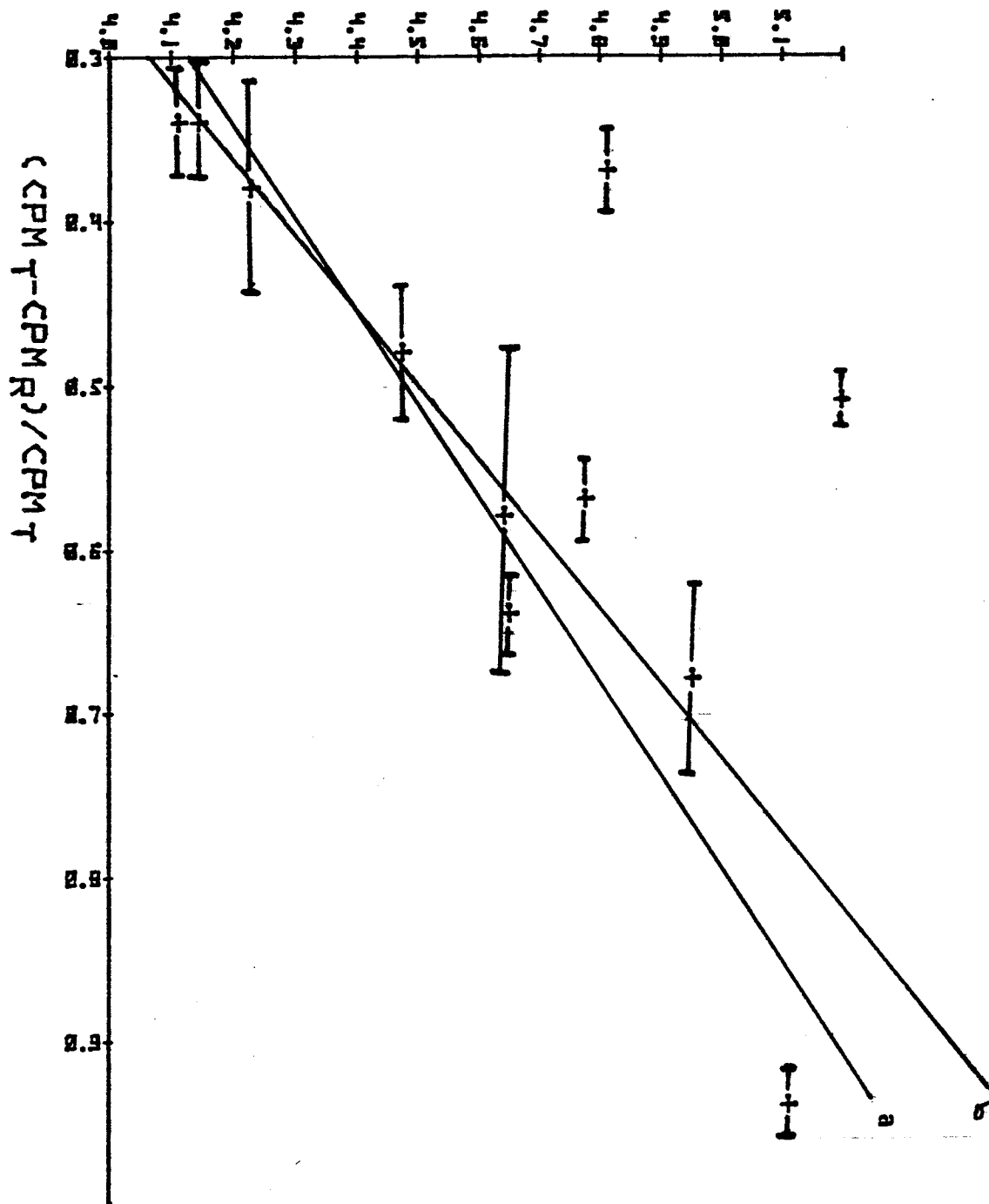
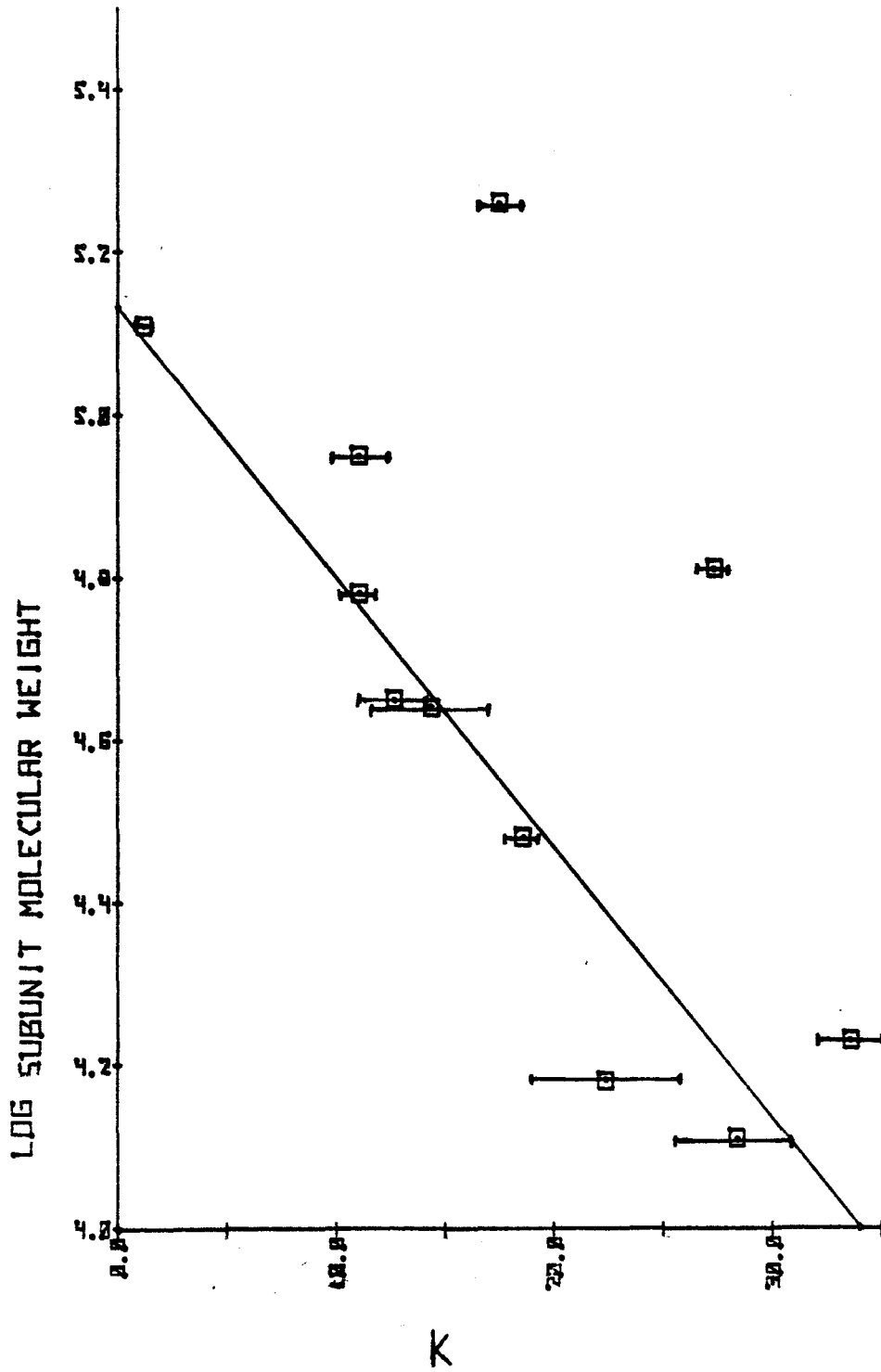


Figure 25. Plot of the apparent first order rate constant vs the log of the subunit molecular weight of the protein. Each point represents the mean  $\pm$  standard deviation. The least squares line for all points but BSA and thyroglobulin is indicated. Correlation coefficient = 0.928.



reflected the activity of a proteolytic enzyme system within the cell prompted us to investigate additional properties of this system. In order to determine if the rate of degradation of BSA was directly proportional to its concentration in the cells, the specific activity of the  $^{125}$ I-labeled protein was decreased by adding varying amounts of cold BSA to the uptake media. As shown in Table 9 there was no difference in the amount of label released by 30 hours when cells were previously exposed to 0, 0.1, or 1.0 mg/ml of cold BSA along with labeled BSA. This was the result expected for a system in which the rate of degradation of BSA is first order in substrate concentration. In this range of BSA concentration, it was not possible to saturate the degradative enzymes with unlabeled BSA. In a related experiment, different amounts of labeled BSA were added to the uptake media. The amounts were 0.2, 0.4, 0.6, 0.7, 1.2, and  $1.5 \times 10^7$  cpm/ml. In all cases, the amount of TCA soluble label released with time was directly proportional to the amount of label taken up by the cells (Table 10).

Cells were simultaneously loaded with dextran-sulfate (1 mg/ml) and  $^{125}$ I-BSA to determine if the presence

TABLE 9

Release of Label From Cells With  $^{125}$ I-BSA Endocytosed  
in the Presence of Cold BSA<sup>a</sup>

Cold BSA Added (mg/ml)	$\frac{\text{cpm}_t - \text{cpm}_r}{\text{cpm}_t}$ at 30 Hours
0	0.31, 0.30
0.1	0.28, 0.29
1.0	0.30, 0.30

<sup>a</sup> Uptake was performed in media with no FCS for 18 hours.  
The  $^{125}$ I-BSA was present in the media at  $1 \times 10^7$   
cpm/ml (0.005mg/ml).



TABLE 10

Release of Label From Cells Incubated With Varying  
Amounts of Labeled BSA<sup>a</sup>

<u>cpm/ml x 10<sup>6</sup> <sup>125</sup>I-BSA</u>	<u>cpm<sub>t</sub> - cpm<sub>r</sub> cpm<sub>t</sub> at 10 hours</u>
2	0.48, 0.60
4	0.54, 0.62
6	0.50, 0.62
7	0.48, 0.62
12	0.51, 0.57
15	0.56, 0.58

<sup>a</sup> Uptake was performed in media with 50% FCS for 19 hours.

of dextran-sulfate in the uptake media could affect the subsequent degradation of  $^{125}$ I-BSA. This experiment was performed because we contemplated using dextran-sulfate as a carrier for introducing proteinase inhibitors into these cells. As can be seen from Figure 26, dextran-sulfate can itself inhibit the release of TCA-soluble radioactivity when administered simultaneously with the substrate. In contrast, when dextran-sulfate was added to the release media after uptake of  $^{125}$ I-BSA alone, it had no effect on the rate of degradation of BSA.

The effect of varying the concentration of fetal calf serum in the release media was also investigated. In Figure 27 it can be seen that the release of TCA soluble radioactivity from  $^{125}$ I-BSA laden macrophages and fibroblasts is inhibited in the presence of 50% FCS. In addition, the percentage of label regurgitated as TCA insoluble radioactivity increases from about 25% to 50%. To determine if these differences could be due to changes in the precipitability of labeled products in the presence of varying levels fetal calf serum,  $^{125}$ I-BSA laden fibroblasts were allowed to release label into

Figure 26. Release of radioactivity from macrophages incubated overnight with  $1 \times 10^7$  cpm/ml  $^{125}$ I-BSA (\*) and  $^{125}$ I-BSA plus dextran-sulfate (□). All values represent averages from 2 samples.

