

DEGRADATION OF PROTEINS

STUDIES ON THE DEGRADATION OF DIHYDROFOLATE REDUCTASE AND
OTHER INTRACELLULAR PROTEINS BY MAMMALIAN CELLS

by

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ABSTRACT

The mechanisms by which mammalian cells degrade intracellular proteins have been investigated. Radioactively labelled proteins of rat hepatoma tissue culture cells were incubated in cell-free systems in an attempt to identify the degrading processes. Although proteins in intact cells are degraded to their constituent amino acids, little acid-soluble material was produced from the labelled proteins at neutral pH, even in cell homogenates which contained all the cellular components.

Dihydrofolate reductase from a subline of the L1210 lymphoma was radioactively labelled and used as a model protein so that changes in a single protein could be investigated. The enzyme was inactivated during incubation with homogenates of the same cell line. Little inactivation was produced by the 100,000 x g supernatant while inactivation was rapid during incubation with the 27,000 x g pellet which contains mitochondria, lysosomes and microsomes, or with the 100,000 x g pellet which contained microsomes.

Column chromatography on Sepharose 2B showed that inactivation of dihydrofolate reductase was accompanied by its incorporation into material of high molecular weight. Since inactive enzyme was recovered in several fractions of the column eluate corresponding to the elution volume of the proteins of the subcellular membranes, this material probably represented enzyme which was inactive and bound to these membranes. Greatest binding occurred with membranes of the microsomal fraction.

The formation of inactive enzyme of high molecular weight did not depend on prior denaturation of the enzyme nor on prior proteolytic cleavage since no significant production of acid-soluble material, change in molecular weight or change in the charge could be demonstrated. Treatment of the inactive enzyme with urea resulted in reactivation, again suggesting that extensive modification of the enzyme by proteolysis did not occur.

Binding of the enzyme to microsomal membranes was not reversed by dilution or by the addition of cofactor and methotrexate. Methotrexate, an inhibitor of dihydrofolate reductase, increases the levels of the enzyme in the cells of patients with cancer treated with the drug, possibly by stabilization against degradation. When methotrexate was present during incubation of dihydrofolate reductase with the subcellular membrane fraction inactivation and binding of the enzyme to microsomal membranes was decreased.

The suggestion is made that binding to microsomal membranes could represent the initial step in the degradation of proteins in intact cells. Lysosomes contain proteases and are a likely site of degradation of intracellular proteins. The failure to demonstrate degradation in broken cell preparations could be due to proteins not being able to enter lysosomes. These organelles are formed from vesicles of the Golgi apparatus. It is suggested that by binding to membranes of the microsomal fraction in intact cells, intracellular proteins could be incorporated into lysosomes at the time of their formation.

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
DHF	7,8-dihydrofolate (dihydropteroylglutamic acid)
DHFR	dihydrofolate reductase
dpm	disintegrations per minute
DTT	dithiothreitol (Claland's Reagent)
EDTA	ethylene diamine tetra-acetate
HTC	hepatoma tissue culture
MTT	3-(4,5-dimethylthiazdyl-2)-2,5-diphenyl tetrazolium bromide
MTX	methotrexate (formerly amethopterin) (4-amino-N ¹⁰ - methyl pteroylglutamic acid)
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
THF	tetrahydrofolate
Tris	tris(hydroxymethyl)-amino methane

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1. INTRODUCTION

The activity of enzymes in cells can be regulated by stimulation or inhibition of existing protein molecules (Frieden, 1971; Holzer and Duntze, 1971) and by changes in the number of these molecules (Schimke and Doyle, 1970). The intracellular proteins of mammalian cells are being synthesized continuously so regulation of the synthesis of proteins can control their levels. Proteins are also degraded continuously and the amount of protein present can be controlled by changes in the rate of degradation. While rapid advances have been made in our knowledge of the mechanism and control of protein synthesis, much less is known about the processes by which proteins are degraded as indicated in recent reviews (Goldberg and Dice, 1974; Schimke and Kutsumura, 1975; Goldberg and St. John, 1976).

1.1 CHARACTERISTICS OF PROTEIN DEGRADATION

Proteins are degraded continuously in both dividing and resting mammalian cells (Eagle et al., 1959; Tanaka and Ichihara, 1976). The rate of degradation of general cellular proteins has been measured in cultured mammalian cells by the release of acid-soluble radioactivity from proteins pre-labelled with radioactive amino acids. Such studies have shown that proteins are degraded at 2-3% an hour in cultured rat hepatoma cells (Hershko and Tomkins, 1971; Gelehrter and Emanuel, 1974). Not all proteins are degraded at the same rate, however. The rate of degradation of individual proteins can differ greatly from the average. In rat liver, for example, the average half life of proteins is 2 1/2 - 3 days (Schimke, 1964). The half-life of ornithine decarboxylase in this

tissue is 11 min (Russell and Snyder, 1969), while that of isozyme 5 of lactate dehydrogenase is 16 days (Fritz et al., 1969).

The rate at which proteins are degraded can be altered by various physiological changes. During starvation the rate of degradation of liver and muscle proteins increases (Millward, 1970). The rate of degradation is influenced by hormones such as glucagon which stimulates protein degradation in perfused rat liver (Woodside et al., 1974) and by insulin which inhibits protein degradation in isolated rat diaphragm (Fulks et al., 1975), perfused rat heart (Rannels et al., 1975) and in perfused liver (Mondon and Mortimore, 1967). Nutritional factors can also alter the rate of protein degradation *in vivo*. Inhibition of degradation by amino acids has been demonstrated in perfused rat liver (Woodside and Mortimore, 1972) and in isolated rat diaphragm (Fulks et al., 1975) and similar results have been obtained in tissue culture systems. Hershko and Tomkins (1971) have shown that removal of serum and amino acids from the medium markedly increased the rate of degradation of HTC cell proteins. Removal of these nutrients also increased degradation in Reuber H35 cells while the addition of insulin prevented this increased rate of degradation (Gunn et al., 1976).

While a physiological change may have an effect on the average rate of degradation of the proteins of a tissue, it does not always produce the same effect on all of the proteins. For example, during starvation the rate of degradation of rat liver proteins increases (Millward, 1970) but the rate of degradation of arginase decreases (Schimke, 1964).

1.2. DETERMINATION OF THE RATE OF DEGRADATION BY PROPERTIES OF THE PROTEIN SUBSTRATE

Whatever the mechanism for degrading intracellular proteins, it has to explain why different proteins are degraded at different rates. It also has to explain how the rate of degradation of individual proteins can be independently altered by changing physiological conditions.

One factor which appears to be important in determining the rate of degradation of a protein is the structure of the protein itself. Evidence for this comes from studies on the degradation of abnormal proteins. Work with bacteria has shown that abnormal proteins resulting from errors during translation or synthesized after exposure to puromycin or amino acid analogues, are degraded more rapidly than normal proteins (Goldberg, 1972). Similar results have been obtained using mammalian cells. Knowles et al., (1975) showed that in Reuber H35 hepatoma cells phosphoenolpyruvate carboxykinase (guanosine triphosphate) synthesized in the presence of medium which contained canavine instead of arginine or 5-fluorotryptophan or 6-fluorotryptophan instead of tryptophan was degraded more rapidly, *in vivo*, than enzyme which was synthesized in the presence of the normal amino acids. Similarly, exposure of human fibroblasts to canavine resulted in an increased rate of degradation of those proteins which were synthesized in the presence of canavine but not of those proteins synthesized in the absence of canavine (Bradley et al., 1976). Rabinovitz and Fisher (1964) have shown that reticulocytes rapidly degrade the haemoglobin which is synthesized in the presence of

analogues of valine or lysine. There is also evidence that proteins synthesized in the presence of mutations are also degraded more rapidly than normal proteins. Haemoglobin Subine, in which a leucine residue is substituted by proline, and haemoglobin Ann Arbor, in which a leucine residue is replaced by arginine, both occur in very low concentrations compared to haemoglobin A. These low levels are due to the rapid degradation of the abnormal proteins (Shaeffer, 1973; Adams et al., 1972). Capecchi et al., (1974) have shown that L cells degrade several missense mutant enzymes of hypoxanthine-guanine phosphoryl transferase more rapidly than the wild type enzyme. The rate of degradation of general proteins in all the mutants was the same.

The reason that abnormal proteins are degraded more rapidly could be that these proteins have an altered conformation as a result of the change in their primary sequence. Observations of the effects of ligands on the rate of degradation of proteins provide evidence that the conformation of a protein is indeed an important factor in determining its rate of degradation.

The conformation of a protein is highly flexible (Englander et al., 1972) and can be altered by interaction with ligands (Yankeeelov and Koshland, 1965; Ludwig et al., 1967 and Citri, 1973). Such interactions have been shown to protect protein molecules from proteolysis, presumably by stabilizing the proteins in conformations in which susceptible bonds are not accessible to the degrading enzymes (Markus, 1965; O'Connell, 1969 and Tanluchi and Anfinsen, 1969).

Interactions of proteins with ligands may be important in

stabilizing proteins against degradation in intact cells. When tryptophan is fed to rats, the rate of degradation of tryptophan pyrrolase in rat liver decreases (Schimke et al., 1965). Since tryptophan prevents digestion of the enzyme by trypsin and chymotrypsin *in vitro*, its effects *in vivo* could be due to stabilization of tryptophan pyrrolase. Administration of thymidine to rats results in increased levels of thymidylate kinase in rat liver and kidney (Hiatt and Bojarski, 1960). It is likely that this results from stabilization of the enzyme against degradation since the enzyme is stabilized by thymidine in homogenates of rat liver (Bojarski and Hiatt, 1960).

Interactions of proteins with other factors may also determine their rate of degradation. Dunaway and Segal (1974, 1976) have described a peptide which specifically stabilized an isozyme of phosphofructokinase against thermal inactivation and against proteolysis by lysosomal enzymes. When the dietary intake of rats was decreased and then glucose administered, the levels of the peptide in rat liver increased. These increased levels were demonstrated before a corresponding increase in the levels of the isozyme of phosphofructokinase occurred (Dunaway and Segal, 1976).

Other factors which may be involved in determining the rate of degradation of proteins include their molecular weight and the isoelectric point. Larger proteins are degraded more rapidly than smaller ones (Dehlinger and Schimke, 1970; Dice et al., 1973; Aplers, 1973; Gurd and Evans, 1973) and acidic proteins more rapidly than neutral or basic ones (Dice and Goldberg, 1975a). Since exceptions to both of these correlations have been demonstrated (Dice and Goldberg, 1975; Dice and Goldberg, 1975a).

the size and charge of proteins could be involved in determining the rate of degradation in an indirect way, for example, by affecting the conformation of a protein (Goldberg and St. John, 1976).