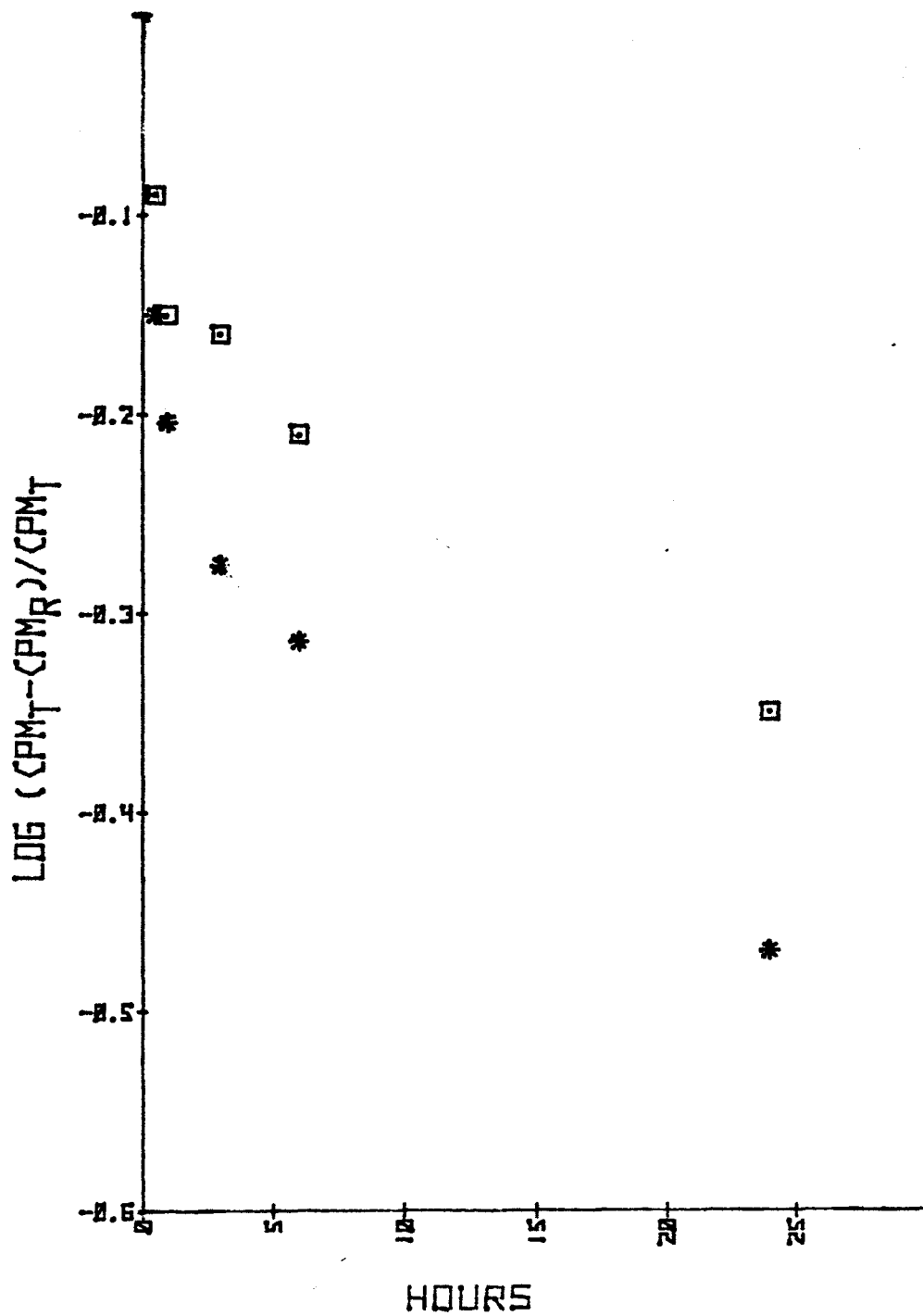
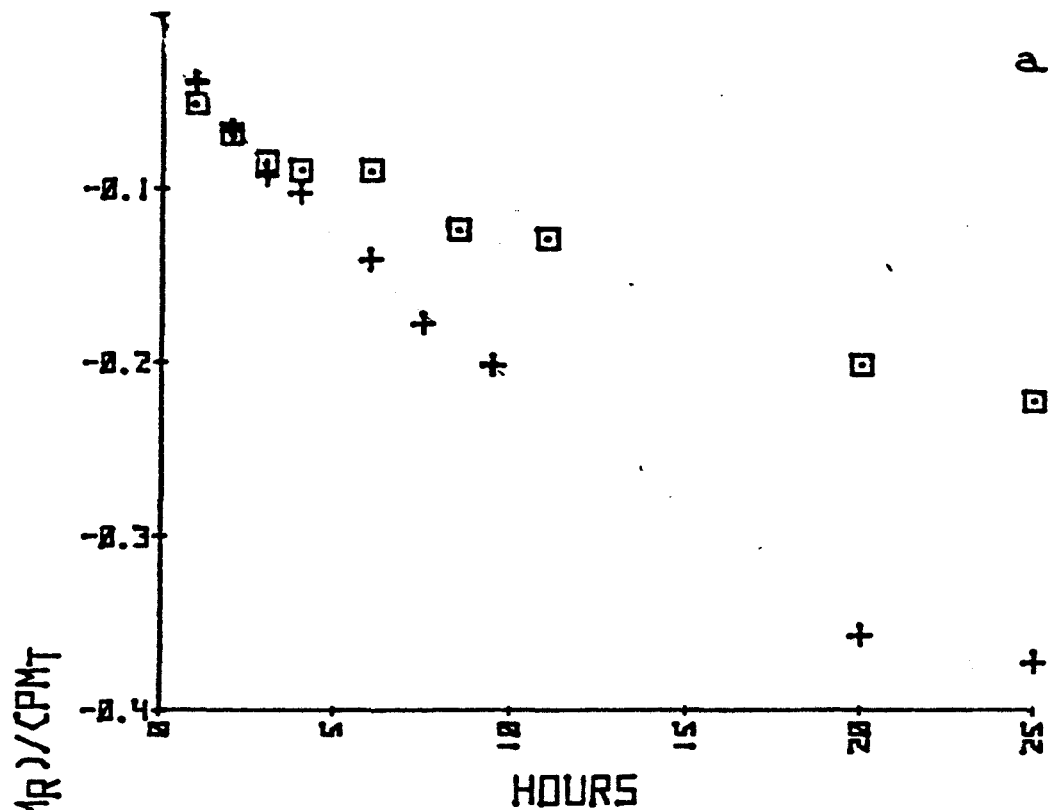


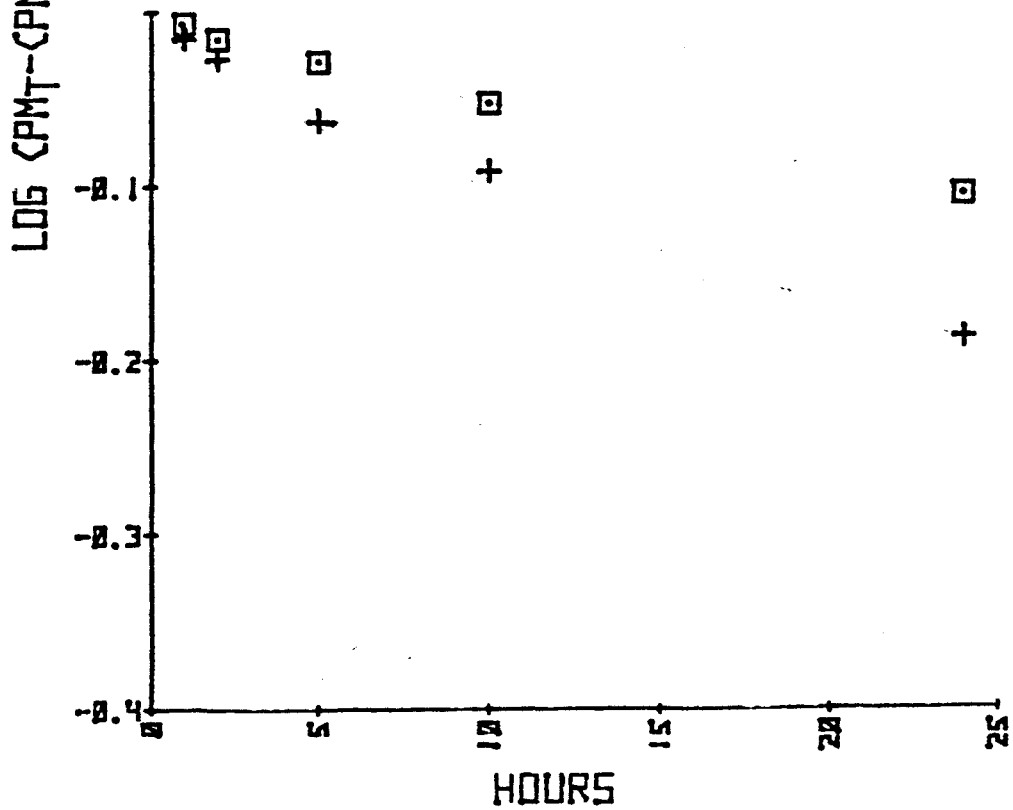
Figure 27. Release of TCA soluble radioactivity from macrophages and fibroblasts incubated overnight with  $1 \times 10^7$  cpm/ml  $^{125}$ I-BSA in 50% FCS. The labeled cells were incubated in media with 1% FCS (+) or 50% FCS (□) and media samples removed at each time point for analysis. Each point is the average value from 2 duplicate plates.

- a) Macrophages
- b) Fibroblasts

a



b



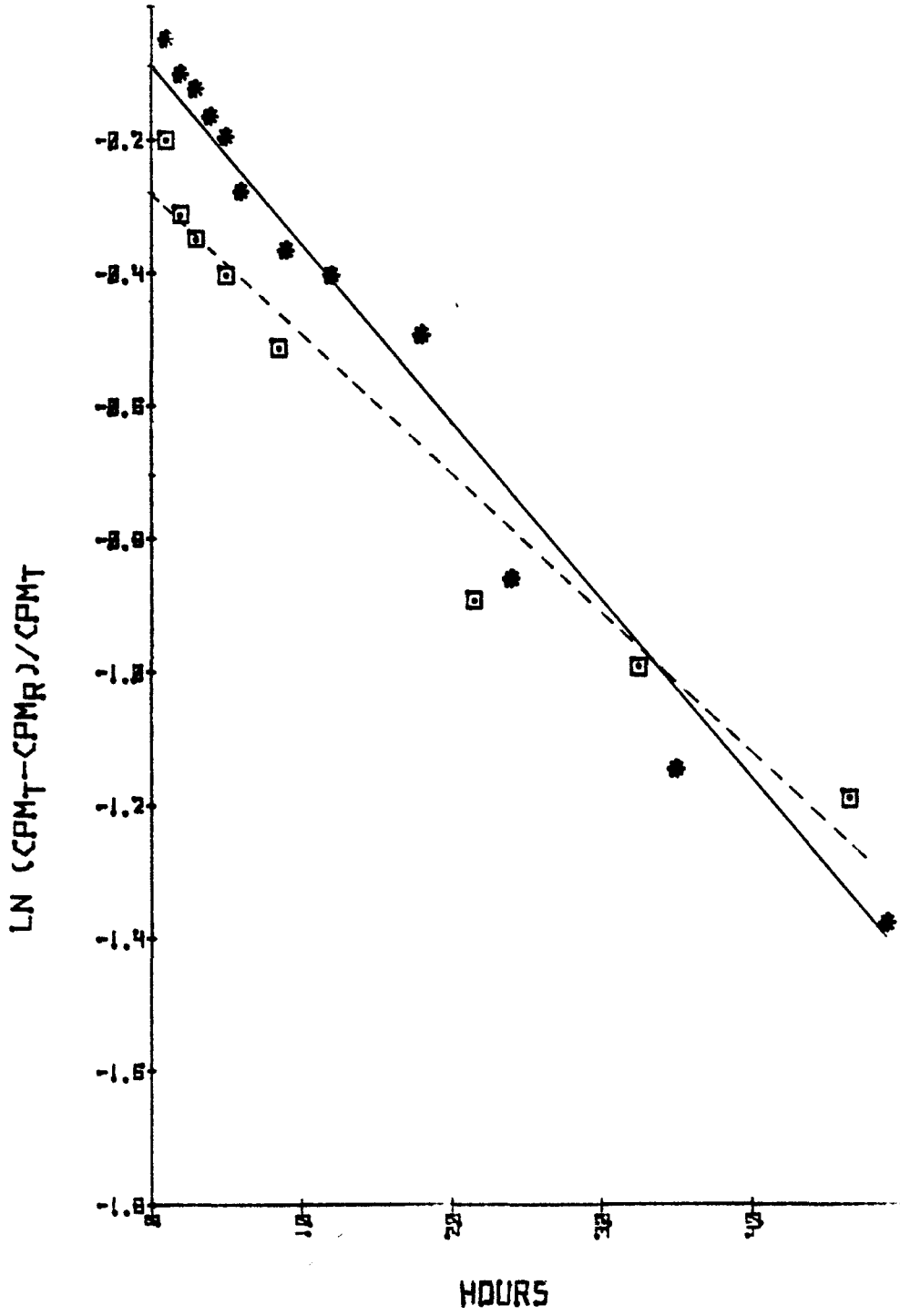
media containing 1% fetal calf serum. Media samples were removed at varying times and fetal calf serum was added to half of each sample before precipitation with TCA. The addition of fetal calf serum had no effect on the amount of TCA insoluble radioactivity present in each sample indicating that the increase in precipitable radioactivity seen in release experiments in 50% fetal calf serum was due to an increase in the amount of incomplete or non-digested protein regurgitated by the cells and not to an increased precipitability of labeled protein in 50% fetal calf serum.

#### H. Effect of Protein Conformation on Protein Degradation

In order to determine the effect of denaturation on the rate of degradation of pinocytosed proteins, preparations of  $^{125}\text{I}$ -BSA which had been boiled for 10 minutes were preincubated with macrophages and the rate of release of TCA soluble radioactivity measured. The denaturation of BSA by this method was verified by analyzing the boiled  $^{125}\text{I}$ -BSA with the Affi-gel blue affinity column. The percent bound by the column decreased from 80% to 30% after boiling. Denatured BSA was degraded at a rate only slightly faster than that of the native protein (Figure 28). This suggests that, for BSA, protein

Figure 28. Release of TCA soluble activity from macrophages incubated overnight in  $1 \times 10^7$  cpm/ml  $^{125}$ I-BSA (+) or  $2 \times 10^6$  cpm/ml heat-treated  $^{125}$ I-BSA (□). Each point represents the average of 2 experiments for the heat-treated BSA and 7 experiments for the untreated BSA. The least squares line for each is indicated.





denaturation may not be the rate determining step in its degradation in lysosomes. During the course of our studies, it was observed that solutions of  $\beta$ -galactosidase which had been frozen and thawed were degraded more rapidly than samples not treated in this manner. This is shown in Figure 29. This progressive increase in the rate of degradation with successive cycles of freezing and thawing was shown to be correlated with a loss in enzymatic activity (Table 11). Clearly, for  $\beta$ -galactosidase, a protein normally degraded slowly in macrophages, denaturation produced a marked increase in its lysosomal degradation rate. This effect of freezing and thawing on  $\beta$ -galactosidase was not observed with the other proteins studied when they were frozen and thawed.

#### I. Relation Between Time of Uptake and Degradation Rate

If the nonlinear release curves are due to the presence in protein substrates of peptide bonds with different susceptibilities to cleavage by proteinases, then one would expect that partially degraded intermediate species to accumulate in cells. Since the release experiments described in the previous sections were performed after 18 hours of uptake of label, one would expect that

Figure 29. Release of TCA soluble radioactivity from cells incubated overnight in freshly labeled  $^{125}\text{I}$ - $\beta$ -galactosidase (+) and frozen and thawed  $^{125}\text{I}$ - $\beta$ -galactosidase (  $\square$  ). Each sample was run in duplicate. The least squares line for each is indicated.

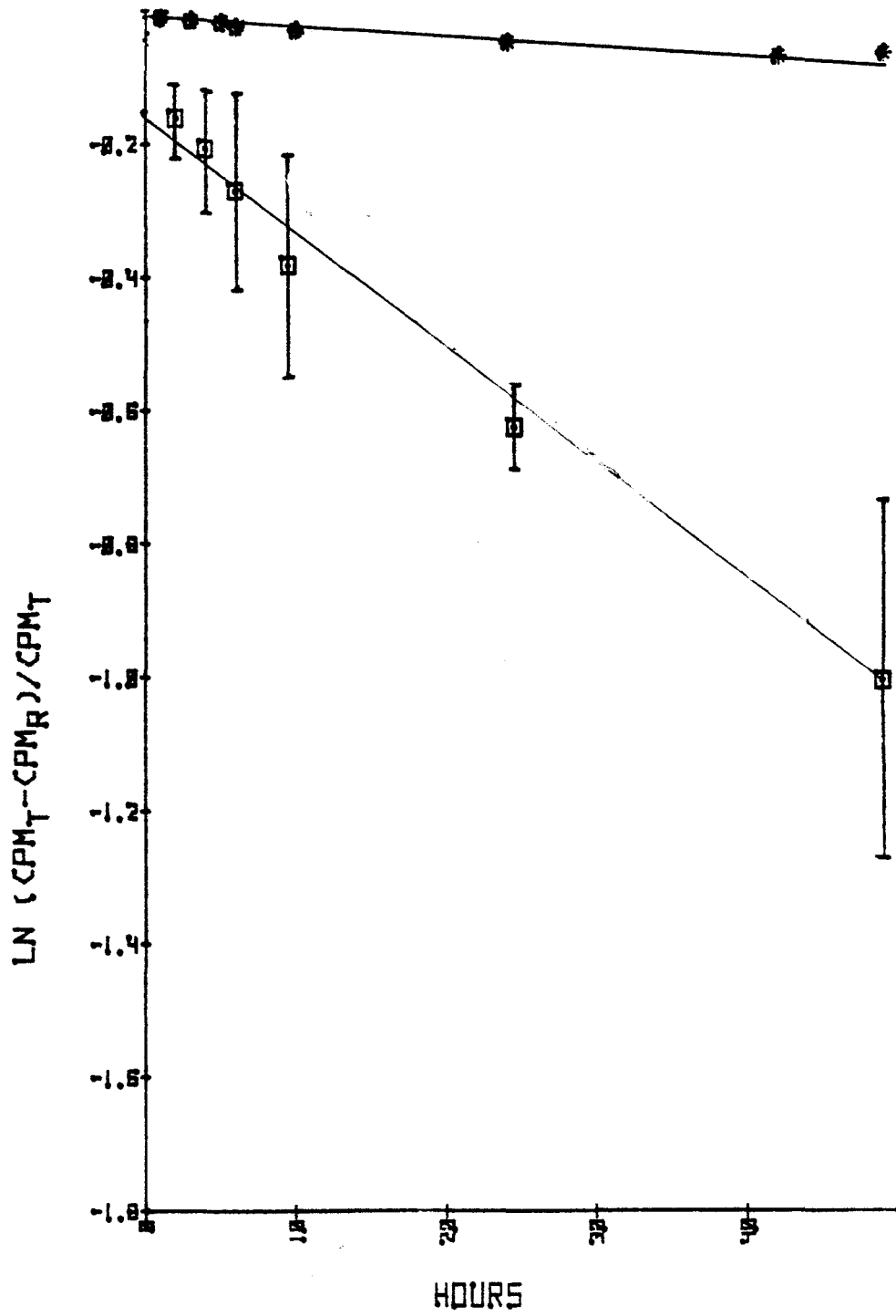


TABLE 11

Effect of Freezing on the Activity of  $\beta$ -Galactosidase

Sample	A/t x 10 <sup>-4</sup> <sup>a</sup>	% of Initial Activity
Before freezing	15.2(15.4,14.9) <sup>b</sup>	-
After freezing	5.9 $\pm$ 0.3 <sup>c</sup>	38.8

<sup>a</sup> Activity is expressed as the change in absorbance at 410nm per second times 10<sup>-4</sup>. The  $\beta$ -galactosidase (100  $\mu$ l of a 0.2  $\mu$ g/ml solution) is preincubated for 5 minutes with 300  $\mu$ l mercaptoethanol (1 M) before the addition of o-nitrophenyl,  $\beta$ -D-galactopyranoside.

<sup>b</sup> Average of 2 determinations. Two values obtained in parentheses.

<sup>c</sup> Mean of 4 determinations  $\pm$  standard deviation.

that these cells had accumulated quantities of polypeptide fragments more resistant to further degradation having already released amino acids from the most susceptible regions of the molecule. To confirm this hypothesis, cells were allowed to accumulate  $^{125}$ I-BSA for just one hour. The cells were then washed and incubated in 1% fetal calf serum and the rate of release of TCA soluble radioactivity measured. With a one hour uptake, the initial rate of release of TCA soluble label into the media is more rapid, and the nonlinearity of the logarithmic plot is more apparent (Figure 30). This was observed in both macrophages and fibroblasts. In contrast, the percentage of the label released in the TCA insoluble form is the same after a one hour uptake as after the overnight uptake. The degradation of heat denatured  $^{125}$ I-BSA after one hour uptake was also studied (Figure 31). Again, the TCA soluble label released after a 1 hour uptake is initially more rapid and a greater percentage of label is released in the rapid phase.

The effect of a one hour uptake on the rate of release of TCA soluble label was studied utilizing the protein substrates described previously (Figures 32, 33). It was found that phosphorylase b and thyroglobulin each showed an increased rate of degradation and an increase

Figure 30. Release of TCA soluble radioactivity from macrophages incubated overnight (●) or 1 hour (■) with <sup>125</sup>I-BSA. The 1 hour data is presented as the mean ± standard deviation for 3 experiments.

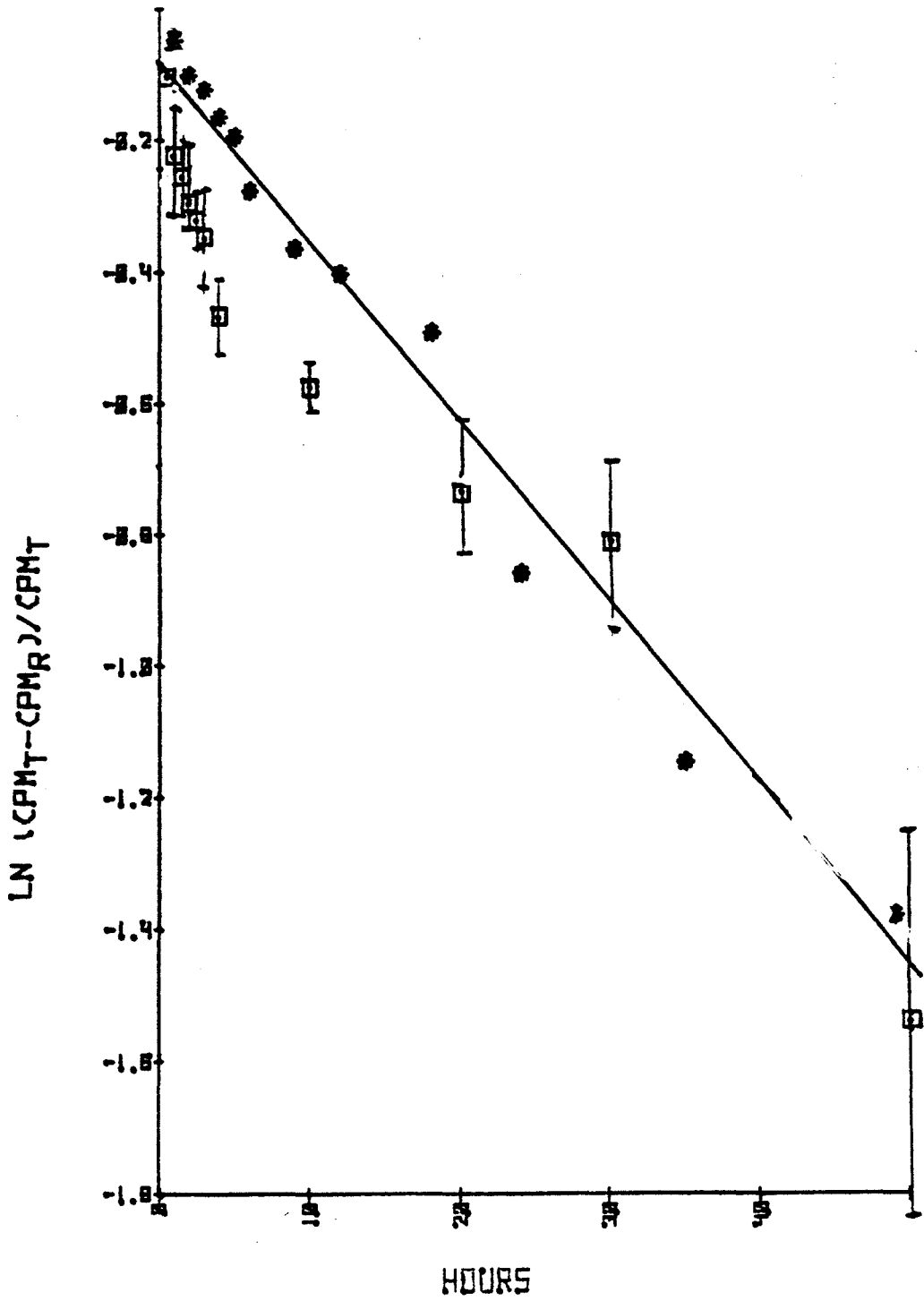




Figure 31. Release of TCA soluble radioactivity from macrophages incubated overnight ( ● ) or 1 hour ( ■ ) with heat-treated <sup>125</sup>I-BSA. The 1 hour data is presented as the mean ± standard deviation for 3 experiments.