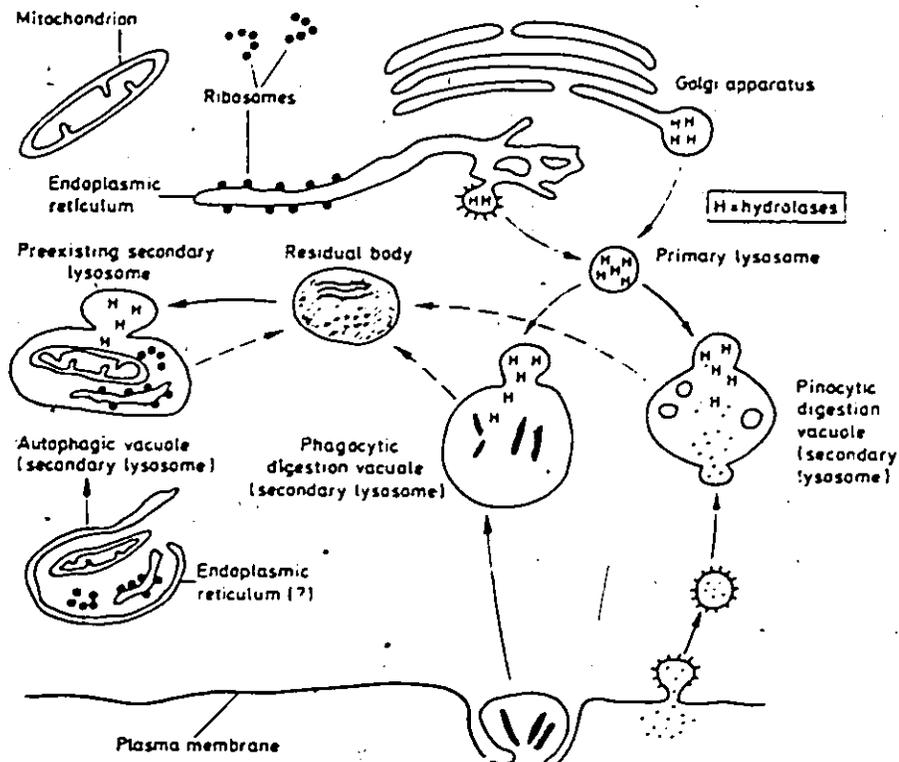
The way in which properties of a protein determine its rate of degradation is not known. Conceivably the rate could depend on the susceptibility of a particular protein conformation to the degrading system. The conformation of individual proteins could be altered by changing levels of substrates, cofactors or specific factors such as that described by Dunaway and Segal (1974, 1976). There is little information on this possibility since the nature of the degrading system is not known. Possible systems are considered in the following sections.

1.3 NATURE OF THE DEGRADING SYSTEM

1.3.1. Lysosomes

Mammalian cells contain lysosomes which are membrane bound structures containing many hydrolytic enzymes (deDuve and Wattiaux, 1966). These include a carboxypeptidase, cathepsin A; endopeptidases, cathepsin B, cathepsin D and a dipetidyl aminopeptidase, cathepsin C.

The following scheme (Holzmann, 1976) illustrates the mechanism by which lysosomes are formed:



Lysosomal enzymes are synthesized in the rough endoplasmic reticulum and are transported to the Golgi apparatus for packaging (deDuve and Wattiaux, 1966; Goldstone et al., 1973). Primary lysosomes

are formed by budding off of vesicles from the Golgi apparatus (Holzmann, 1976). Lysosomes are known to be involved in the digestion of foreign material which enters cells by phagocytosis or by pinocytosis. Such material is contained in vacuoles which fuse with the primary lysosomes to form secondary lysosomes (Holzmann, 1976). In addition, portions of the cytoplasm, often containing recognizable cellular structures such as mitochondria; become surrounded by membranes to form autophagic vacuoles. These vacuoles also fuse with primary lysosomes to form secondary lysosomes (Holzmann, 1976).

The use of radioactively labelled molecules has shown that material which gains access to lysosomes by pinocytosis is digested inside these organelles. When ribonuclease labelled with [^{125}I] was injected into mice, label was rapidly lost from the blood and accumulated in kidney cells. Subsequently a loss of radioactivity from the kidney occurred (Davidson et al., 1971). Studies on cell-free systems showed that the radioactive protein was associated with the lysosomal fraction. When the lysosomes were incubated *in vitro*, production of acid-soluble material from the [^{125}I]-labelled ribonuclease was demonstrated (Davidson, 1973; Davidson, 1975a).

In similar experiments [^{131}I]-labelled albumin was injected into rats. The radioactive protein accumulated in lysosomes of rat liver and production of acid-soluble material could be demonstrated during *in vitro* incubation of isolated lysosomes (Mego, 1973).

- a) Evidence that lysosomes are involved in the degradation of intracellular proteins

Since lysosomes contain proteases and are known to degrade foreign proteins, they are a likely site of degradation of intracellular proteins (deDuve and Wattiaux, 1966; Holzmann, 1976; Schimke and Doyle, 1970). Some evidence that lysosomes are involved in protein degradation has come from the use of various inhibitors. Wibo and Poole (1974) have treated cultured rat fibroblasts with chloroquine. This drug is taken up by the cells and accumulates within lysosomes (Wibo and Poole, 1974; deDuve et al., 1974). Treatment with chloroquine resulted in an inhibition of protein degradation possibly as a result of the inhibition of cathepsin B₁ by chloroquine (Wibo and Poole, 1974). Similar results have been obtained using pepstatin, a pentapeptide which inhibits cathepsin D (Barrett and Dingle, 1972) but does not affect any other known protease of rat liver (Dean, 1975b). This drug inhibits the degradation of cytoplasmic proteins by lysosomal proteases *in vitro* (Dean, 1976). Since pepstatin does not cross cell membranes, effects on protein degradation in intact cells were not demonstrated until recently when Dean (1975b) perfused rat liver with medium containing pepstatin incorporated into lipid vesicles. Such vesicles can cross cell membranes and molecules incorporated into them are accumulated inside lysosomes (Gregoriadis and Ryman, 1972; Gregoriadis, 1974; Gregoriadis et al., 1974; Weissman et al., 1975). Dean showed that while pepstatin alone or lipid vesicles alone had no effect on the rate of degradation of the proteins of rat liver, perfusion with medium containing pepstatin incorporated in vesicles resulted in a decreased rate of protein degradation.

Other circumstantial evidence that lysosomes may be involved in

the degradation of intracellular proteins comes from observations of lysosomes and their enzymes under conditions in which the rate of protein degradation is altered. Denervation leads to an increased rate of protein degradation (Goldberg, 1969) and an increased amount of lysosomal enzymes (Wienstock and Iodice, 1969). In muscular dystrophy the levels of lysosomal proteases are markedly increased (Wienstock and Iodice, 1969; Kar and Pearson, 1972). During perfusion of rat liver with an unsupplemented medium, protein degradation is stimulated while lysosomes become larger and more fragile (Mortimore and Neely, 1975). If insulin, amino acids or cycloheximide is added, there is a corresponding decrease in the rate of protein degradation and in the size and fragility of the lysosomes (Neely et al., 1974; Mortimore and Neely, 1975).

Studies on the degradation of cytoplasmic proteins *in vitro* confirm that lysosomal proteases can hydrolyze these proteins. Hayashi et al., (1973) showed significant degradation of the proteins of rat liver during incubation with lysosomes. Their incubations were performed below pH 5.0, the optimum pH for lysosomal proteases, and under conditions in which the lysosomes were largely disrupted (Huisman et al., 1974). Dean (1975) and Segal et al., (1974) have used lysosomes from rat liver at acidic pH's and in the presence of Triton X-100, a detergent which disrupts lysosomal membranes. They have shown that soluble proteins of rat liver are rapidly degraded under these conditions. They labelled the proteins by the dual-isotope method of Arias et al., (1969). This method involves exposing the cells to an amino-acid such as [¹⁴C]-

leucine for a period of time. The isotope is then removed and the cells are exposed for a shorter period of time to the same amino-acid labelled with [^3H]. In this way intracellular proteins are labelled with both [^{14}C] and [^3H] and have [$^3\text{H}/^{14}\text{C}$] ratios which differ depending on the rate of degradation of the proteins. Those proteins which are rapidly degraded have a higher ratio than do those proteins which are degraded more slowly. Using proteins labelled in this way, they showed that liver proteins with short half-lives *in vivo* were degraded more rapidly by the lysosomal enzymes than were those proteins with longer half-lives *in vivo*. Although these results confirm that lysosomal enzymes can degrade intracellular proteins if they come into contact with them at a suitable pH, they do not prove that these are the enzymes which are responsible for degradation in intact cells. Similar correlations between the rate of degradation *in vivo* and the rate of proteolysis by a variety of different proteases have been shown. Dice et al., (1973) labelled proteins of rat liver by the dual-isotope method (Arias et al., 1969) in such a way that those proteins which were rapidly degraded *in vivo* had a high $^3\text{H}/^{14}\text{C}$ ratio. The acid-soluble material produced during incubation of these proteins with trypsin or pronase, *in vitro*, had a high ratio of $^3\text{H}/^{14}\text{C}$ indicating that those proteins which were rapidly degraded *in vivo* were also rapidly degraded by these non-lysosomal proteases. Bond (1971) has measured the rate of degradation of five enzymes of rat liver by trypsin and chymotrypsin and has shown that the rate of degradation by these proteases correlates with the rate of degradation of the five

enzymes *in vivo*.

More convincing evidence that lysosomes are involved in degrading intracellular proteins would be the demonstration that intracellular proteins were degraded by intact lysosomes, *in vitro*, at neutral pH. Hayashi et al., (1973) could show no production of acid-soluble material from proteins of rat liver during incubation with lysosomes of rat liver at this pH, nor could Huisman et al., (1973) show degradation of serum albumin by lysosomes of rat liver.

b) Access of intracellular proteins to lysosomal proteases

If lysosomes are involved in the degradation of intracellular proteins, intracellular proteins must either enter lysosomes or lysosomal proteins must leak from these organelles.

1) Entry of substrate into lysosomes

If proteins are degraded inside lysosomes, a method for entry into these organelles must exist. As already discussed (1.2) the structure of proteins appears to be important in determining their susceptibility to the degrading system. It is difficult to explain why this is so on the basis of degradation of proteins inside lysosomes.

Intracellular proteins can be incorporated into lysosomes as a result of formation of autophagic vacuoles (deDuve and Wattiaux, 1966; Deter, 1971; Buckley, 1973; Lockshin and Beaulaton, 1974). If formation of such vacuoles simply involves the uptake of a section of cytoplasm, then there would presumably be no selective uptake of individual proteins.

Various models have been proposed to explain how lysosomes could degrade proteins in a selective manner. Segal et al., (1974) suggest that proteins enter lysosomes by a process such as autophagy which is non-specific and that the rate limiting step is the susceptibility of the protein to degradation by lysosomal proteases. This model requires that proteins which are more resistant to these enzymes can leave the lysosomes (Segal et al., 1965; Segal and Dunaway, 1975). As pointed out by Behlinger and Schimke (1970) this model could explain how proteins of lower molecular weight are degraded less rapidly than those of higher molecular weight, if the smaller proteins are able to pass out of lysosomes more readily than larger proteins. The evidence for this hypothesis is based on the observation that the rate of degradation of soluble proteins by lysosomal proteases *in vitro* correlates with the rate of degradation *in vivo* (Segal et al., 1974). As mentioned previously (1.3.1), this does not provide proof that lysosomal enzymes are responsible for the degradation in intact cells since similar correlations have been shown with other non-lysosomal proteases (Bond, 1971; Dice et al., 1973). There is no evidence that proteins can escape undigested from lysosomes (Gordon, 1973).

deDuve and Wattiaux (1966) have suggested that autophagic vesicles could selectively engulf altered proteins. They suggest that denaturation of proteins could be the first step in degradation and that denatured proteins either induce autophagy or are transported to a region of the cell where autophagy occurs. Evidence that selective modification of proteins may occur prior to their total hydrolysis is presented later (1.3.2.).