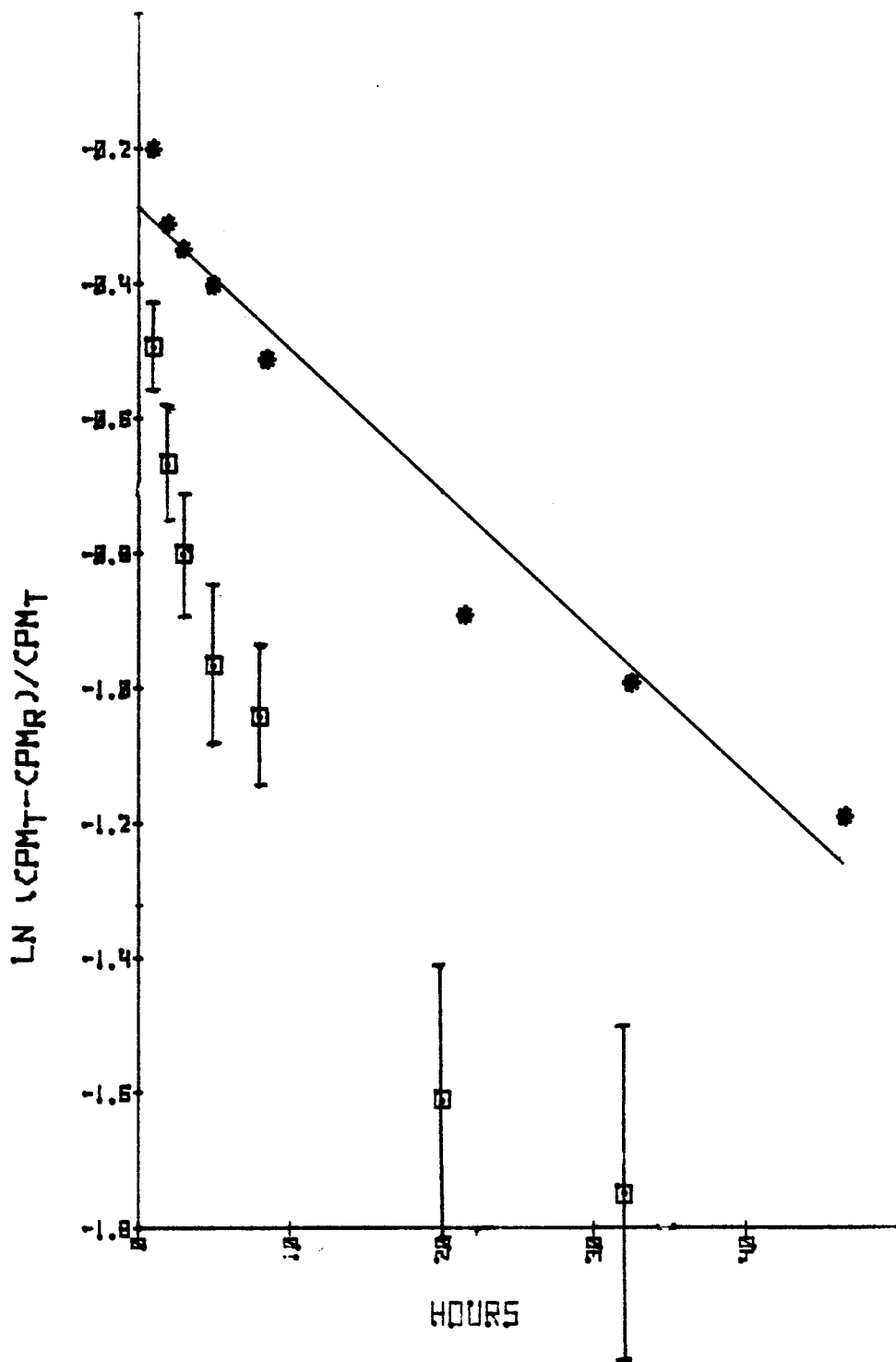


Figure 32. Release of TCA soluble radioactivity from macrophages incubated overnight ( ● ) or 1 hour ( ■ ) with <sup>125</sup>I-phosphorylase b. The 1 hour data is presented as the mean ± standard deviation for 3 experiments.

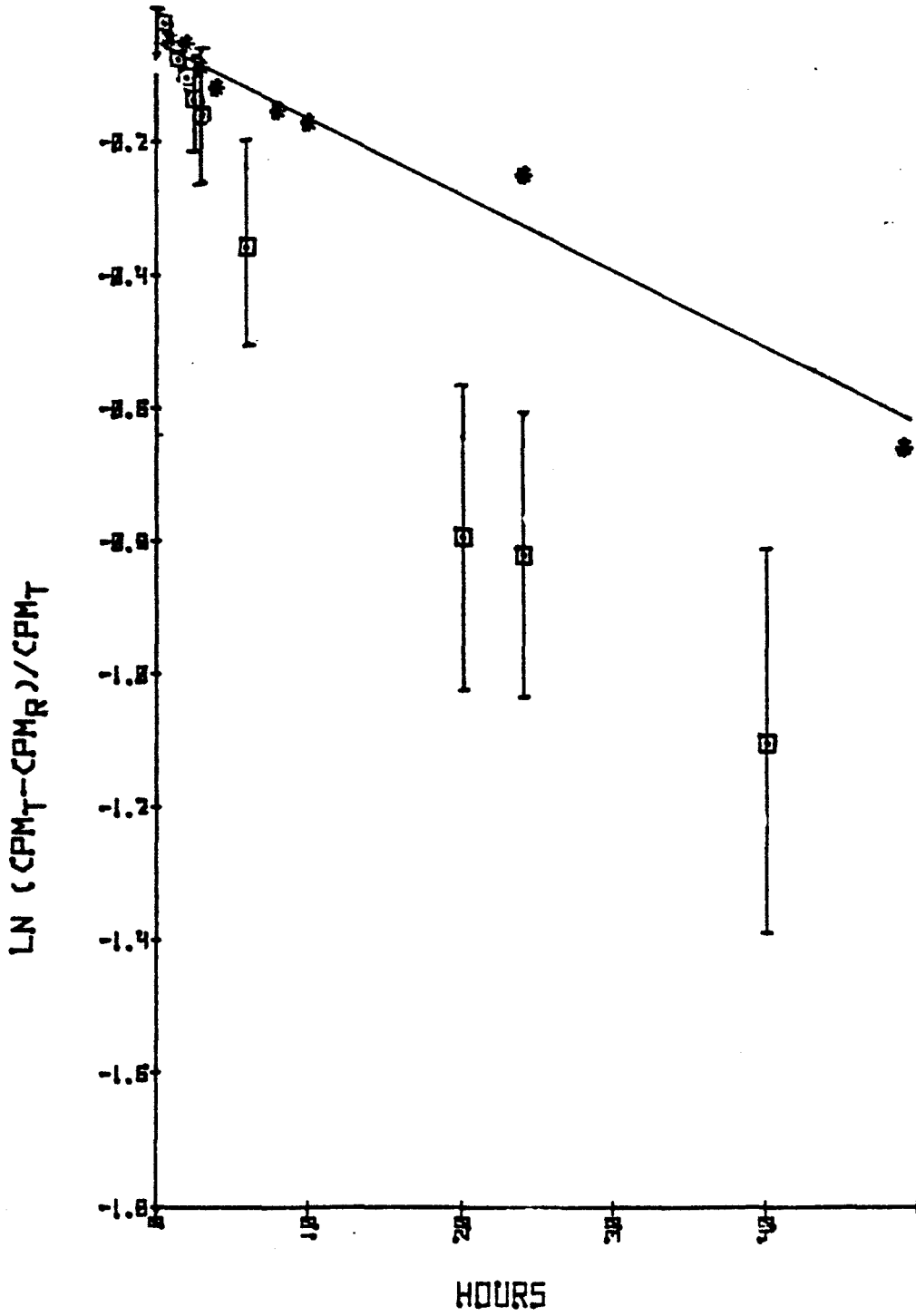
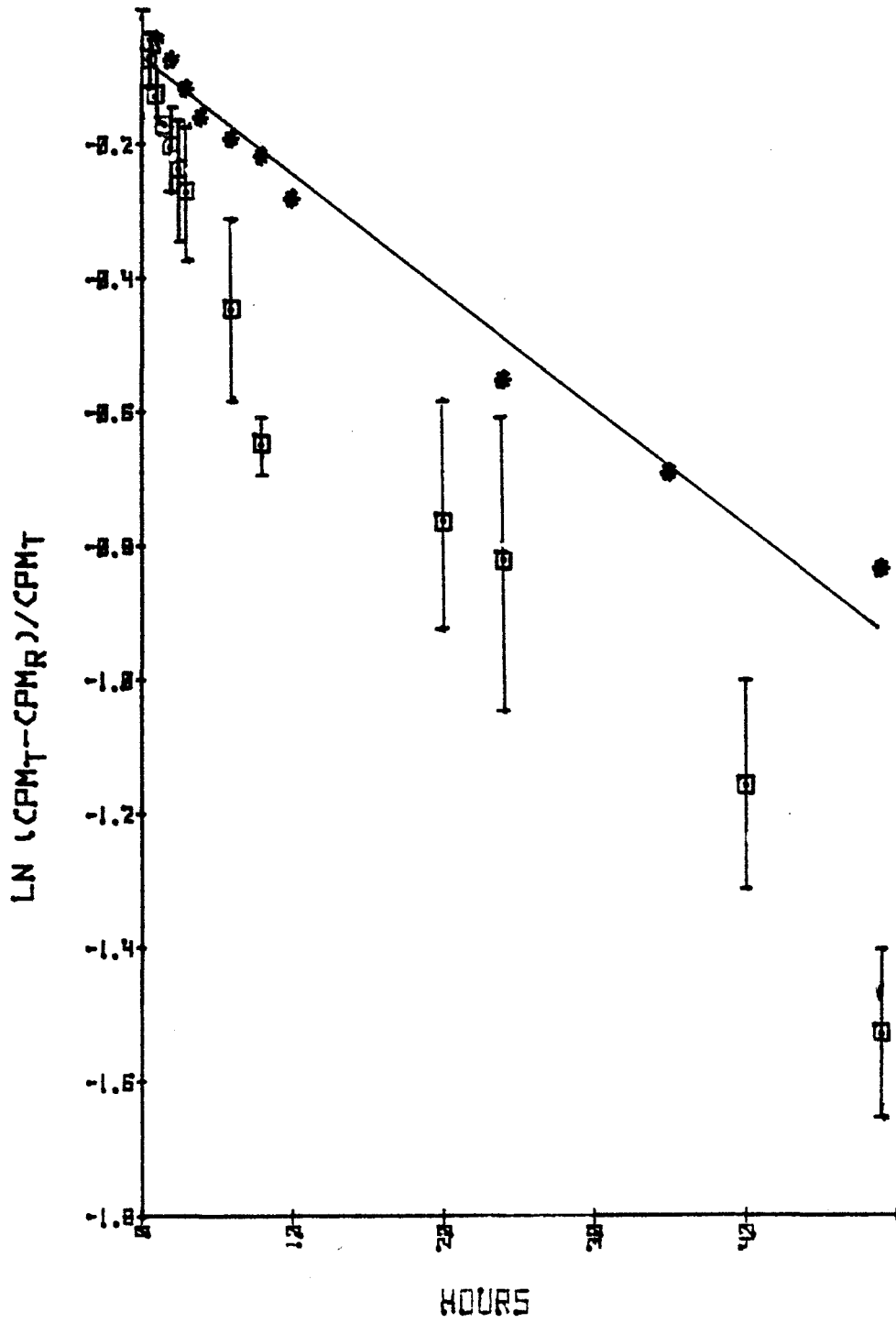


Figure 33. Release of TCA soluble radioactivity from macrophages incubated overnight (✱) or 1 hour (□) with  $^{125}\text{I}$ -thyroglobulin. The 1 hour data is presented as the mean  $\pm$  standard deviation for 3 experiments.



in the proportion of label released during the rapid phase. In contrast, the degradation rates of  $^{125}\text{I}$ -ovalbumin were not much affected. However, in most cases, the proportion of the total labeled protein released at the slower rate had been decreased.

Rate constants for the release of labeled proteins endocytosed for 1 hour were calculated as before. The results are shown in Table 12.

#### J. Inhibitors

Finally, the effect of protease inhibitors on the release of label from endocytosed  $^{125}\text{I}$ -BSA was studied. The inhibitors studied included leupeptin and antipain for cathepsin B and pepstatin for Cathepsin D. An altered release of label was observed only when the inhibitors were added to the uptake media along with the labeled BSA for the overnight incubation. There was no effect observed when the inhibitor was added to the release media only. This result is understandable for pepstatin since it is only taken up by pinocytosis (161) and does not enter the cell quickly. The explanation for this result for leupeptin and antipain is not known since these inhibitors are reported to be able to permeate the plasma membranes of macrophages (22).

TABLE 12

Calculated Rate Constants and Y-Intercepts For  
1 Hour and Overnight Uptake Experiments

Protein	Apparent First Order <sup>a</sup> Rate Constant (X 10 <sup>3</sup> )		Y-Intercept <sup>b</sup>	
	Overnight	1 Hour	Overnight	1 Hour
Ribonuclease	22.3 ± 3.7	26.6 ± 2.0	- .333	- .558
Arginase	18.5 ± 0.8	16.5 ± 1.3	- .136	- .216
Ovalbumin	12.6 ± 1.6	7.8 ± 0.6	- .185	- .164
BSA	27.3 ± 0.8	22.6 ± 2.0	- .236	- .628
Phosphorylase b	11.0 ± 1.2	28.6 ± 1.9	- .029	- .454
Thyroglobulin	17.5 ± 0.9	27.0 ± 1.2	- .135	- .388
Denatured BSA	21.2 ± 1.9	31.6 ± 3.7	- .430	- .972

<sup>a</sup>Calculated from all data points. Value given is mean ± standard deviation.

<sup>b</sup>Calculated from all data points after 8 hours.