Another model is one in which proteins enter lysosomes by a process of pinocytosis (Poole, 1975; Dean, 1975a). Pinocytosis is the process by which cells ingest molecules which are first adsorbed onto the cell membrane. They are incorporated into the cell when the membrane folds inwards forming a vesicle which is pinched off. This process is a highly specific one. When cells are exposed to a mixture of proteins, the proteins enter at very different rates, presumably as a result of their differing affinities for receptors on the plasma membrane (Jacques, 1969; Williams et al., 1975). Poole (1975) suggests that intracellular proteins could enter lysosomes by an analogous process. He proposes that proteins bind to lysosomal membranes to different degrees and become incorporated into these organelles following invagination of the lysosomal membrane. In *in vitro* experiments, Dean (1975a) has shown that proteins become adsorbed to the membranes of a lysosomal preparation and that there is a correlation between the degree of binding and the rate of degradation of the protein in intact cells. There is little evidence, however, that extensive pinocytosis of lysosomal membranes occurs. One report shows that ferritin incorporated into the cytoplasm of macrophages following ingestion of erythrocytes is removed from the cytoplasm and incorporated into lysosomes by a process which might involve pinocytosis of lysosomal membranes. Electron microscope studies suggest that while some ferritin becomes incorporated into autophagic vacuoles, other molecules enter lysosomes directly in vesicles which are formed by invagination of lysosomal membranes (Fedorko et al.,

1973). Another study (Buckley, 1973) has shown by light microscopy that lysosomes are continually putting out extensions. Buckley suggests that these extensions could be involved in the formation of autophagic vacuoles. If this were so, proteins selectively bound to the lysosomal membranes would be selectively incorporated. The general occurrence of this lysosomal movement and its role in the formation of autophagic vacuoles, however, are not known.

ii) Leakage of lysosomal proteases

Lysosomal proteases have pH optima in the acidic range (Barrett, 1972). The pH inside lysosomes has generally been assumed to be acidic (deDuve and Wattiaux, 1966). Some evidence that this is so has been obtained from the observation that weakly basic dyes accumulate inside lysosomes (deDuve, 1969). If it is assumed that these dyes cross membranes only in the uncharged form, accumulation would be explained by protonation inside the acidic organelles (Reijngoud and Tager, 1973). Further evidence that the pH is acidic comes from the use of yeast which was stained with indicators. After phagocytosis by human polymorphonuclear neutrophils, this material was incorporated into lysosomes where the acidic pH could be demonstrated by observation of the dye by light microscopy (Mandell, 1970). If intracellular proteins are degraded inside lysosomes, therefore, the environment will be at a suitable pH for digestion to proceed. If intracellular proteins are degraded outside lysosomes, however, the proteases must be active at neutral pH. Neutral proteases of lysosomal origin have been reported in some tissues including rabbit polymorphonuclear leucocytes (Davies et al.,

1971), human leukocytes (Oransky et al., 1973) and human spleen (Starkey and Barrett, 1976). At present there is no evidence that these proteases occur in lysosomes from all cell types.

Degradation in the cytoplasm could also be produced by leaked lysosomal proteases which are optimally active at acidic pH's but which have some activity at neutral pH. Under some conditions in which the rate of degradation is altered, changes in the fragility of lysosomes can be demonstrated. During perfusion of rat liver, large lysosomes are formed which are more sensitive to osmotic disruption *in vitro* than are control lysosomes. This change is accompanied by an increased rate of protein degradation (Neely et al., 1974; Mortimore and Neely, 1975). Perfusion of isolated rat hearts results in an increased rate of protein degradation and an increase in lysosomal enzymes in the soluble fraction after homogenization (Rannels et al., 1975). The increase in free levels of enzyme was not accompanied by an increase in the total amount of lysosomal enzymes. Addition of insulin to the perfusion media decreased the rate of protein degradation and also decreased the amount of free, but not total, lysosomal enzymes (Rannels et al., 1975).

Although indicating that leakage of lysosomal proteases could be involved in the degradation of proteins, the increased fragility of lysosomes has not been demonstrated *in vivo*. Some lysosomes are always broken during homogenization of cells (Holzmann, 1976) so it is possible that perfusion of rat liver and heart produces changes in the lysosomes, such as the increased size (Mortimore and Neely, 1975), which make them more susceptible to rupture during homogenization. This interpretation

is supported by findings on the effect of vitamin A on lysosomes. Vitamin A stimulates the release of lysosomal enzymes during incubation of lysosomes *in vitro* (Dingle, 1961; Roels, 1969). It has been suggested that it also decreases the stability of lysosomes in intact cells since administration of excess vitamin A to rats causes an increased amount of free lysosomal enzymes in homogenates of rat liver (Dingle et al., 1966; Sudhakaran and Kurup, 1974). It has recently been shown by Kim et al., (1976) that although treatment of rats with vitamin A resulted in increased lysosomal enzymes in the soluble fraction of homogenized liver, the rate of degradation of the proteins of rat liver was unaltered by treatment with vitamin A. Leakage of the lysosomal proteins in vitamin A treated livers could, therefore, occur only during homogenization or, if cathepsins do leak from lysosomes in intact cells, they probably do not contribute to the degradation of proteins.

1.3.2. Extralyosomal proteases

Several nonlysosomal proteases have been described. Katunuma's group has described group-specific proteases, in liver, skeletal muscle and small intestine of rats. They have described one enzyme which specifically inactivates pyridoxal-phosphate dependent enzymes, another, nicotine adenine dinucleotide dependent enzymes and a third, flavin adenine nucleotide dependent enzymes (Katunuma et al., 1971; Katunuma et al., 1971a; Kominami et al., 1972; Katunuma et al., 1972; Katunuma et al., 1973; Katunuma, 1973). Unlike lysosomal proteases, these enzymes are

optimally active at neutral pH. They do not degrade proteins to their constituent amino acids. The enzyme which inactivates pyridoxal phosphate dependent enzymes inactivates ornithine transaminase without producing any detectable change in the molecular weight of the protein molecule. The release of ninhydrin positive material from the molecule was very small suggesting that inactivation resulted from very limited proteolysis (Katunuma, 1975; Kominami et al., 1975). It was shown that after inactivation the N-terminal amino acid of ornithine transaminase was altered, indicating that the group-specific protease had cleaved the protein close to the N-terminal end (Kominami et al., 1975).

Katunuma's group suggests two ways in which these specific proteases may be involved in the regulation of the degradation of intracellular proteins. First, they suggest that the level of an individual enzyme could be altered by changes in the activity or amount of the group specific protease which inactivates it. They have described inhibitors of these enzymes, the levels of which change under various physiological conditions (Katunuma et al., 1972). They also report that the levels of the proteases themselves change under different conditions (Katunuma et al., 1971; Katunuma et al., 1971a; Katunuma et al., 1972; Katunuma et al., 1973; Katunuma, 1973; Banno et al., 1975).

Secondly, they have shown that inactivation of enzymes by the group specific proteases is prevented by the presence of the appropriate cofactor (Katunuma et al., 1971; Katunuma et al., 1971a; Kominami et al., 1972). They suggest that an important factor determining the rate of degradation of an intracellular protein is the availability of cofactors,

substrates or other ligands which protect the enzyme against inactivation by the group specific proteases. Their model proposes that the rate limiting step in the degradation of a protein is the initial alteration by these proteases and that once altered, the protein is degraded to its component amino acids by other non specific enzymes, perhaps lysosomal in origin (Katunuma et al., 1972; Katunuma, 1973; Katunuma, 1975).

Although Katunuma's group has argued that these group specific proteases are not lysosomal enzymes, they have recently shown that the enzyme specific for pyridoxal phosphate dependent enzymes occurs in mitochondria rather than in the soluble fraction of the cell (Kominami et al., 1975; Katunuma et al., 1975). There is therefore still no evidence that these enzymes exist for the alteration of cytosolic proteins.

Related to the model of Katunuma in which alteration of proteins outside the lysosomes is the initial step in degradation is the suggestion that spontaneous denaturation of a protein is the rate limiting step (Li and Knox, 1972; Ballard et al., 1974). Such a mechanism would explain how the conformation of a protein, as determined by its size, charge, primary sequence and availability of ligands, could determine its rate of degradation. In *E. coli* there is evidence that denaturation may indeed be an early step in the degradation of abnormal proteins. These rapidly degraded proteins accumulate in insoluble aggregates before being hydrolyzed to their constituent amino acids (Prouty et al., 1975). In mammalian cells, however, evidence for this mechanism is limited. Li and Knox (1972) have shown that the catalytic activity of tryptophan oxygenase and the immunological activity was lost from soluble fractions

of rat liver during *in vitro* incubation. They suggest that this represented spontaneous denaturation of the enzyme although they did not eliminate the possibility that the enzyme was altered by a soluble enzyme. Ballard et al., (1974) have shown that phosphoenolpyruvate carboxykinase (guanosine triphosphate) was lost from the soluble fractions of rat liver homogenates, without detectable proteolysis of the enzyme. They suggest that the loss of solubility resulted from denaturation. Although they have since reported that the enzyme becomes bound to microsomal membranes under these conditions (Ballard and Hopgood, 1976) they still argue that denaturation of the enzyme probably occurred first.

1.3.3. Multiple mechanisms

There is evidence that more than one mechanism may be involved in the degradation of intracellular proteins.

Hershko and Tomkins (1971) showed that protein degradation was increased in HTC cells in culture by removing amino acids or serum from the medium. The increased rate of degradation was decreased by inhibitors of protein synthesis while the rate in control cells was unaffected. Similar results were obtained in cultured Reuber H35 cells (Gunn et al., 1976). Poole and Wibo (1973), studying the rate of degradation of proteins labelled by the dual-isotope method (Arias et al., 1969), showed that iodoacetic acid, fluoride and chloroquine decreased the rate of protein degradation in rat fibroblasts in culture. The rate of degradation of proteins of both long and short half-lives was inhibited to the same

extent. The addition of fresh medium to the cells also reduced the rate of degradation of those proteins with long half-lives, but had no effect on the rate of degradation of those proteins with shorter half-lives.

Poole (1975) suggests that inhibition of the degradation of proteins with both long and short half-lives by three different inhibitors (Poole and Wibo, 1973), indicates that all proteins have some degradative steps in common. Since the degradation of some proteins but not of others was decreased by the addition of fresh medium, he proposes that some of the steps in degradation are different for different proteins. Holzmann (1976) suggests that all proteins may be degraded inside lysosomes while differences may exist in the mechanism by which they enter the lysosomes.

Knowles and Ballard (1976), on the other hand, suggest that protein degradation occurs by two different mechanisms, one of which is the formation of autophagic vacuoles and incorporation into lysosomes. The other mechanism may involve degradation of proteins outside of lysosomes.

1.4. RATIONALE AND EXPERIMENTAL DESIGN

It is apparent that although there have been various models put forward for the mechanisms of protein degradation, there is little evidence favouring one over the other (1.3.).

Lysosomes appear to be a likely site of degradation of intracellular proteins, mainly because they contain the necessary proteases. Whether or not the specificity of protein degradation is exerted at the level of the lysosomes or whether there is an initial process such as denaturation or alteration by enzymes such as those described by Katunuma is not known. Alternatively proteins may be degraded to their amino acids by unknown non-lysosomal enzymes.

In an attempt to elucidate the processes involved in degradation, I have used cell-free preparations of mammalian cells and measured the production of acid-soluble material from radioactively labelled proteins in various cellular fractions. Since the initial step in degradation may involve limited cleavage of proteins (1.3.2.), polyacrylamide-SDS gel electrophoresis was used to see whether any changes in the molecular weight of proteins could be detected. In some experiments radioactively labelled proteins of HTC cells were used.

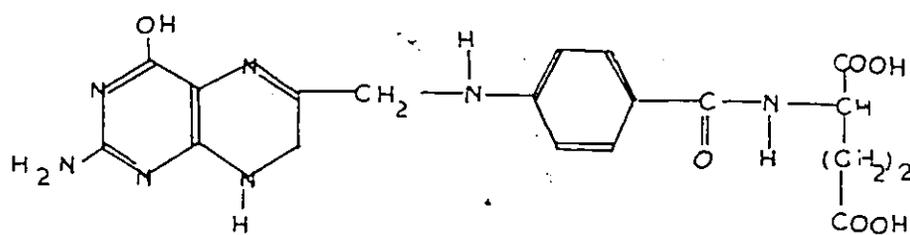
In other experiments a single protein was used so that any changes which occurred during the cell-free incubations could be more readily detected. Dihydrofolate reductase (DHFR) was used as a model protein since purified enzyme could be obtained relatively easily and its degradation appears to be altered by drugs used in the chemotherapy of cancer.

Dihydrofolate reductase is an enzyme which catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by reduced nicotinamide adenine dinucleotide phosphate. The reaction is represented in the following equation:

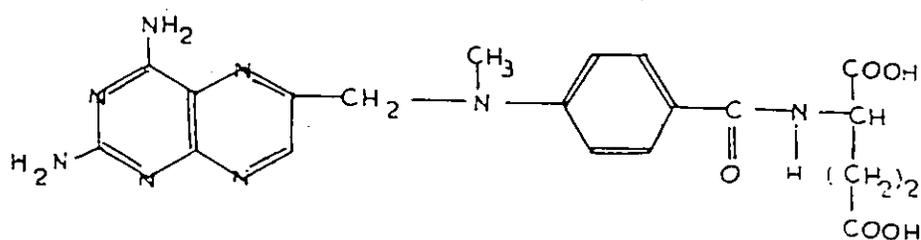


The enzyme is important in dividing cells since it maintains the pool of reduced folate which is essential as the donor of carbon units in the synthesis of purines and in the formation of thymidylic acid from deoxyuridylic acid (Blakley, 1969).

Dihydrofolate reductase is the target enzyme for various folic acid analogues such as methotrexate (MTX) which are used in the chemotherapy of cancer. The similarity between the normal substrate and the analogue can be seen in the following structures:-



DHF



MTX

As well as inhibiting DHFR, methotrexate increases the total amount of enzyme in the cells of patients. Increased enzyme levels are also produced in cultured cells which are treated with the drug (Bertino et al., 1963; Hillcoat et al., 1967). Since MTX stabilizes the enzyme in crude extracts of cultured cells (Hillcoat et al., 1971), the increased levels seen in intact cells possibly result from decreased degradation of the enzyme.

Inhibition of DHFR is an effective method of treating cancer so it is important to understand the processes involved in regulating levels of this enzyme in cells. Since the increased levels produced during treatment with MTX are probably the result of decreased degradation it is especially important to understand the mechanism by which the enzyme is degraded. In the experiments reported here cell-free systems have been used in an attempt to characterize the degrading process. Inactivation of the enzyme by various cell fractions has been studied. In addition, the enzyme was radioactively labelled to determine whether inactivation was accompanied by the production of acid-soluble material, a change in molecular weight, a change in the charge or a change in the solubility of the enzyme.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Chemicals

Adenosine-5'-triphosphate, dithiothreitol, MTT, sodium dodecylsulphate, trichloroacetic acid, nicotinamide adenine dinucleotide phosphate (reduced form), phosphoenolpyruvate, pyruvate kinase, cytochrome c, β -glycerophosphate, phenolphthalein glucuronide, Sepharose 2B and haemoglobin were purchased from Sigma Chemical Company, Saint Louis, MO.; bovine serum albumin and folic acid were from Calbiochem, San Diego, CA.; ethylene diamine tetra-acetate, tris(hydroxymethyl)-amino methane, sodium borohydride, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene from Fisher Scientific, Fairlawn, N.J.; ammonium sulphate (special enzyme grade) and urea from Schwarz-Mann, division of Beckton, Dickinson and Company, Orangebury, N.Y.; Triton X 100, NCS Tissue Solubilizer and naphthalene from Amersham/Searle, Arlington Heights, IL.; methotrexate was from Lederle products department of Cyanamid of Canada Limited, Montreal; acrylamide, N,N'-methylene bisacrylamide, riboflavin, N,N,N' N'-tetramethylethylenediamine and 2-mercaptoethanol from Eastman Kodak Company, Rochester, N.Y.; dioxane, scintillation grade, from Mallinckrodt, Saint Louis, MO., and ammonium persulphate from the McArthur Chemical Company, Montreal.

Fetal calf serum, viokase and the ingredients for RPMI 1640 medium (without sodium bicarbonate) were purchased from the Grand Island

Biological Company, Grand Island, N.Y.

Dihydrofolate was prepared in this laboratory by reducing folic acid with dithionite as described by Blakley (1960).

2.1.2 Radioactive Materials

L-[4,5-³H]-leucine, specific activity 60 curies/ μ mole, [3',5',9(n)-³H] methotrexate, sodium salt, specific activity 5.3 curies/ μ mole and adenosine 5'[\gamma-³²P]triphosphate, ammonium salt, specific activity 15.06 curies/ μ mole were obtained from Amersham/Searle.

L-[¹⁴C(U)]-leucine, 275 millicuries/ μ mole, and [¹⁴C] labelled formaldehyde, 10 millicuries/ μ mole, were from New England Nuclear.

2.2. METHODS

2.2.1. Growth and Maintenance of Mammalian Cells

HTC cells from the rat, originally supplied by Dr. G. Tomkins, were grown in monolayer in 75 cm² plastic flasks (Falcon) in RPMI 1640 medium, supplemented with 10% fetal calf serum. Cells were maintained by subculture of 1×10^5 cells/ml every four days.

Mouse LM cells were obtained from a subline (LM4) of the L1210 lymphoma as described by Hillcoat (1971). When isolated this subline had high levels of DHFR and was resistant to MTX. The cells were passaged every six days in the ascites form by injection of 4×10^6 cells in 0.2 ml of RPMI 1640 medium into the peritoneal cavity of BDF₁ mice.

A model F Coulter counter was used to count cells of both lines.

2.2.2. Preparation of Subcellular Fractions of HTC Cells

HTC cells were radioactively labelled by two methods. In some experiments culture 24 h old were exposed to fresh medium containing 1 μ curie, L-[4,5-³H]-leucine/ml, for a period of 1 h or to medium containing 5 μ curies L-[¹⁴C(U)]-leucine/ml, for a period of 48 h. Cells were harvested by the addition of 2 ml of 0.25% viokase and incubation at 37°C until the cells lifted from the flasks. They were washed once in saline and resuspended in 0.1M Tris-HCl, pH 7.4. After sonication for two periods of 20 sec each, they were centrifuged at 20,000 x g for 15 min and the unincorporated radioactive leucine removed from the supernatant on a column of Sephadex G10 (2.5 x 20 cm), previously equilibrated with 0.1M Tris-HCl, pH 7.4. Fractions containing protein were detected by the absorbance at 280 nm, then pooled and stored frozen at -20°C for periods

of up to three months. In other experiments, proteins from HTC cells were labelled by exposure of cultures 24 h old to fresh medium containing μ curie L-[4,5- 3 H]-leucine/ml for a period of 24 h. The medium was then replaced by fresh unlabelled medium for a period of 1 h. Cells were harvested with a rubber policeman, washed in saline and suspended in 0.02M Tris-HCl, pH 7.4, at a concentration of $5 - 6 \times 10^6$ /ml. After 10 min at room temperature, the cells were homogenized 15 times in a Dounce homogenizer fitted with a loose pestle. The homogenate was immediately brought to 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, by the addition of 1.0M sucrose, 0.02M Tris-HCl, pH 7.4 and centrifuged at $600 \times g$ for 15 min. The pellet was discarded and the $600 \times g$ supernatant was centrifuged for 20 min at $27,000 \times g$. The supernatant was used as radioactive protein substrate, and freshly prepared for each experiment.

Unlabelled $27,000 \times g$ lysosomal pellet from HTC cells was prepared from cells grown in 153 cm^2 glass bottles for a period of 49 h without any change of medium. They were harvested, broken and fractionated by the methods used in the preparation of the [3 H]-labelled $27,000 \times g$ supernatant. The $27,000 \times g$ lysosomal pellet was suspended in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, with a Dounce homogenizer and freshly prepared each day.

2.2.3. Preparation of Subcellular Fractions of LM Cells

LM cells were harvested from the peritoneal cavity of mice by rinsing with saline. They were suspended, at a concentration of 4×10^8 cells/ml, in 0.02M Tris-HCl, pH 7.4. After 10 min at room temperature the cells were homogenized five times in a Dounce homogenizer fitted with

a loose pestle. The homogenate was immediately brought to 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, by the addition of 1.0M sucrose, 0.02M Tris-HCl, pH 7.4, centrifuged at 600 x g for 15 min and the supernatant centrifuged at 27,000 x g for 20 min. The 27,000 x g pellet was used as the lysosomal pellet. The 100,000 x g light microsomal pellet was obtained by centrifuging the 27,000 x g supernatant at 100,000 x g for 30 min. Each pellet was resuspended at a concentration of 5 - 10 mg of protein/ml, in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, with five strokes of a Dounce homogenizer.

In some experiments mitochondria-rich, lysosome-rich or heavy microsome-rich fractions were used instead of the total 27,000 x g pellet. The mitochondria-rich pellet was obtained by centrifuging the 600 x g supernatant at 3,000 x g for 20 min. The lysosome-rich pellet was obtained by centrifuging the 3,000 x g supernatant at 8,000 x g for 20 min. The heavy microsome-rich fraction was prepared by centrifuging the 8,000 x g supernatant at 27,000 x g for 20 min. Each pellet was resuspended in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, at a concentration of 3 - 10 mg/ml.

When [³H]-labelled fractions of LM cells were used they were prepared from cells grown in mice injected with L-[4,5-³H]-leucine. Mice were injected intraperitoneally with 25 μ curies, zero, one, two, three and four days after injection of the cells.

Boiled fractions were prepared by placing samples of 0.2 - 0.4 ml in sealed disposable glass culture tubes (10 x 75 mm) in a boiling water bath for 5 min. The tops of the tubes were then broken off and the samples sonicated for 15 sec.

2.2.4. Preparation and Labelling of DHFR

DHFR was purified from LM cells by affinity chromatography on a column of Sepharose-MTX (Gauldie and Hillcoat, 1972). Cells harvested from 100 mice and stored frozen were suspended in 2 volumes of 0.05M Tris-HCl, pH 7.5, containing 10^{-5} M NADPH, and stirred at 4°C for 1 h. After centrifugation at 20,000 x g for 15 min, the supernatant, with an enzyme activity 1 - 8 units/mg, was treated with ammonium sulphate and the fraction precipitating between 45% and 90% saturation dissolved in a minimum volume of distilled water containing 1×10^{-5} M NADPH. This solution was desalted on a column of Sephadex G-10 (2.5 x 25 cm), the active fractions pooled and then applied to a column of AH-Sepharose-4B (1 x 9 cm), to which MTX had been coupled. The column was washed with 0.1M Tris-HCl, pH 7.5, containing 1×10^{-5} M NADPH and then with 0.2M Tris-glycine, pH 9.5, containing 2M potassium chloride and 10^{-5} M NADPH until the absorbance at 280 nm of the eluate fell to a low level. DHFR was then eluted with 0.2M Tris-glycine, pH 9.5, containing 2M potassium chloride and 10^{-5} M DHF. The active fractions were pooled and passed through a column of Sephadex G-75 (2.5 x 20 cm), previously equilibrated with 0.05M Tris-HCl, pH 7.5.

DHFR was labelled with [14 C] by methylation of the ϵ amino groups of lysine residues (Rice and Means, 1971). Purified enzyme in 0.05M Tris-HCl, pH 7.5, was lyophilized and then dissolved in 0.25M sucrose at a concentration of 50 - 600 units/ml. It was brought to pH 9.0 with 1.0M sodium hydroxide, then 100 μ l of 0.04M [14 C]-labelled formaldehyde were immediately added to 1 ml of the enzyme solution. Sodium borohydride,

20 μ l, 50 mg/ml in distilled water, was added 30 sec, 50 sec, 70 sec and 90 sec after addition of the formaldehyde. After another 60 sec, 100 μ l of sodium borohydride solution were added. All these procedures were carried out at 4°C.

The labelled enzyme solution was dialyzed for 24 h against 4 changes of 1,000 volumes of 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, to reduce the acid-soluble counts to a low level.

During lyophilization, 40% of the enzyme activity was lost, while during the labelling procedure 60% of the remaining activity was lost. Methylation of the enzyme was probably not the cause of the loss of activity during the labelling procedure, since the same amount of activity was lost during dialysis of a control sample.