Figure 34 illustrates the release of label in the presence of the various inhibitors. Pepstatin ( $8.8 \times 10^{-5}M$ ) retarded the release of TCA soluble radioactivity from the macrophages. Leupeptin ( $6.6 \times 10^{-4}M$ ) and antipain ( $4.9 \times 10^{-4}M$ ) which inhibit lysosomal serine proteinases, appeared to cause an increased release of label. The percentage of label released in the initial rapid phase decreased with pepstatin and increased with leupeptin and antipain. The experiment was repeated using chloroquine ( $1 \times 10^{-4}M$ ), a lysosomotropic agent, which is known to become concentrated within the lysosomes where it disrupts intralysosomal pH and may additionally inhibit cathepsin B and other lysosomal proteases. It is also known to induce autophagy (143). Chloroquine greatly inhibited the release of label into the media. Dextran-sulfate also inhibited the release of label into the media (Figure 35). Its effects on the proteases papain, cathepsin B, and trypsin are shown in Figure 36.

The effect of inhibitors on regurgitation of TCA insoluble labeled protein into the media was also investigated (Table 13). Leupeptin and pepstatin caused a slight decrease in the percentage of TCA insoluble

Figure 34. Release of TCA soluble radioactivity from macrophages incubated with  $1 \times 10^7$  cpm/ml  $^{125}$ I-BSA incubated in the presence of pepstatin, 60  $\mu$ g/ml ( $\odot$ ), chloroquine, 50  $\mu$ g/ml ( $\Delta$ ), leupeptin, 290  $\mu$ g/ml ( $\blacklozenge$ ), and antipain, 290  $\mu$ g/ml ( $\blacksquare$ ). All values from 3 experiments are given for the inhibitor studies. The rate constants calculated for the degradation of  $^{125}$ I-BSA in the presence of inhibitors are 0.047 (antipain), 0.036 (leupeptin), 0.011 (pepstatin), and 0.006 (chloroquine).

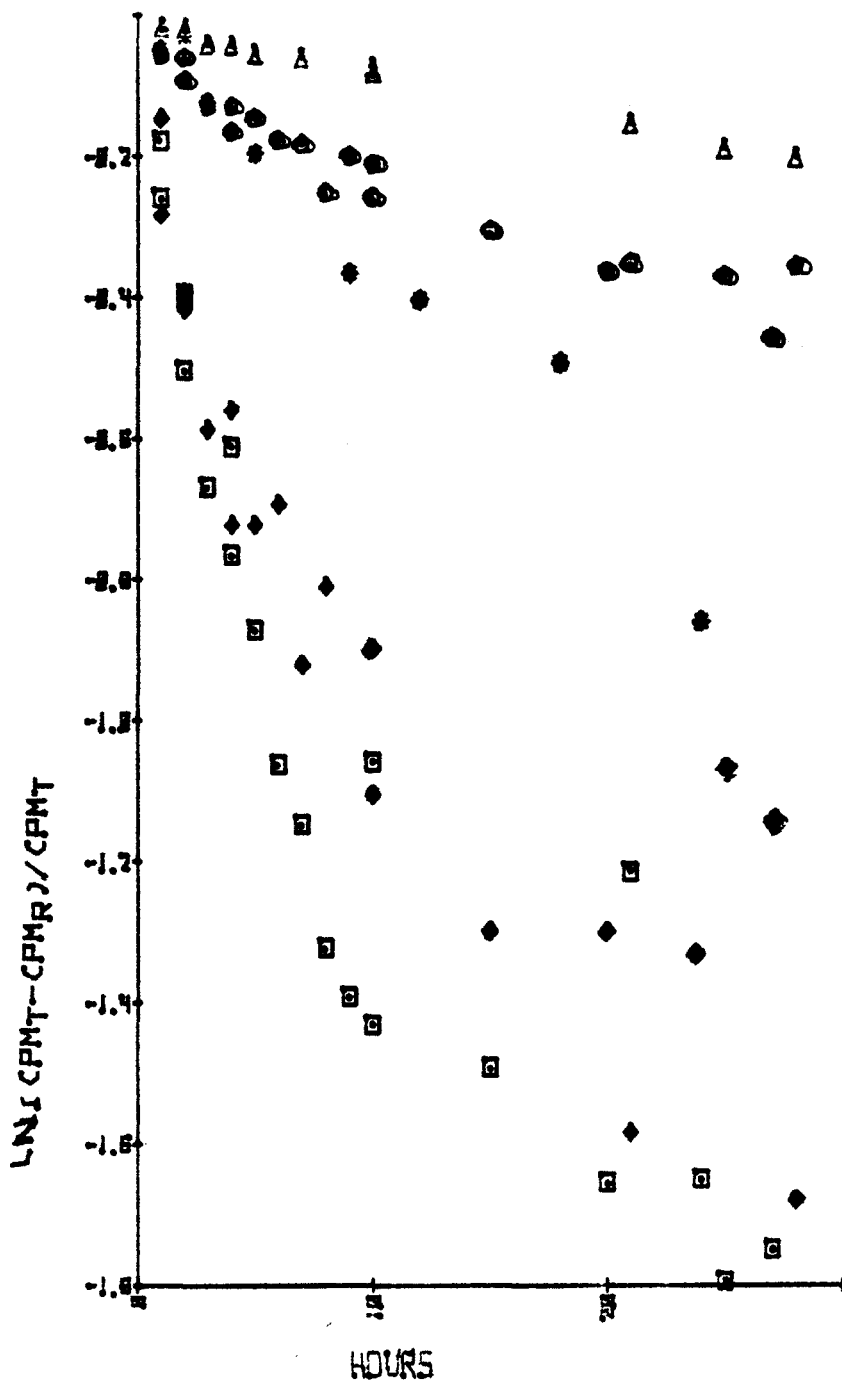


Figure 35. Release of TCA soluble radioactivity from macrophages incubated overnight with  $1 \times 10^7$  cpm/ml  $^{125}$ I-BSA alone (\*) or in the presence of dextran (◆) or dextran-sulfate (■) at 1 mg/ml. The control and dextran experiments were performed in duplicate. The dextran-sulfate data represents the average of 4 samples. Plotted is the % of total counts per minute released during the initial rapid phase vs time.

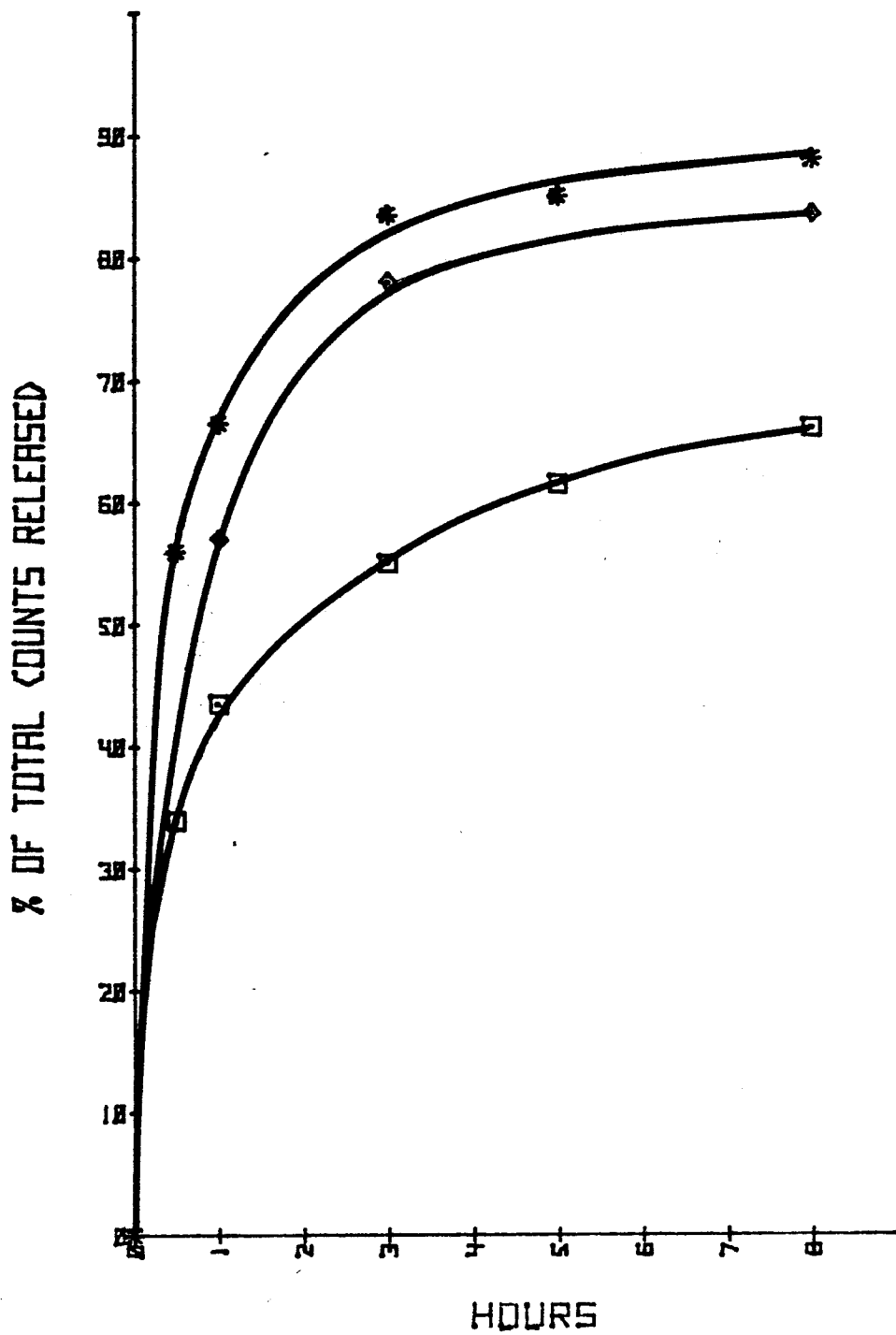


Figure 36. The inhibition of papain (\*), cathepsin B (■), and trypsin (○) by dextran-sulfate. The concentration of dextran-sulfate is plotted against the percent of the unhibited activity remaining at that concentration.

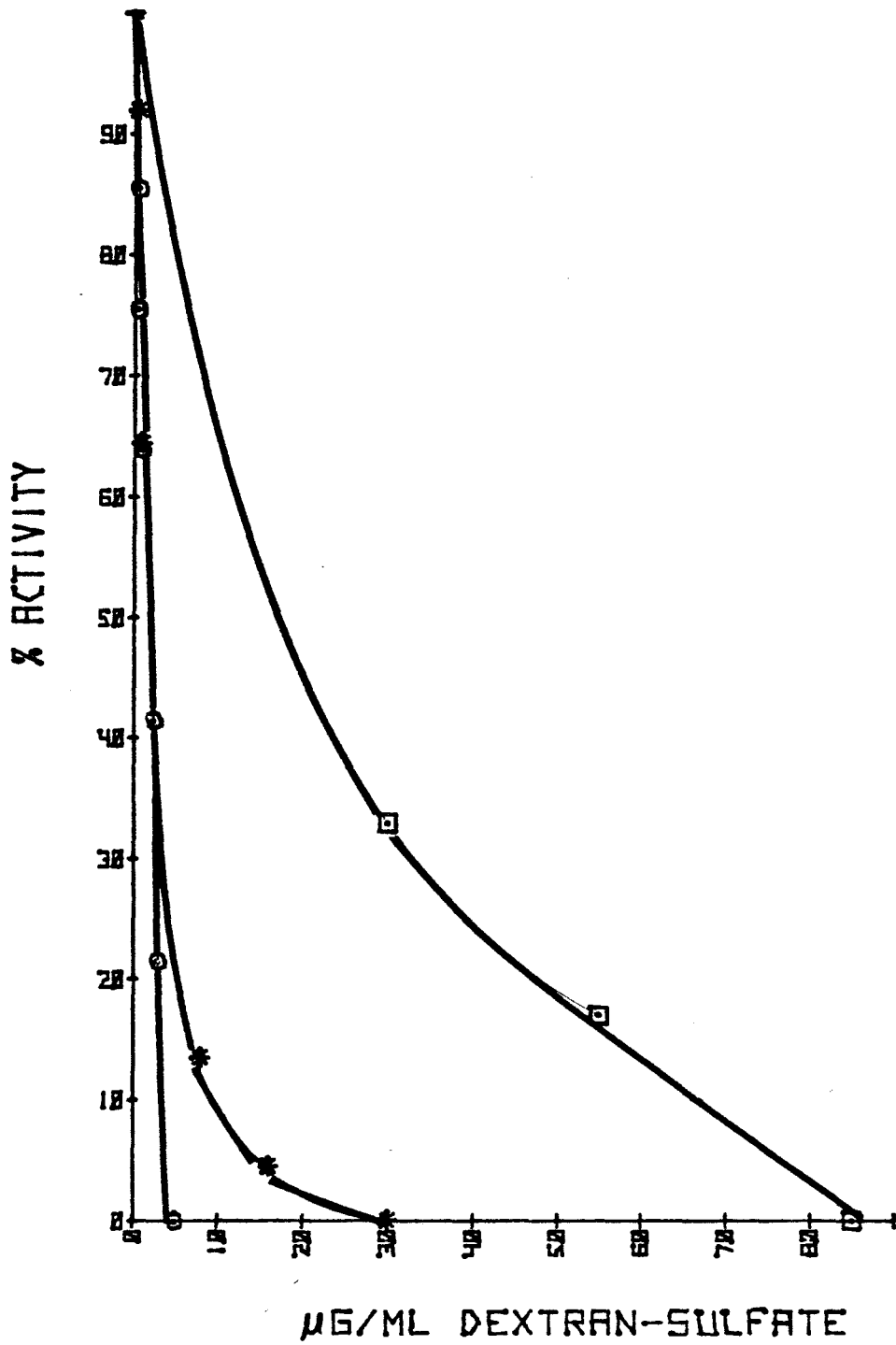


TABLE 13

Release of TCA Insoluble Radioactivity From Endocytosed  
<sup>125</sup>I-BSA in the Presence of Inhibitors<sup>a</sup>.