Labelled enzyme was centrifuged at 27,000 x g to remove any insoluble enzyme and stored at -20°C for periods of up to two months. The labelled enzyme gave a single band of radioactivity after polyacrylamide-SDS gel electrophoresis. On polyacrylamide gels, in the absence of SDS, it gave a single band of radioactivity which corresponded to the position of enzyme activity.

2.2.5. Assay of DHFR

DHFR was assayed at pH 7.5 by a continuous recording spectrophotometric method (Bertino et al., 1965). The activity is measured as the change in absorbance at 340 nm in an assay mixture which contains 1×10^{-4} M NADPH, 1×10^{-4} M DHF, 1×10^{-3} M 2-mercaptoethanol, 0.15M potassium chloride, 0.1M Tris-HCl, pH 7.5. When MTX was present, assays were performed at pH 8.5. At this pH the inhibition of the enzyme by

MTX is partially reduced (Hillcoat et al., 1971).

A unit of enzyme activity was taken as that which reduced 1×10^{-2} μ moles of DHF per min.

The protein concentration of DHFR was calculated from a turnover number of 2090 moles dihydrofolate reduced per min per mole of enzyme at pH 7.5 and 37°C (Gauldie and Hillcoat, 1972). The molecular weight of the enzyme was determined as 18,000 from polyacrylamide-SDS gel electrophoresis. Little activity was lost during storage, freezing or thawing of the enzyme, so inaccuracies in the calculation of the protein concentration due to formation of inactive soluble enzyme were not significant.

2.2.6. Inactivation of DHFR

DHFR was incubated with fractions of LM cells in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. All incubations were at 37°C. Aliquots were removed at intervals and the enzyme activity measured. The activity at zero time was measured as soon as enzyme was added to the cellular fraction, immediately before the sample was put into a waterbath at 37°C.

Purified, frozen enzyme was used in these assays. It was shown that freezing and thawing the enzyme up to 4 times did not alter the rate of inactivation of the enzyme by the 27,000 x g pellet. The enzyme was routinely centrifuged at 27,000 x g before use in order to remove any insoluble denaturated enzyme.

Equivalent amounts of DHFR, as determined by enzyme activity, were added to each incubation. When cell fractions containing 100,000 x g supernatant were used, the endogenous activity was measured and the amount of purified enzyme reduced so that the final activity was the same

in all incubations.

When an adenosine-5'-triphosphate (ATP) regenerating system was present, the incubation mixture contained 7 mM potassium sulphate, 7 mM magnesium sulphate, 1.4 mM ATP, 20 mM phosphoenolpyruvate and 1.2 units of pyruvate kinase at pH 7.4.

2.2.7. Production of Acid-Soluble Material

Acid-soluble material was measured by adding 10% trichloroacetic acid, (TCA), to a final concentration of 5%. Bovine serum albumin (BSA), 1 mg/ml, was added as carrier protein. The samples were left overnight at 4°C, centrifuged at 600 x g for 10 min and aliquots of the supernatant counted in Bray's scintillation fluid (Bray, 1960).

2.2.8. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Ornstein (1964) and Davis (1964) as described by Hiebert et al., (1972). Aliquots of 200 µl in 7.5% sucrose were applied to gels of 7% acrylamide, pH 8.9. Bromphenol blue, 0.001% was applied as a tracking dye to separate gels since it has been shown to interact with DHFR during electrophoresis (Hiebert et al., 1972). The gels, 8 cm long, were run at 1 mA/gel for 30 min and then at 3 mA/gel for 6 - 7 h in the BioRad model 300 gel electrophoresis cell.

When urea was incorporated into the gels it was at a final concentration of 8 M. Samples were incubated in 8 M urea, 1.2 mM 2-mercaptoethanol, for 60 min at room temperature before being applied to the gels.



DHFR activity was detected at the end of electrophoresis by incubating gels at 37°C, in the dark, in an assay mixture which contained 1×10^{-4} M NADPH, 1×10^{-4} M DHF, 0.15M potassium chloride, 0.4 mg/ml MTT, 0.1M Tris-HCl, pH 7.5. The enzyme was detected as a blue band formed by reduction of MTT, a tetrazolium dye, by the tetrahydrofolate produced during the enzyme reaction (Mell et al., 1968).

Polyacrylamide-SDS gel electrophoresis was carried out by the method of Fairbanks et al., (1971). This method uses 5.6% acrylamide gels, pH 7.4, containing 1% SDS. Samples were incubated at 37°C for 30 min in 1% SDS, 7.5% sucrose, 0.01M Tris-HCl, pH 8.0, 1 mM ethylene diamine tetra-acetate (EDTA), 40 mM dithiothreitol (DTT), and 10 µg pyronin Y/ml. Aliquots of 200 µl were applied to the gels which were run at a constant current of 8 mA/gel until the pyronin Y was 1 - 2 cm from the bottom of the gel. Ovalbumin, β-galactosidase, BSA, pepsin, cytochrome c and myoglobin were used to calibrate the gels for molecular weight.

Protein was detected on the 7% polyacrylamide gels and on the 5.6% polyacrylamide-SDS gels with Coomassie blue (Fairbanks et al., 1971). Gels were scanned at 550 nm in a Gilford model 2410 gel scanner.

Gels containing labelled proteins were fractionated into 2 mm slices using a Gilson Aliquogel Fractionator. Slices were treated at 50°C for 2 h with approximately 0.5 ml of NCS Tissue Solubilizer and the radioactivity in each slice was counted in 10 ml of Bray's liquid scintillation fluid.

2.2.9. Detection of High Molecular Weight [¹⁴C]-labelled
DHFR by Column Chromatography

DHFR was incubated with fractions of LM cells at 37°C in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, for 2 h. A sample of 0.25 ml of the incubation mixture, or of the 27,000 x g supernatant after centrifugation for 20 min, was applied to a column of Sepharose 2B (1 x 18 cm), previously equilibrated with 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The column was eluted with the same buffer at 4°C. Fractions of 0.65 ml were collected and the radioactivity in 0.45 ml counted in Bray's liquid scintillation fluid.

2.2.10. Purification of Cathepsin D

Cathepsin D was partially purified from both rabbit liver and LM cells by the method of Barrett (1967). The method involves preparation of an acetone dried powder from the lysosomal-mitochondrial pellet, extraction with an acidic buffer and ion exchange chromatography on Sephadex A-50. Although the enzyme prepared from rabbit liver eluted from the ion-exchange column in 0.05M sodium chloride, as described by Barrett for the rabbit liver enzyme, 0.5M sodium chloride was needed to elute the enzyme of LM cells.

2.2.11. Assay of Marker Enzymes

Partially purified cathepsin D, prepared as described in Section 2.2.10, was used to develop an assay for cathepsin D. Haemoglobin was methylated using [¹⁴C]-labelled formaldehyde and sodium borohydride (Rice and Means, 1971) and used as a substrate for cathepsin D.

Incubations contained 80-100 μg of labelled haemoglobin and 0.25 μg of partially purified cathepsin D in 0.2 ml, 0.1 M sodium acetate buffer, pH 4.0. Reactions were stopped by the addition of 10% TCA to a final concentration of 5% and unlabelled haemoglobin, 1 mg/ml was added as a carrier. Samples were centrifuged at 600 x g for 15 min and aliquots of the supernatant counted in 10 ml of Bray's liquid scintillation fluid. The production of acid-soluble material was shown to be linear with time and with the amount of cathepsin D in the incubation. The stability of the lysosomes in the 27,000 x g pellet was determined by the amount of the total cathepsin D which was in the 27,000 x g supernatant after the 27,000 x g pellet was removed by centrifugation. The total cathepsin D was measured by dissolving a sample in Triton X-100 at a final concentration of 0.1%. Since Triton X-100 was found to inhibit the production of acid-soluble material it was also added to the samples of 27,000 x g supernatant. In this way the activity in the 27,000 x g supernatant could be expressed as a percentage of the total activity.

Acid phosphatase was measured by the release of inorganic phosphate from β -glycerophosphate (Barrett, 1972).

β -glucuronidase was measured by the release of phenolphthalein from phenolphthalein glucuronide (Gianetto and deDuve, 1955).

Cytochrome c oxidase was measured by the method of Cooperstein and Lazarow (1951). The concentration of cytochrome c used in this assay was increased to 3.1×10^{-5} M. It was reduced with sodium dithionite which was added as a powder, to a final concentration of 0.1%.

NADPH-cytochrome c reductase was measured by the method of Williams and Kamin (1962).

2.2.12. Protein Determination

Protein concentrations were measured by the method of Lowry et al., (1951), using BSA as a standard.

3. RESULTS

3.1. DEGRADATION OF HTC CELL PROTEINS

In intact cells proteins are degraded to their constituent amino acids. Radioactively labelled proteins of HTC cells were therefore used in *in vitro* incubations and attempts made to characterize the protein degrading system by measuring the release of radioactive amino acids and changes in the molecular weights of the labelled proteins.

3.1.1. Production of acid-soluble material

Lysosomes are the most likely site of degradation of intracellular proteins so the degradation of HTC cell proteins by these organelles was investigated first. It is difficult to obtain large amounts of lysosomes from cultured HTC cells so in these preliminary studies lysosomes were obtained from LM cells. Passage of LM cells in the peritoneal cavity of mice allows cells to be harvested in much greater amounts than is practical with HTC cells grown in tissue culture.

The fraction of LM cells which sediments between 600 x g and 27,000 x g was used as the lysosomal fraction. This pellet contains lysosomes, mitochondria and heavy microsomes. The pellet was suspended in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, an isotonic buffer (osmolality 255 mOsmoles/kg), intended to maintain the lysosomes intact. Incubations were in the same buffer. Using cathepsin D and β -glucuronidase as lysosomal marker enzymes, it was shown that only 6-10% of the lysosomal

enzymes were soluble at the beginning of the incubations. After 2 h at 37°C, 16-21% were soluble.

The radioactive substrate used in these experiments was the 20,000 x g supernatant from HTC cells which were grown for 1 h in [³H]-leucine and broken by sonication.

When the [³H]-labelled proteins of HTC cells were incubated with lysosomes of LM cells at pH 7.4, in the isotonic sucrose buffer, there was little increased production of acid-soluble material from the proteins of HTC cells as compared to a control which contained an equivalent amount of BSA instead of the LM lysosomes (Table 1). Nor was the rate of production of acid-soluble material increased when the lysosomal pellet was sonicated.

Incubations were also performed at pH 7.2 in phosphate buffered saline (PBS), an isotonic buffer (osmolality, 291 mOsmoles/kg) of greater ionic strength than the sucrose-Tris buffer. Again there was little production of acid-soluble material whether the lysosomes were sonicated or intact (Table 1).

Since lysosomal proteases have pH optima in the acidic range, these incubations were repeated in an isotonic (osmolality, 284 mOsmoles/kg) phosphate-citrate buffer, pH 5.0 (Gomori, 1955). Under these conditions lysosomes were much less stable. At the beginning of the incubation, 37% of the cathepsin D activity was soluble. This rose to 42% after 3 h at 37°C. When [³H]-labelled proteins from HTC cells were incubated with the 27,000 x g lysosomal fraction of LM cells in this buffer, significant production of acid-soluble material occurred (Table 2). Degradation was increased by sonication of the 27,000 x g pellet.