Inhibitor Added	% Released as TCA Insoluble
None	27.6 ± 3.8 <sup>b</sup>
Antipain	25.3 ± 4.4 <sup>c, d</sup>
Leupeptin	15.7 ± 3.0 <sup>c, e</sup>
Pepstatin	14.3 ± 3.0 <sup>c, e</sup>
Chloroquine	36.5 (30.6, 42.1) <sup>f</sup>

<sup>a</sup>Release of Label was performed for 28 hours in media containing 1% FCS.

<sup>b</sup>Average of 4 determinations

<sup>c</sup>Average of 3 determinations

<sup>d</sup>Difference from control not statistically significant

<sup>e</sup>Differences from control statistically significant  
(p < .025)

<sup>f</sup>Average of 2 determinations.

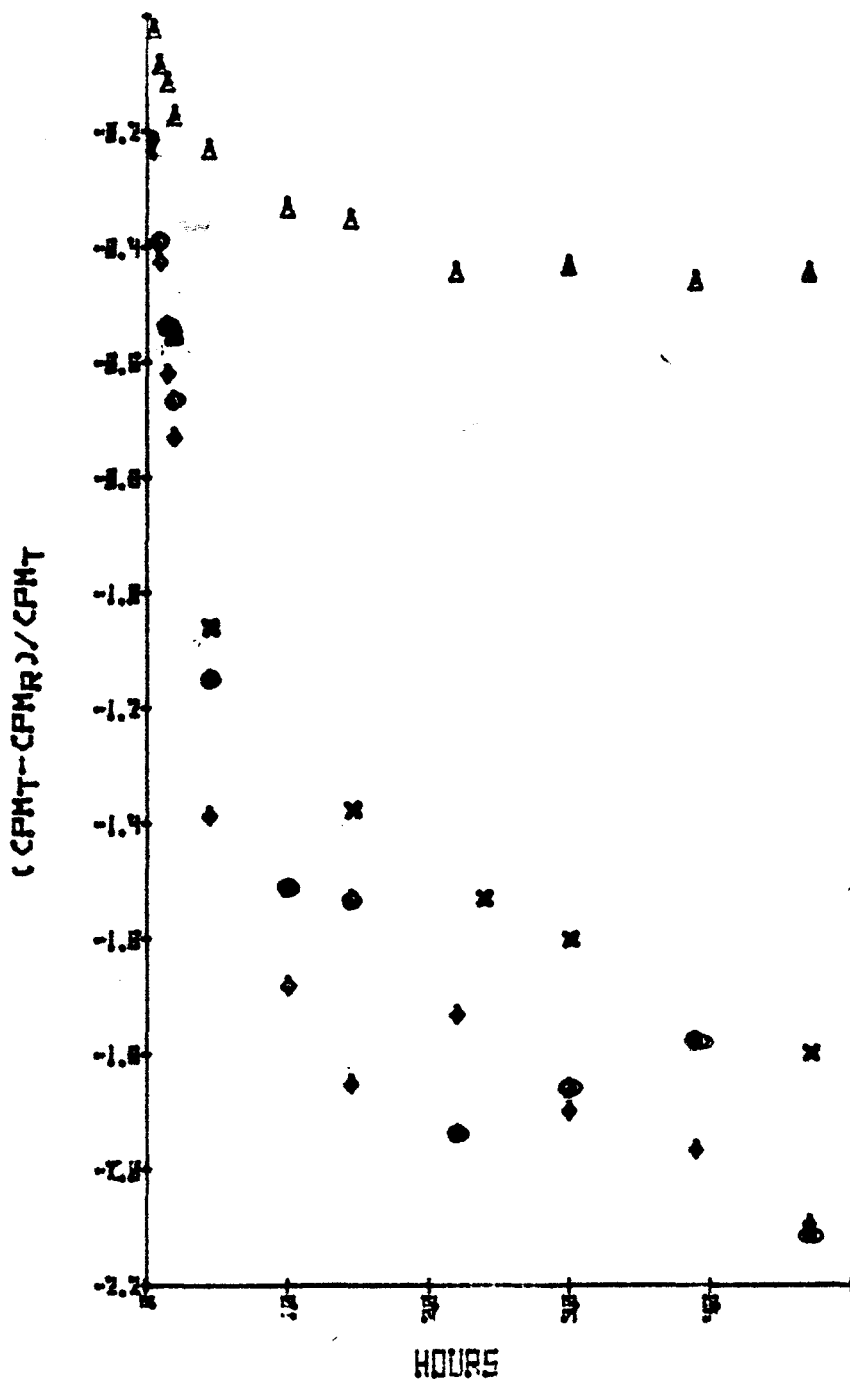
Data not sufficient to determine statistical significance of the difference from control values.



radioactivity released while chloroquine caused a slight increase. Antipain had almost no effect.

These inhibitor experiments were also repeated with cells which had been loaded with labeled protein and inhibitor for only 1 hour. Inhibitors were also present in the release media. The results are shown in Figure 37. Leupeptin and pepstatin now both appear to have little effect. Chloroquine, however, caused an inhibition of the release of label.

Figure 37. Release of TCA soluble radioactivity from macrophages preincubated for 1 hour with  $4 \times 10^7$  cpm/ml  $^{125}$ I-BSA in the presence of pepstatin, 60  $\mu$ g/ml (◆), leupeptin, 290  $\mu$ g/ml (○), and chloroquine, 50  $\mu$ g/ml (△). The release with no inhibitors present is also shown (+). From 2 experiments are given for the inhibitor studies.



## CHAPTER IV

### DISCUSSION

#### A. Experimental Techniques

The uptake and digestion of extracellular proteins by macrophages was studied by Ehrenreich and Cohn (104). In their experiments, cells were maintained in glass T-flasks after collection and protein uptake and degradation studies were performed in these flasks. The general procedure was as follows: Iodinated HSA, in a concentration of  $10^6$ - $10^7$  cpm/ml, was added to approximately  $8 \times 10^6$  cells in medium 199 containing 50% newborn calf serum. After appropriate uptake times, cells were washed with isotonic saline. Cell associated radioactivity was then collected by repeated freezing and thawing in distilled water. Protein degradation studies were performed by adding fresh media after the washing step and collecting all the cells in a flask for each time point in the experiment. They also collected the media at each time point and measured TCA soluble and insoluble radioactivity present. They reported that the increase in the TCA soluble radioactivity in the media with time was about equal in magnitude to the decrease in cell-associated radioactivity.

The amount of TCA insoluble label in the media showed no consistent change and was attributed to intact protein remaining from the pulse. Because of the large quantities of cells required for T-flasks, we attempted to adapt the methodology of Cohn and his associates to quantities of cells which could be grown on glass coverslips immersed in media or on the bottom of small glass culture dishes. These efforts were unsuccessful. In attempting to locate the sources of error in the methodology, we found that glass culture plates and coverslips were the main causes of our difficulties. It was observed that iodinated proteins were nonspecifically adsorbed to glass surfaces. This radioactivity was firmly bound and was not totally removed with subsequent washing. However, on incubating these surfaces with fresh media, the radioactivity was slowly released. Some of this labeled protein could also be released by repeated freezing and thawing of the plates. This led to anomalously high estimates of the amounts of labeled protein taken up by the cells, of the amounts of labeled protein subsequently released by the cells, and of the amounts of cell-associated radioactivity remaining at the end of the release experiment. These

errors were significantly reduced by performing all experiments in plastic culture dishes. Also, it was found that sonicating the plastic culture plates, in contrast to repeated freezing and thawing, could release the cell associated radioactivity in preference to the non-specifically bound label. Following sonication of the plates, microscopic examination revealed very few if any attached intact cells remaining.

Our method for measuring protein uptake and degradation, therefore, relied on the use of plastic multiplates with sonication to release the cell associated labeled protein. Degradation studies were performed by collecting aliquots from the media at various times during the experiment and by collecting the cell associated radioactivity remaining at the end of the experiment. To distinguish radioactivity arising from protein degradation from the release of intact protein, each aliquot of media was treated with TCA-phosphotungstic acid and the soluble radioactivity measured. With this technique, all the data points in a single experiment could be obtained from a single culture plate. This resulted in improved precision because it was not necessary to apply uncertain

corrections for differences in cell numbers between plates. Uncertainties arising from differences between plates in the amounts of labeled protein taken up by the cells were also avoided. In addition, the sensitivity was sufficiently great so that an entire experiment could be done with  $4 \times 10^6$  cells. This made it possible for several experiments to be performed concurrently using pooled cells from 4 to 8 animals.

The media samples collected were precipitated with TCA-phosphotungstic acid and the soluble and insoluble radioactivity determined for each time point. In agreement with Ehrenreich and Cohn, we found that the TCA-soluble counts increased with time. However, in contrast to their results, we also found a significant time dependent release of TCA insoluble radioactivity. This difference may be due to the fact that the other workers were unable to measure small changes in the level of TCA insoluble radioactivity against a large background resulting from the release of TCA insoluble labeled protein non-specifically adsorbed to their glass culture flasks. In our experiments, we were able to observe that the sum of the TCA insoluble and soluble counts released equaled, within

experimental error, the amount of label lost by the cells.

The reproducibility of this technique is demonstrated in Table 14. This table summarizes the data obtained for  $^{125}\text{I}$ -BSA, our most thoroughly studied protein.

#### B. Uptake of Substrates

The uptake by cultured macrophages of the non-digestible labeled polymer,  $^{14}\text{C}$ -dextran-sulfate, was found to increase with time with its concentration in the medium. We were able to calculate an endocytotic index (EI) (105) which is defined as the volume of media in order for them to have accumulated a measured amount of radioactive material. For a non-receptor mediated uptake, the endocytotic index should be independent of the concentration of labeled polymer in the medium. For receptor mediated uptake, the endocytotic index should appear to decrease with increasing concentration of the label. In agreement with the literature (112), the endocytotic index for dextran-sulfate was found to be greater than that measured for dextran and was also observed to be concentration independent.

Studies on the uptake of  $^{125}\text{I}$ -BSA by cultured macrophages appeared to indicate that the process was



Table 14

<sup>125</sup>I-BSA Release Experiments

Cell Batch Number	<sup>125</sup> I-BSA Batch Number	$(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t$							
		<u>1 Hr</u>	<u>2 Hr</u>	<u>3 Hr</u>	<u>4 Hr</u>	<u>5 Hr</u>	<u>6 Hr</u>	<u>9 Hr</u>	<u>24 Hr</u>
1	1	.963	.905	.875	.834	-	.740	.667	.341
1	1	.941	.907	.866	.837	-	.760	.676	.343
1	1	.967	.877	.855	.824	-	.703	.581	.229
1	1	.934	.873	.859	.790	-	.694	.633	.283
2	2	.972	.891	.895	-	.853	-	-	.552
2	2	.943	.856	.856	-	.728	-	-	.421
2	2	.970	.897	.900	-	.825	-	-	.496
2	2	.933	.908	.944	-	.841	-	-	.425
6	3	.962	.936	.920	.904	.840	.814	.721	.420

Table 14 (Cont'd.)

<sup>125</sup>I-BSA Release Experiments

Cell Batch Number	<sup>125</sup> I-BSA Batch Number	$(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t$							
		<u>1 Hr</u>	<u>2 Hr</u>	<u>3 Hr</u>	<u>4 Hr</u>	<u>5 Hr</u>	<u>6 Hr</u>	<u>9 Hr</u>	<u>24 Hr</u>
6	3	.972	.939	.902	.861	.835	.815	.708	.459
12	4	.974	.929	-	.856	-	.827	-	.467
14	4	-	.911	.936	.885	.838	-	.700	.483
15	4	.943	-	.934	.915	.848	-	.744	.356
15	4	.983	.937	.922	.902	.832	-	.828	.500
15	4	.961	-	-	-	.858	-	.779	.513
15	4	.966	.934	.919	.858	.845	-	.769	.465
15	4	.958	.949	.931	.899	.816	-	.700	.393
16	4	-	-	-	-	-	.830	.779	-

Table 14 (Cont'd.)