TABLE 1
 PRODUCTION OF ACID-SOLUBLE MATERIAL FROM [³H]-LABELLED PROTEINS
 OF HTC CELLS DURING INCUBATION WITH THE 27,000 x g
 LYSOSOMAL PELLET FROM LM CELLS AT pH 7.4

Incubation	% Acid-soluble at zero time	% Acid-soluble after 3 h	Change
27,000 x g pellet in sucrose-tris buffer	3.6	4.6	1.0%
BSA in sucrose- tris buffer	3.4	4.2	0.8%
Sonicated 27,000 x g pellet in sucrose- tris buffer	2.9	4.4	1.5%
27,000 x g pellet in PBS	3.4	6.1	2.7%
BSA in PBS	2.7	4.8	2.1%
Sonicated 27,000 x g pellet in PBS	3.3	5.7	2.4%

Incubations contained 0.98 mg of BSA or protein from the 27,000 x g pellet and 0.06 mg [³H]-labelled HTC cell proteins in a final volume of 1.2 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 or 1.2 ml, PBS, pH 7.2. The total dpm in incubations was 40,406. cpm measured ranged from 51 to 82. Results given represent the mean of duplicate incubations. Similar results were obtained in seven different experiments using sucrose-tris buffer and two experiments using PBS.

TABLE 2

PRODUCTION OF ACID-SOLUBLE MATERIAL FROM RADIOACTIVELY LABELLED
 PROTEINS OF HTC CELLS DURING INCUBATION WITH THE 27,000 x g
 LYSOSOMAL PELLET FROM LM CELLS AT pH 5.0

Incubation	Total dpm in incubation	% Acid-soluble at zero time	% Acid-soluble after 3 h	Change
27,000 x g pellet and [³ H] proteins	40,406	4.14	27.35	23.21%
BSA and [³ H] proteins	40,406	4.22	10.14	5.92%
Sonicated 27,000 x g pellet and [³ H] proteins	40,406	2.18	34.59	32.41%
27,000 x g pellet and [¹⁴ C] proteins	50,295	2.54	15.17	12.63%
BSA and [¹⁴ C] proteins	50,295	2.09	6.90	4.81%
Sonicated 27,000 x g pellet and [¹⁴ C] proteins	50,295	2.21	22.04	19.83%

Incubations contained 0.96 mg BSA or protein from the 27,000 x g pellet of LM cells and 0.06 mg of [³H]-labelled or 0.085 mg of [¹⁴C]-labelled proteins of HTC cells in a final volume of 0.92 ml phosphate-citrate buffer, pH 5.0. Proteins labelled with [³H] were from cells exposed to [³H]-leucine for 1 h. Proteins labelled with [¹⁴C] were from cells exposed to [¹⁴C]-leucine for 48 h *in vivo*. Results given are the mean of duplicate incubations. Similar results were obtained in four different experiments. cpm measured ranged from 30 to 1123.

The proteins of HTC cells which are labelled during exposure of intact cells to [^3H]-leucine for 1 h are mainly proteins with short half-lives (Arias et al., 1969). Proteins which turn over more slowly in intact cells can be labelled by exposure of the cells to radioactive amino acid for a longer period of time. When the rate of degradation of proteins of HTC cells labelled with [^3H]-leucine for 1 h was compared to the rate of degradation of proteins which had been labelled with [^{14}C]-leucine for 48 h, under the above conditions of acid pH, it was found that the [^3H]-labelled proteins were degraded to a greater extent than the [^{14}C]-labelled proteins (Table 2).

These results indicate that the 27,000 x g lysosomal fraction of LM cells contains proteases which are able to degrade proteins of HTC cells. Moreover, there is a correlation between the rate of degradation by these proteases *in vitro* and the half lives of the proteins in the intact cell. The 27,000 x g lysosomal pellet does not degrade the proteins at neutral pH. One possible reason for this lack of degradation at neutral pH is that the substrate proteins and the 27,000 x g pellet are from different cell lines. The incubations at neutral pH were, therefore, repeated using the 27,000 x g pellet from HTC cells as the source of lysosomes. The substrate protein in these experiments was the 27,000 x g supernatant obtained from the same cells which had been exposed to [^3H]-leucine for 24 h and broken with a Dounce homogenizer. Again little acid-soluble material was produced during incubation of the [^3H]-labelled HTC proteins with the lysosomal fraction at neutral pH (Table 3).

To see whether some other component is necessary for degradation

TABLE 3

PRODUCTION OF ACID-SOLUBLE MATERIAL FROM [³H]-LABELLED PROTEIN
FROM HTC CELLS BY THE 27,000 x g PELLETT OF HTC CELLS

	% Acid-soluble at zero time	% Acid-soluble after 3 h	Change
27,000 x g pellet	0.79	1.55	0.76
Boiled 27,000 x g pellet	0.79	0.93	0.14

Incubations contained 0.214 mg of protein from the [³H]-labelled 27,000 x g supernatant and 0.06 mg of protein from the 27,000 x g pellet of unlabelled HTC cells, in a final volume of 0.30 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4.

The total dpm in incubations was 173,025. Results given are the mean of triplicate incubations. Similar results were obtained in five different experiments. cpm measured ranged from 126 to 300.

to occur at neutral pH, the incubations were carried out with all the cellular components present. The cells were simply broken by homogenization and the total homogenate incubated in the isotonic sucrose-Tris buffer. The rate of production of acid-soluble material was very low, less than 0.3% an hour (Table 4). Only 0.05% per hour of the proteins were degraded by a heat sensitive process (Table 5). Some of this degrading activity was lost when intact cells and nuclei were removed by centrifugation at 600 x g and the majority was lost when the 27,000 x g pellet was removed by centrifugation (Table 4). The addition of EDTA which stabilizes lysosomes *in vitro* (Davidson, 1975) did not increase the degradation of [³H]-labelled proteins of HTC cells (Table 5).

3.1.2. Changes in the molecular weights of proteins from HTC cells during *in vitro* incubations

Since little acid-soluble material was produced in broken cell preparations, polyacrylamide-SDS gel electrophoresis was used to see whether less extensive degradation occurred. The substrate used in these experiments was the [³H]-labelled 27,000 x g supernatant of HTC cells, prepared from cells grown for 24 h in the presence of [³H]-leucine. There was no change in the positions of the protein peaks on the gels after incubation of the proteins with the 27,000 x g lysosomal pellet as compared with a control which contained boiled 27,000 x g pellet (Fig. 1).

To investigate whether soluble proteins already present in the [³H]-labelled 27,000 x g supernatant rather than proteins in the 27,000 x g pellet cleave proteins of the soluble fraction during *in vitro* incubations, the molecular weight distribution of the proteins of

TABLE 4
 PRODUCTION OF ACID-SOLUBLE MATERIAL FROM SUBCELLULAR FRACTIONS
 OF [³H]-LABELLED HTC CELLS

	Total dpm in incubation	Total dpm/mg protein	% Acid soluble at 0 time	% Acid- soluble after 3 h	Change
Total homogenate	533,812	635,490	0.67	1.44	0.77
600 x g supernatant	274,136	637,525	0.75	1.31	0.55
27,000 x g supernatant	225,936	610,638	0.85	1.04	0.19

Incubations contained 0.82 mg of protein from the total homogenate, 0.36 mg of protein from the 600 x g supernatant or 0.32 mg of protein from the 27,000 x g supernatant of HTC cells in a final volume of 0.20 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. Results given are the mean of triplicate incubations. Similar results were obtained in three different experiments. cpm measured ranged from 183 to 747.

TABLE 5

PRODUCTION OF ACID-SOLUBLE MATERIAL FROM THE [³H]-LABELLED PROTEINS
OF HTC CELLS IN THE PRESENCE OF EDTA

	Total dpm in incubation	% Acid-soluble at zero time	% Acid-soluble after 3 h	Change
Total homogenate	391,825	0.50	1.25	0.75%
Total homogenate boiled	391,825	0.50	1.10	0.60%
Total homogenate + EDTA	442,900	0.53	1.20	0.67%
Total homogenate boiled + EDTA	442,900	0.51	1.13	0.52%

Incubations contained 0.53 mg of protein from the total homogenate in a final volume of 0.20 ml, 0.25 M sucrose, 0.02 M Tris-HCl pH 7.4. Where present EDTA was at a final concentration of 10^{-4} M. Results given are the mean of duplicate incubations. cpm measured ranged from 198 to 516.

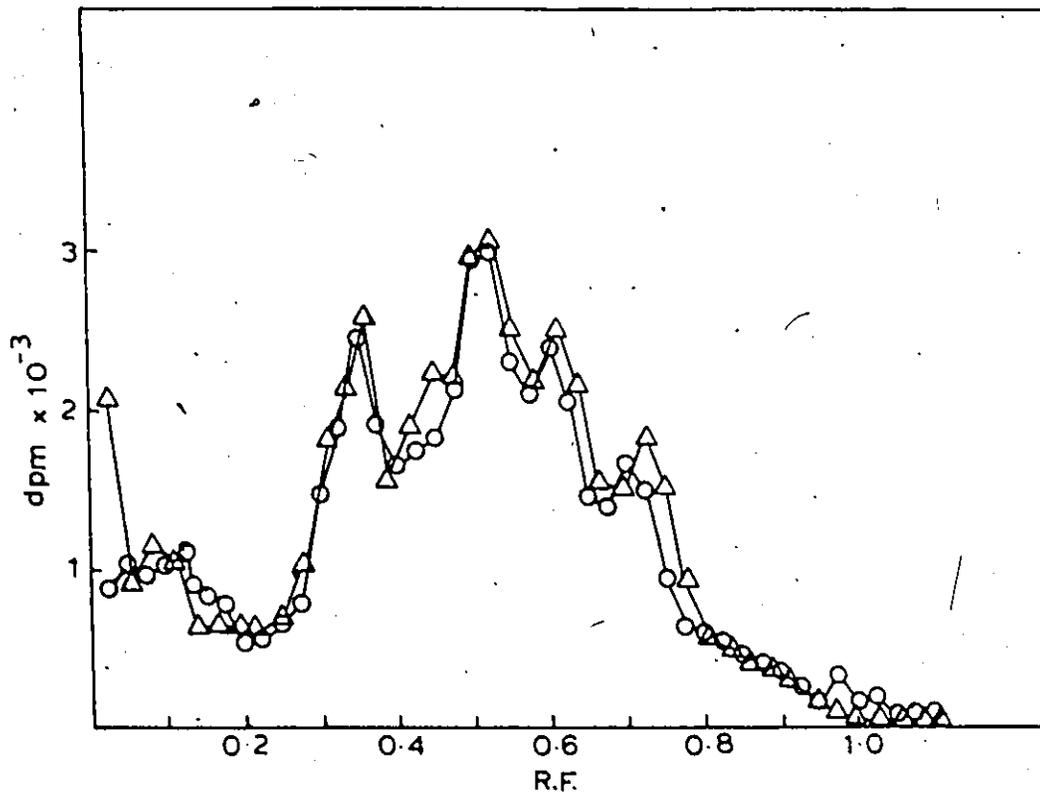


Fig. 1. Polyacrylamide-SDS gel electrophoresis of [³H]-labelled proteins which remain in the 27,000 x g supernatant after incubation *in vitro*

Incubations were at 37°C and for 3 h. They contained 570 µg of [³H]-labelled protein, prepared as described in the text, and 100 µg of unlabelled protein from the 27,000 x g pellet of HTC cells in a final volume of 0.35 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The volume of sample applied to each gel was that which contained 84 µg of protein from the 27,000 x g supernatant at the beginning of the incubation.

[³H]-labelled 27,000 x g supernatant incubated with 27,000 x g pellet, ○—○; [³H]-labelled 27,000 x g supernatant incubated with boiled 27,000 x g pellet, △—△.

Similar results were obtained in seven different experiments.

the 27,000 x g supernatant before and after incubation were compared. Again there was no change in the positions of the radioactive peaks. Although there was a general decrease in each of the peaks, this was not accompanied by a corresponding increase in proteins of lower molecular weight (Fig. 2). A general decrease in the amount of radioactive protein in the peaks was also seen after incubation of intact cells for a further 3 h. This also occurred without an increase in proteins of lower molecular weight.

The production of acid-soluble material during incubation *in vitro* is much too small to account for this loss of soluble proteins. The most probable explanation is that these proteins were removed from solution during the incubations. Labelled proteins were indeed found in the 27,000 x g pellet at the end of the incubation. The molecular weight distribution of these proteins on polyacrylamide-SDS gels is shown in Fig. 3. There was no detectable difference in the molecular weight distribution of the proteins which sedimented with the 27,000 x g pellet as compared to those which remained soluble.