<sup>125</sup>I-BSA Release Experiments

Cell Batch Number	<sup>125</sup> I-BSA Batch Number	$(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t$							
		<u>1 Hr</u>	<u>2 Hr</u>	<u>3 Hr</u>	<u>4 Hr</u>	<u>5 Hr</u>	<u>6 Hr</u>	<u>9 Hr</u>	<u>24 Hr</u>
18	4	-	-	.848	.793	-	.727	.629	.447
19	4	.912	.864	.804	.784	-	.703	.604	-
19	4	.922	.850	.810	.784	-	.724	.590	.406
20	5	-	.910	-	-	.770	-	-	.399
20	5	.933	-	.820	-	.793	-	-	.481
Mean		.953	.904	.884	.848	.823	.758	.694	.423
Standard Deviation		.020	.030	.044	.047	.047	.054	.074	.079

not receptor mediated. This was supported by the observation that the uptake mechanism could not be saturated with increasing concentrations of labeled BSA in the media (Table 2) and by the observation that increasing amounts of cold BSA did not inhibit the uptake of label (Table 3). In both cases, the endocytic index measured after an overnight uptake was independent of BSA concentration.

The EI for BSA was less than those for the non-digestible substrates. This could be due to ongoing protein degradation during uptake since the amount of label accumulated by cells in a 24 hour period increases in the presence of protease inhibitors. The uptake of BSA was also increased in the presence of dextran sulfate. This might have been due to the inhibition of degradation since dextran sulfate was found to inhibit release of TCA soluble radioactivity (Figure 26). It could also have been due to an increase in pinocytosis by cells in the presence of dextran sulfate (112) or to the formation of non-covalent dextran sulfate-protein conjugates which are taken up by the cells (144).

The uptake of BSA is also increased following denaturation by boiling. Similar results have been reported for formalin-treated BSA with rat yolk-sac (107). This

increased uptake is not due to decreased degradation in the lysosomes as the rate of release of TCA soluble radioactivity from the denatured BSA was slightly greater than that from native BSA (Figure 12). Instead, it may have been due to the exposure of hydrophobic regions of the protein on denaturation with a concomitant increase in non-specific adsorption to the plasma membrane of the cells. In contrast,  $\beta$ -galactosidase denatured by freezing and thawing had a decreased overnight uptake, but this was probably due to its much increased rate of degradation after denaturation (Figure 29).

### C. Release of Labeled Substrates

Cells loaded with non-digestible substrates were found to release label in a rapid initial phase followed by a very slow steady release which continued for at least 24 hours (Figure 8). This is in agreement with studies previously reported (105).

Cells loaded with labeled protein were found to release both TCA insoluble and soluble radioactivity with time (Figure 9). The time course of the release of TCA insoluble label resembled that of the release of

<sup>14</sup>C-dextran-sulfate by cells; there was a rapid initial release followed by a slow continuous release of label.

This TCA insoluble label in the media was shown by gel exclusion chromatography to be associated with a large molecular weight fraction (Figure 11). It has previously been suggested that macrophages are capable of regurgitating the contents of some of the endocytic vesicles back into the media (105). The amount of TCA insoluble label released is greater when the release media contains 50% FCS as compared with 1% FCS (Figure 27). This could be due to an increased membrane activity induced by fetal calf serum (110, 111).

Because of this release of TCA insoluble label, all media samples collected were precipitated with TCA so that the counts due to TCA insoluble and soluble label could be distinguished. In order to assess the true rate of lysosomal protein degradation, it was necessary to correct for the release of intact labeled protein from the cells as described in the results section.

The TCA soluble label released by cells preloaded with <sup>125</sup>I-BSA was also analyzed by column chromatography (Figure 11) and was found to be composed of low molecular weight labeled products (MW > 300) in agreement with the results of others (104, 126-129). When data from the time

course of release of TCA soluble labeled products were analyzed graphically for a first order reaction, the experimental points did not appear to fall on a straight line. Thus, the rates of release of soluble products during the late intervals in the reaction were slower than expected on the basis of the data collected during the early stages. This was particularly apparent in those experiments in which the cells had been pre-exposed to labeled substrates for only a short period (1 hour). Compare, for example, Figures 30 through 33. Similar non-linear semi-log plots have been obtained when native proteins are treated with proteinases in vitro, and is probably due to a heterogeneity of the microenvironments in which the labeled amino acids residues reside in the intact protein. This causes the labeled residues to be released from the protein at different rates.

Other possible explanations for the non-linearity of the semi-log plots of the release data were also considered. These include heterogeneity in the cell populations, heterogeneity in the lysosomal populations, and heterogeneity in the substrates themselves. If either the first or second explanation was correct, the findings that the rate of release of label during the late stage, as

well as the relative proportion of label released during the early and late stages varied in a systematic way with the size of the protein substrate would require mechanisms for the selective uptake of large and small proteins into different cell types or the selective sequestration of large and small proteins into endocytic vesicles of different hydrolytic capacities. While the possibilities can not be excluded, the absence of any precedents for such behavior together with difficulties in conceptualizing how such mechanisms might work makes these possibilities seem less likely. The possibility that the substrates themselves are heterogeneous is not consistent with the finding that the shapes of the curves obtained with BSA were similar between the various batches of BSA used, were only slightly affected by heat denaturation, and could not be eliminated by subsequent purification of the protein by affinity chromatography. Similarly, the apparent biphasic degradation patterns observed with some of the smaller protein substrates, which exhibited a substantial fast phase, would require a substantial heterogeneity which was not observed when these proteins were subjected to either agarose or polyacrylamide gel electrophoresis prior to labeling. In contrast, the apparent biphasic degradation



patterns observed with large molecular weight proteins, which characteristically exhibited a small initial rapid phase, might have been due to the presence of low levels of denatured protein or other protein contaminants.

A final possibility which must be considered is that the method of labeling of protein results in a heterogeneous population of labeled protein. This will be discussed in greater detail in reference to differences in the rates of degradation of large and small proteins. For now it is sufficient to say that no differences in the apparent degradation rates of protein were observed when they were labeled by either the chloramine T method or the lactoperoxidase method. Moreover, even if the distribution of label within a protein were not the same for each molecule, the non-linear semi-log plots still imply that different labeled residues are released at different rates.

The existence of apparent biphasic degradation curves for the various labeled proteins employed in these experiments implies that cells can accumulate with time labeled materials which are more resistant to subsequent proteolysis than the original protein substrate. Since the period of uptake of the various labeled proteins was usually about 24 hours, one might expect that these labeled cells had accumulated significant amounts of

resistant peptide fragments by the time the degradation studies were initiated. By decreasing the time of uptake of labeled substrates to one hour, one might expect to see a significant increase in the apparent rate of degradation and in the extent of the initial rapid phase. In addition, this effect should be more exaggerated for the large molecular weight proteins which had exhibited the greatest differences in the rates and extents of the fast and slow degradation phases. A comparison of the results in Figures 30, 32, and 33 show that these expectations were realized.

As a further test of the hypothesis that cells can accumulate labeled polypeptide intermediates, the cell associated radioactivity following a 20 hour uptake of labeled BSA was subjected to gel exclusion chromatography. The elution pattern was characteristic of a polydispersed system containing high, intermediates, and low molecular weight radioactive material (Figure 11).

The effect of protease inhibitors on the release of TCA soluble radioactivity was studied. Pepstatin, which inhibits cathepsin D was added to the uptake media at a concentration of 60  $\mu\text{g/ml}$  and was found to inhibit the degradation of  $^{125}\text{I}$ -BSA (Figure 34). No effect was

observed when pepstatin was added only to the release media after the initial 20 hour uptake period. Similarly, pepstatin had no effect on degradation when added to the uptake media when the duration of uptake was limited to one hour. These results are consistent with the conclusion that the uptake of pepstatin into cells is by endocytosis rather than by passive diffusion. Chloroquine, a lysosomotropic reagent which alters intralysosomal pH and which inhibits cathepsin B and other lysosomal proteases (143), also inhibited the degradation of  $^{125}$ I-BSA which had been taken up by macrophages (Figure 34). This inhibition occurred irrespective of whether chloroquine was added during the uptake period or during the release period in agreement with earlier findings that chloroquine can rapidly penetrate the cell by passive diffusion (145). In contrast to these results, leupeptin and antipain, inhibitors of cathepsin B<sub>1</sub> and other lysosomal thiol dependent proteases, actually enhanced the apparent rate of degradation of  $^{125}$ I-BSA. However, the concentrations of inhibitors used in these experiments were high (290 µg/ml) and part of their effect could be due to other actions on the cells. Since the plasma membranes of some cells are permeable to these inhibitors it

is not possible to rule out an extra lysosomal site of action. In addition, the presence of these inhibitors in long-term culture of newborn heart cells has been found to actually cause an increase in the level of cathepsin B in these cells (151). As a consequence of these considerations, the results we obtained with leupeptin and antipain are difficult to interpret.

There is a substantial body of evidence in the literature which indicates that denatured proteins are degraded more rapidly by proteases in vitro than are native proteins. This may be due to an increase in the accessibility of previously buried peptide bonds in the denatured state or to an increased conformational flexibility of the peptide backbone in the denatured state. Intuitively, therefore, one might expect denaturation to have a greater effect on the degradation rates of large proteins, since these contain a greater proportion of buried peptide bonds and are degraded more slowly in the native state in vivo. A comparison of the degradation rates for native and denatured BSA and  $\beta$ -galactosidase in Figures 28 and 29 confirm this expectation. Thus, while the denatured protein was degraded more rapidly than the native protein in both cases, the effects of denaturation were

much greater for the larger  $\beta$ -galactosidase than for BSA.

One complication in interpreting the results of such denaturation studies is that we have no assurance that the denatured states of these two proteins in dilute aqueous solution are in any way equivalent, since our definition of denaturation is simply a loss of biological activity. For BSA, the absence of biological activity was judged by the failure of the heat treated protein to bind the ligand conjugated to agarose in the affi-gel blue column. For  $\beta$ -galactosidase, denaturation was judged by the loss of enzymic activity following repeated freezing and thawing of dilute aqueous solutions of the enzyme.

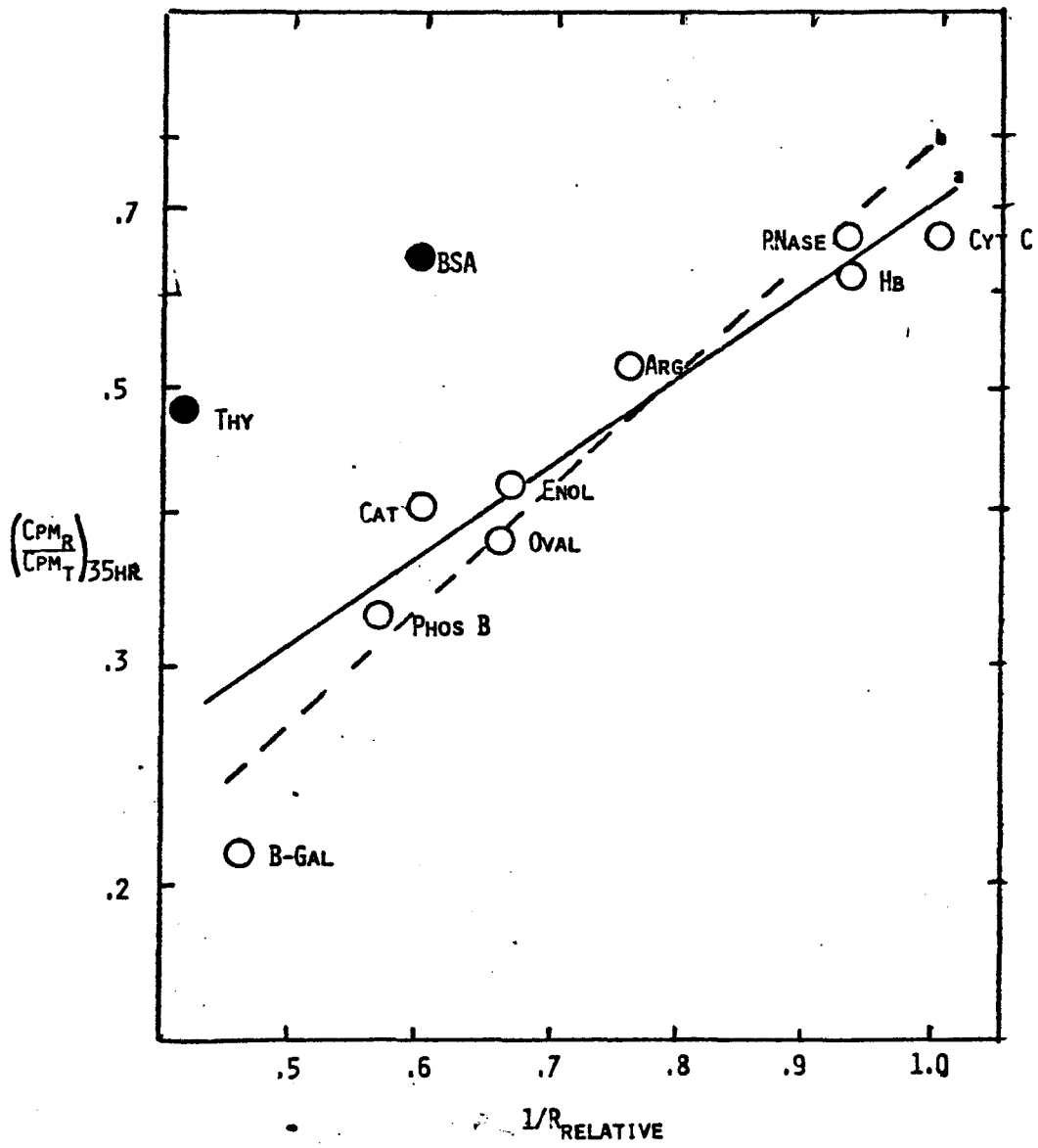
#### D. Protein Structure and Degradation Rate