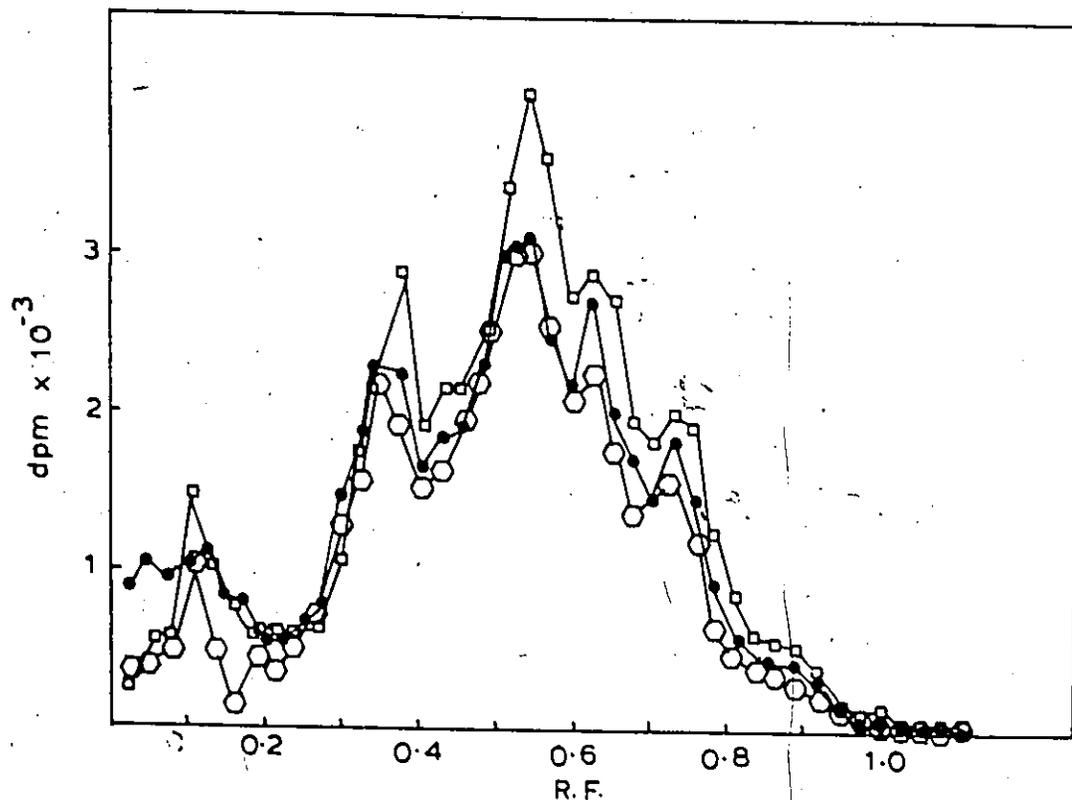


Fig. 2. Polyacrylamide-SDS gel electrophoresis of the [³H]-labelled proteins from the 27,000 x g supernatant of HTC cells before incubation, after incubation *in vitro* and after incubation of intact cells

Aliquots of 27,000 x g supernatant which contained 84 μ g of protein before incubation or after a further 3 h incubation of intact cells were applied to gels. Incubations *in vitro* were for 3 h at 37°C, they contained 570 μ g protein from the 27,000 x g supernatant. They were terminated by centrifuging at 27,000 x g for 20 min and a volume equivalent to that which originally contained 84 μ g of protein was applied to the gel.

Before incubation, \square — \square ; after incubation of intact cells, \circ — \circ ; after incubation *in vitro*, \bullet — \bullet .

Similar results were obtained in six different experiments.

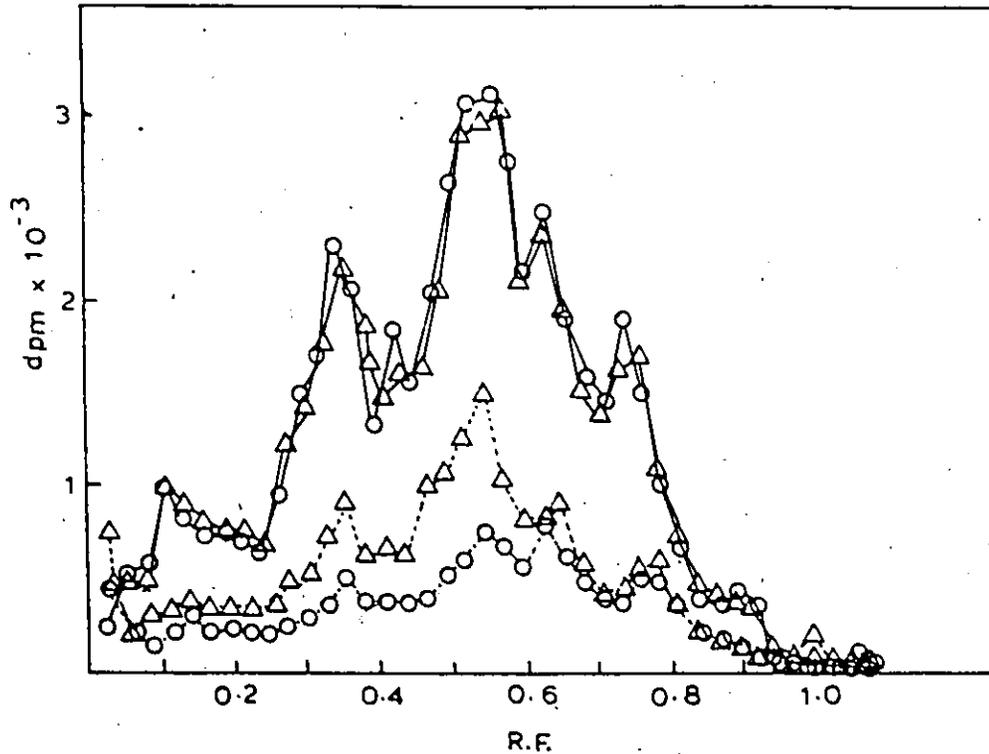


Fig. 3. Polyacrylamide-SDS gel electrophoresis of the [^3H]-labelled proteins from the 27,000 x g supernatant of HTC cells after incubation *in vitro*

Incubations were at 37°C and contained 800 μg of [^3H]-labelled protein from the 27,000 x g supernatant and 272 μg of unlabelled protein from the 27,000 x g pellet of HTC cells, in a final volume of 1.0 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 3 h the 27,000 x g pellet was removed by centrifugation and the [^3H]-labelled proteins in both the supernatant and pellet applied to polyacrylamide-SDS gels. The volume of 27,000 x g supernatant put onto the gels was that which initially contained 70 μg of [^3H]-labelled protein. The amount of 27,000 x g pellet put onto the gel was that arising from a volume of incubation which had initially contained 320 μg of [^3H]-labelled 27,000 x g supernatant protein. The solid lines indicate proteins remaining in the 27,000 x g supernatant, the dotted lines indicate proteins in the 27,000 x g pellet.

[^3H]-labelled 27,000 x g supernatant incubated with 27,000 x g pellet, $\circ\text{---}\circ$; [^3H]-labelled 27,000 x g supernatant with boiled 27,000 x g pellet, $\Delta\text{---}\Delta$.

Similar results were obtained in two different experiments.

3.2 DEGRADATION OF DHFR

Since it was not possible to show any production of acid-soluble material nor change in molecular weight of the proteins of HTC cells *in vitro* at neutral pH, an individual enzyme was used for similar studies so that the fate of a single protein could be followed. DHFR purified from LM cells was incubated with various fractions of these cells. The investigation of the degrading system for this enzyme was begun by attempting to find which cell fraction inactivates the enzyme *in vitro*.

3.2.1. Inactivation of DHFR *in vitro*

a) Initial studies

Purified DHFR is very unstable, rapidly losing activity at 37°C. When the enzyme was incubated alone at 37°C in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, activity was lost by a first order process (Fig. 4). Although the effect of temperature on this process has not been investigated, loss of activity by this mechanism is subsequently referred to as heat denaturation.

The enzyme was protected against heat denaturation by the addition of boiled protein from the 27,000 x g pellet of LM cells (Fig. 5). The greater the amount of protein, the greater the degree of protection. The nature of the protein was also important in determining the degree of protection, for example, BSA was less efficient in stabilizing the enzyme against heat denaturation than boiled protein from the 27,000 x g pellet of LM cells (Fig. 6). When boiled BSA was

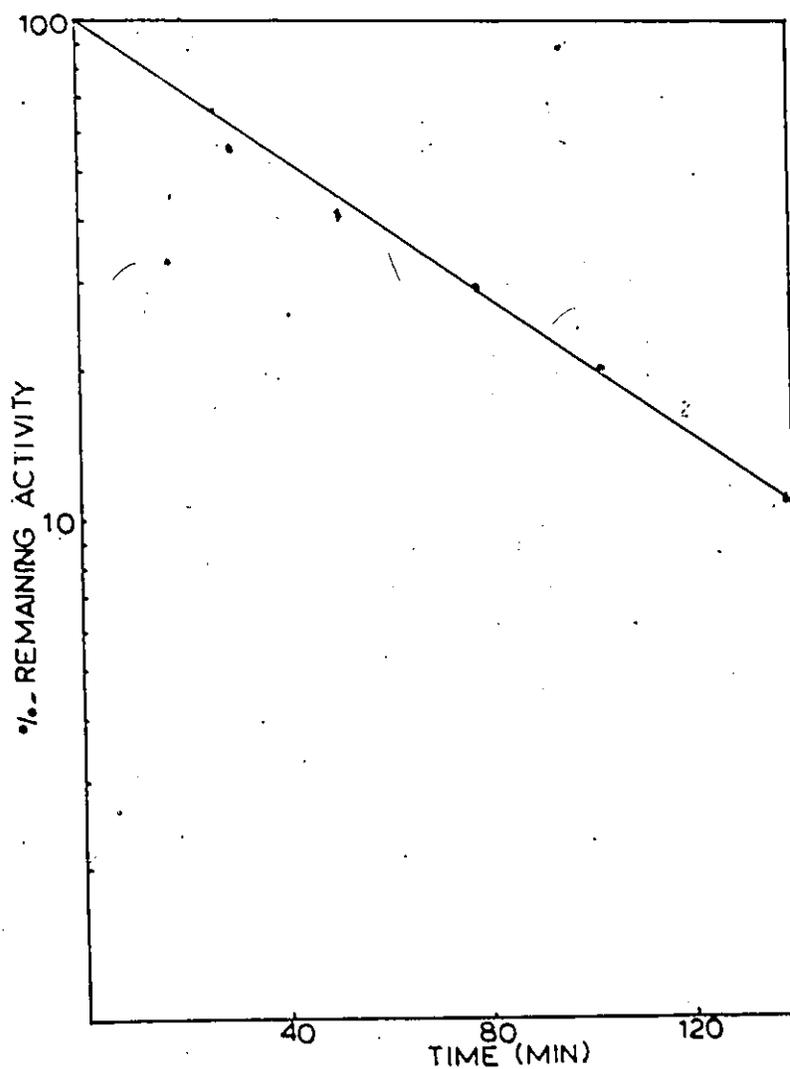


Fig. 4. Loss of activity of DHFR *in vitro*

Incubations contained 10.00 units of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris, HCl, pH 7.4.

Although the rate of loss of activity differed with different enzyme preparations, similar results were obtained in eight experiments.