There appeared to be a relationship between the subunit molecular weights and the degradation rates of the various protein substrates studied (Figure 24). We have previously suggested that the non-linear semi-logarithmic plots of our degradation data were due to differences in the susceptibilities of various peptide bonds to proteolytic cleavage. Moreover, the increase in the rate of degradation of denatured  $\beta$ -galactosidase may have been due to an increased exposure of normally buried peptide bonds in the denatured state. This leads to the

idea that the degradation rates of various proteins should in some way be related to the fraction of the total number of peptide bonds exposed to solvent and thus accessible action of the lysosomal proteases. Since the surface area of a sphere varies with the square of its radius while its volume varies with the cube of its radius, the ratio of surface area to volume should be inversely proportional to its radius ( $A/V \propto 1/R$ ). Therefore, for a series of globular proteins which approximate spheres, one would expect that the fraction of peptide bonds accessible to proteases, and thus the apparent degradation rate might be proportional to the inverse of the subunit radius. Figure 38 shows a plot of  $1/\text{relative subunit radius}$  vs the log of the fraction of undigested labeled material remaining in the cell after 35 hours for the various proteins in this study. In this plot,  $r$  was calculated by assuming that all the proteins or protein subunits were spheres of equal density.

A relative  $r$  was then calculated by setting  $r$  for cytochrome  $c$  equal to 1. As can be seen, a straight line can be drawn through most of the data points. Two proteins whose degradation rates deviate markedly from this line are thyroglobulin and BSA. In each case, both proteins behave as if their subunit molecular weights were

Figure 38. Plot of  $1/\text{relative radius}$  versus the log of  $(\text{cpm}_t - \text{cpm}_r) / \text{cpm}_t$  at 35 hours. Solid line, all the proteins except thyroglobulin, BSA and  $\beta$ -galactosidase; dashed line, all the proteins except thyroglobulin and BSA.





much smaller than expected. The anomalous behavior of thyroglobulin can be readily explained in terms of its structure and biological function. Characterization of thyroglobulin synthesized from thyroglobulin messenger RNA suggests that it is composed of two polypeptide chains of two polypeptide chains of 300,000 to 330,000 molecular weight (146, 147). This protein appears to be highly susceptible to limited proteolysis so that when the isolated 660,000 molecular weight dimer is subjected to SDS disc gel electrophoresis in the presence of mercaptoethanol, a variety of discrete bands are obtained with a range of molecular weights from 19,000 to 330,000 (148). It has been suggested that the monomer consists of a linear array of a repeating peptide unit of molecular weight of approximately 19,000 (148, 149). On this basis, it would be expected that the thyroglobulin subunit could behave in our system as if it were much smaller than 330,000. From our data, thyroglobulin appears to be composed of subunits of average molecular weight of 32,000. The explanation for the anomalous behavior of BSA may be similar. Recent studies have shown that this protein is rapidly cleaved by endopeptidases into smaller fragments

which retain important structural and functional features of the intact protein (150). BSA thus behaves as if it were composed of several globular domains linked by short peptide segments. In our system, such a molecule might be expected to behave as if it were composed of smaller subunits. From our results, BSA appears to have an average subunit molecular weight of 17,000.

#### E. Comparisons With Intracellular Protein Degradation

The work by many authors cited in this dissertation indicate the possible existence of more than one pathway for intracellular protein degradation. One pathway appears to involve lysosomes and is sensitive to inhibition by lysosomal proteinase inhibitors and agents which disrupt lysosomal function. The second may involve an extralysosomal degradative system. However, this conclusion is still controversial and others have proposed that the lysosome is the major site of cellular protein turnover. The work described in this dissertation was undertaken with the belief that the results might permit these two alternatives to be distinguished.

Table 15 summarizes some of the characteristics of intracellular protein turnover which must be explained by any description of this process. Figures 1 and 3 describe

the two types of alternative mechanisms which have been proposed. The model described in Figure 1 assumes that cellular proteins are degraded primarily in lysosomes with the proteolytic steps being rate determining. Thus, the half life of a cellular protein is reported to be related to its inherent susceptibility to the combined action of the various proteolytic and peptidolytic activities in the lysosome. In order to account for differences in turnover rates between proteins, this model further assumes that the transfer of proteins between the lysosome and other cellular compartments can occur in two directions. Thus, stable proteins are those which escape the lysosome before significant degradation can occur, while short lived proteins are those which are almost completely degraded within the period of their residency in the lysosome. Although no compelling evidence has ever been presented to support a two way transfer of proteins into the lysosome, it has been noted that lysosomes themselves turn over, and it is conceivable that at some point near the end of their lifetime they become "leaky." This model predicts that the properties of intracellular protein turnover should strongly resemble those of lysosomal protein degradation.

A second form of model 1 proposes that the rate determining step in cellular protein turnover is the transfer of the substrate into the lysosome. Accordingly, a steady state should exist in which the amounts of various cellular protein substrates within the lysosome are related to the ratios of their rates of transfer into the lysosome and their rates of degradation within the lysosome. This model proposes that differences in turnover rates between proteins are largely due to differences in their rates of sequestration by lysosomes. This model makes the following predictions: Increased protein turnover caused by nutritional deprivation results from an increased rate of transfer of protein into the lysosome and is reflected in an increased size of the lysosomal substrate pool; low concentrations of lysosomal proteinase inhibitors should produce only a transient inhibition which diminishes as the substrate pool within the lysosome increases to a new steady state level; intermediate levels of inhibitors should produce a more persistent inhibition, while high concentrations should block protein turnover and produce an extensive vacuolization of the cell. In agreement, it has been observed that the size and protein content of lysosomes is initially low in the fed state

in perfused rat liver, but increases in proportion to increases in protein turnover during amino acid deprivation (157, 158). In addition, the inhibition of protein degradation in cultured macrophages induced by chloroquine is accompanied by a profound increase in the number and size of vacuoles within the cell (88). The failure of lysosomal proteinase inhibitors to completely block cellular protein turnover may not be incompatible with models 1 and 2, because the large number of proteolytic and peptidolytic activities within the lysosome may preclude the complete inhibition of this system by one or a few agents. A more serious objection may be the apparent lack of additivity of these inhibitions (72).

The third model, described in Figure 3, proposes the existence of at least two pathways of cellular protein degradation. One pathway associated with lysosomes degrades most cellular proteins at a comparable rate which may depend on how rapidly portions of the cytoplasm are sequestered in lysosomes. The second pathway is extralysosomal and degrades various cellular proteins at different rates depending on their accessibility, their inherent susceptibility toward proteolysis, or the ease with which they undergo spontaneous physical or chemical

modification to a more susceptible form. Accordingly, long lived proteins are relatively resistant to the action of this system and are degraded primarily in lysosomes, short lived proteins are sensitive to the action of the extralysosomal system and are degraded by it. Proteins with intermediate half-lives are degraded in varying proportions by both systems. This model can account for differences in degradation rates between proteins, for the inability of inhibitors to completely block protein turnover (Table 15), for differences in the sensitivity of short-lived and long-lived proteins to inhibition of degradation (Table 15), and for the observation that starvation, which increases the contribution of the lysosomal pathway, ameliorates the differences seen in the degradation rates between proteins.

The work described in this dissertation allow a distinction to be made between some of the models described above. Thus, I have observed that small proteins are degraded more rapidly in the lysosome than are large proteins despite the fact that small proteins turnover more slowly in vivo (Table 15). Similarly, I have observed no important differences in the rate of degradation of acidic and basic proteins despite the fact that acidic proteins

TABLE 15

Comparison of Intracellular and Extracellular Protein Degradation<sup>a</sup>  
Physical Properties of Proteins<sup>b</sup>

<u>Intracellular Proteins</u>		<u>Extracellular Proteins</u>	
<u>Long Half-Life</u>	<u>Short Half-Life</u>	<u>Long Half-Life</u>	<u>Short Half-Life</u>
Low Molecular Weight	High Molecular Weight	High Molecular Weight	Low Molecular Weight
Basic pI	Acidic pI	No Relationship Seen Between pI and Degradation Rate	Normal Conformation
Normal Conformation or Amino Acid Composition	Abnormal Conformation or Amino Acid Composition	Normal Conformation	

Effects of Inhibitors on Protein Degradation<sup>c</sup>

<u>Inhibitor</u>	<u>Intracellular Proteins</u>		<u>Extracellular Proteins</u>
	<u>Long Half-Life</u>	<u>Short Half-Life</u>	
Fetal Calf Serum	Inhibits 10% (10%)	No Effect (10%)	Inhibits 29% (50%)
Pepstatin	Inhibits 0-40% (10-100 µg/ml)	No Effect (50 µg/ml)	Inhibits 59% (60 µg/ml)
Antipain	Inhibits 18-28% (50 µg/ml)	No Effect (50 µg/ml)	Increases 74% (290 µg/ml)

TABLE 15 (Cont'd.)

Effects of Inhibitors on Protein Degradation<sup>c</sup>

<u>Inhibitor</u>	<u>Intracellular Proteins</u>		<u>Extracellular Proteins</u>
	<u>Long Half-Life</u>	<u>Short Half-Life</u>	
Leupeptin	Inhibits 20-70% (10-50 µg/ml)	No Effect (10 µg/ml)	Increases 33% (290 µg/ml)
Chloroquine	Inhibits 20-70% (10-50 µg/ml)	Inhibits 12-45% (10-50 µg/ml)	Inhibits 78% (50 µg/ml)

a The values for extracellular protein represent the values for our data at 24 hours degradation time.

b References 17 - 33, 152.

c References 22, 46, 68, 72, 73, 77, 85, 90, and 151.



appear to turnover more rapidly in vivo than do basic proteins (Table 15). However, our samples were too limited to fully test the effect of pI on degradation rate. These observations indicate that the rate determining step in protein turnover is not proteolysis in the lysosome, arguing against model 1. My results also suggest that the rate of lysosomal protein degradation is more sensitive to the effects of chloroquine and pepstatin than is the overall rate of turnover of cellular protein. The inhibitions by these reagents, which I have observed, more closely resemble those seen for the degradation of long-lived proteins, and are clearly much greater than those observed for short-lived proteins (Table 15), supporting model 3.

#### F. Summary

This study characterized the processes of uptake and degradation of extracellular proteins by cultured macrophages and fibroblasts. It was found that labeled BSA was taken up by the macrophages at a rate that was proportional to its concentration in the medium and was independent of the amount of unlabeled BSA present. This result suggests that BSA uptake was either not receptor mediated or that it was not possible to saturate the receptors under the experimental conditions used.

Cells loaded with labeled proteins released TCA insoluble and soluble radioactivity into the medium. The appearance of TCA insoluble label may have been due to regurgitation of undegraded protein and its release was virtually complete by 6 hours. TCA soluble labeled products were released during the entire course of the experiment and was due to degradation of the labeled protein within the cells.

The initial rate of release of the TCA soluble label was proportional to the amount of label taken up by the cells and therefore appeared to be first order in substrate. However, first order plots of the release data were non-linear over time. This was interpreted to mean that labeled amino acid residues in different regions of the protein were cleaved at different rates. It was further hypothesized that the slower degradation observed at later times was due to the accumulation of protein fragments more resistant to cleavage than the intact protein. In confirmation of this conclusion, it was observed that short uptake periods resulted in increased rates of release of soluble degradation products.

The release of TCA soluble label was inhibited by 50% FCS, chloroquine, and pepstatin. This inhibition is

consistent with degradation having occurred in the lysosome.

The overall rate of degradation of a protein was inversely proportional to its subunit size. This observation can be explained if it is assumed that the degradation rate of protein is proportional to the fraction of its peptide bonds exposed on the surface. For a spherical protein, this fraction should be inversely proportional to its molecular radius. In confirmation, it was shown that prior denaturation produced a greater increase in the degradation rate of a large protein than of a small protein.

Finally, a comparison of our results with those obtained for cellular protein degradation argues against a model for the latter in which the rate determining step is proteolysis in the lysosome. Instead, a model involving both lysosomal and extra-lysosomal degradative systems in cellular protein turnover is favored.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

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

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