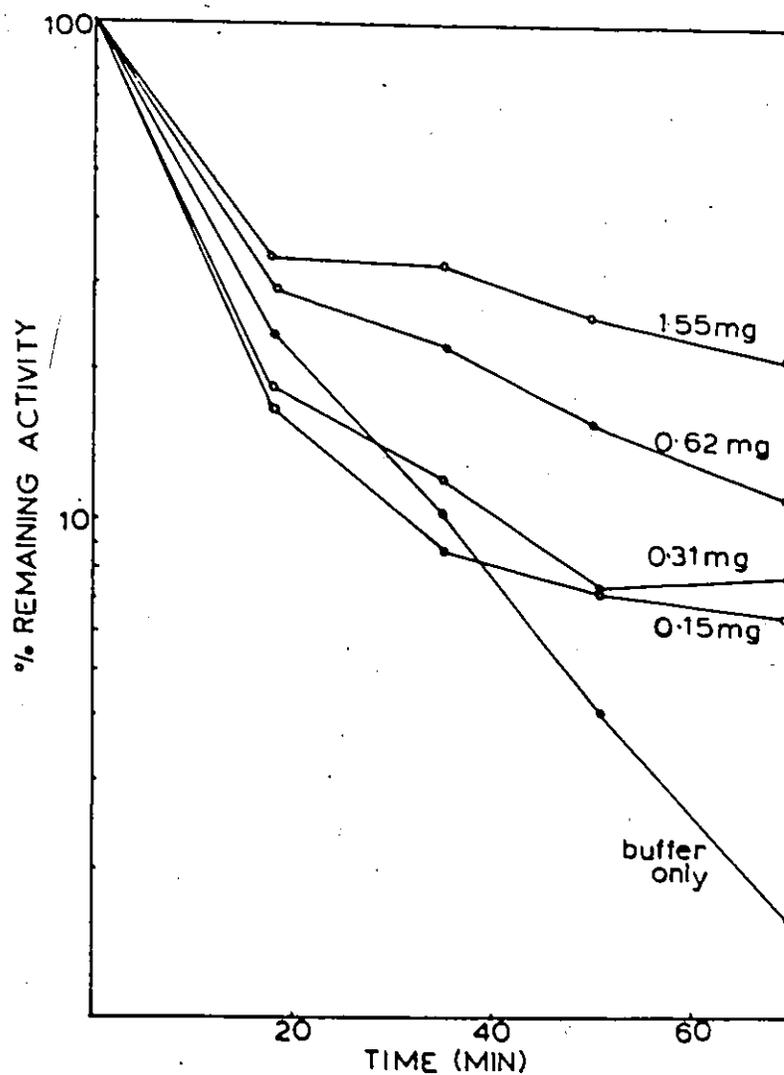


Fig. 5. Loss of activity of DHFR in the presence of varying amounts of boiled protein from LM cells

Incubations contained 3.5 units of DHFR and varying amounts of boiled protein from the 27,000 x g pellet of LM cells in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Similar results were obtained in four different experiments.

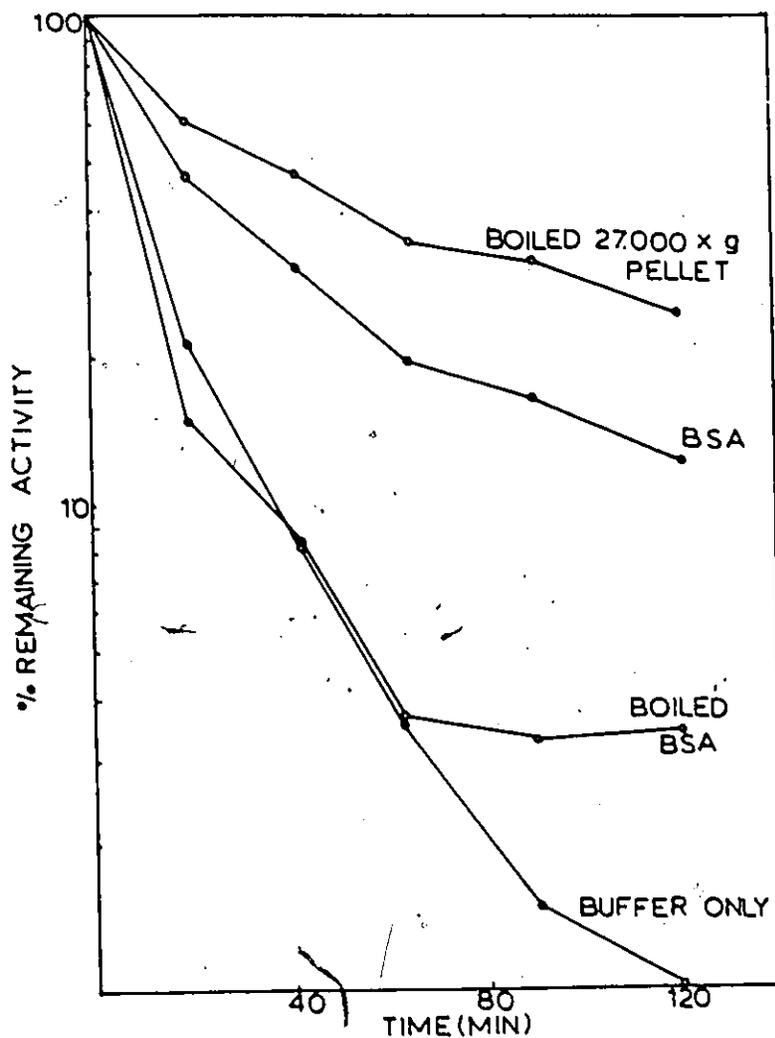


Fig. 6. Loss of activity of DHFR in the presence of BSA and boiled protein from the 27,000 x g pellet of LM cells

Incubations contained 0.70 mg of BSA or boiled protein from the 27,000 x g pellet and 4.7 units of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4

Similar results were obtained in four different experiments.

present in the incubation, little protection was provided (Fig. 6), possibly because boiled BSA is less soluble than the non-boiled protein (Table 6).

These results illustrate the problems involved in the choice of suitable controls for studies of the inactivation of DHFR by fractions of LM cells. The degree of protection against heat denaturation appeared to depend on the type of protein, the amount of the protein and the physical state of the protein. It was decided that boiled cellular fractions would be used as controls in these experiments. All boiled fractions were sonicated in order to disperse the proteins. In an attempt to keep the amount of inactivation by simple heat denaturation constant equal concentrations of the various fractions were used. These arrangements were not entirely satisfactory since the amount of soluble material in the different fractions was not the same (Table 6) nor was the amount of soluble protein in the boiled fractions the same as in the unboiled fractions (Table 6).

b) Identification of the fraction which inactivates DHFR *in vitro*

When purified DHFR was incubated with the 600 x g supernatant of LM cells activity was lost in the control which contained boiled protein (Fig. 7). In the incubation containing unboiled 600 x g supernatant, however, the loss of activity was greater. Activity was also lost by a heat sensitive process in incubations which contained

TABLE 6

PROTEIN REMAINING IN THE 100,000 x g SUPERNATANT
AFTER 2 h AT 37°C

Sample	Soluble protein after 2 h mg/ml	% Soluble
BSA	1.74	96.92
Boiled BSA	0.09	5.02
27,000 x g pellet	0.46	26.25
Boiled 27,000 x g pellet	0.27	15.36
100,000 x g pellet	0.71	39.94
Boiled 100,000 x g pellet	0.20	11.17

Incubations contained 0.76 mg of protein in a final
volume of 0.39, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4.

Results given are the mean of duplicate samples.

Similar results were obtained in two experiments.

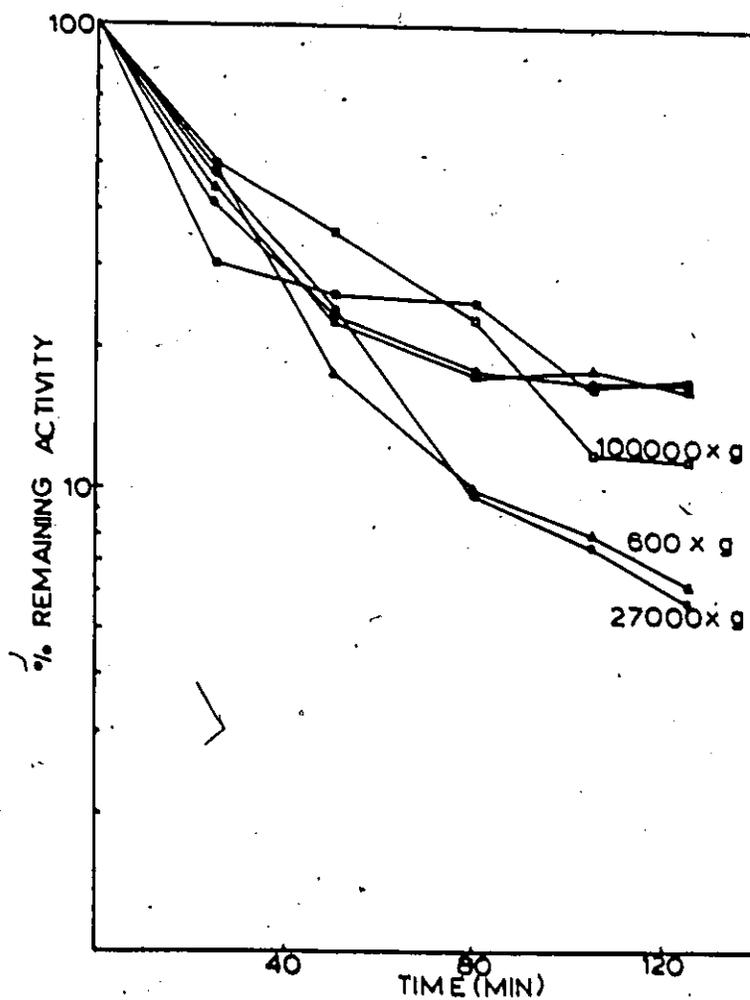


Fig. 7. Inactivation of DHFR in the presence of various fractions of LM cells

Incubations contained 0.47 mg of protein from the fractions of LM cells and 5.6 units of DHFR activity in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The incubation containing the 600 x g supernatant had 4.9 units of purified enzyme, the incubation containing the 27,000 x g supernatant had 4.6 units of purified enzyme and the incubation containing the 100,000 x g supernatant had 4.4 units of purified enzyme. Incubations containing each boiled fraction had 5.5 units of purified DHFR.

The open symbols show the unboiled fractions, the closed symbols show the corresponding boiled fractions.

Similar results were obtained in four different experiments.

27,000 x g supernatant. Little activity was lost by a heat sensitive process in incubations which contained 100,000 x g supernatant. These results indicate that the 100,000 x g pellet of LM cells probably inactivates DHFR *in vitro*. If this were the only fraction which inactivated the enzyme, the inactivation per mg of cellular protein would be expected to increase when the 27,000 x g pellet was removed. Since such an increase was not seen, components of the 27,000 x g pellet may also inactivate the enzyme. This was tested by resuspending the pellets and incubating them with purified DHFR. In the presence of the 27,000 x g pellet activity was rapidly lost by a heat sensitive process (Fig. 8). Activity was also rapidly lost in the presence of the 100,000 x g pellet. This process appeared to be less heat sensitive (Fig. 8).

One explanation for the apparent heat sensitive inactivation of DHFR is that it was simply the result of greater protection of the enzyme against heat denaturation by the boiled than by the unboiled fractions. Evidence against such an explanation is given in Fig. 9. The rate of inactivation of DHFR increased with increasing amounts of unboiled 27,000 x g pellet. This is in contrast to the decreasing rate of inactivation seen with increasing amounts of boiled 27,000 x g pellet (Fig 5.), and indicated that this fraction inactivated DHFR *in vitro*.

The 100,000 x g pellet of LM cells contained light microsomes (Table 7). The 27,000 x g pellet of LM cells contained mitochondria, lysosomes and heavy microsomes. Fractions rich in each of these organelles can be prepared (Table 7). To see which subcellular organelle was responsible for the inactivation of DHFR by the 27,000 x g pellet,

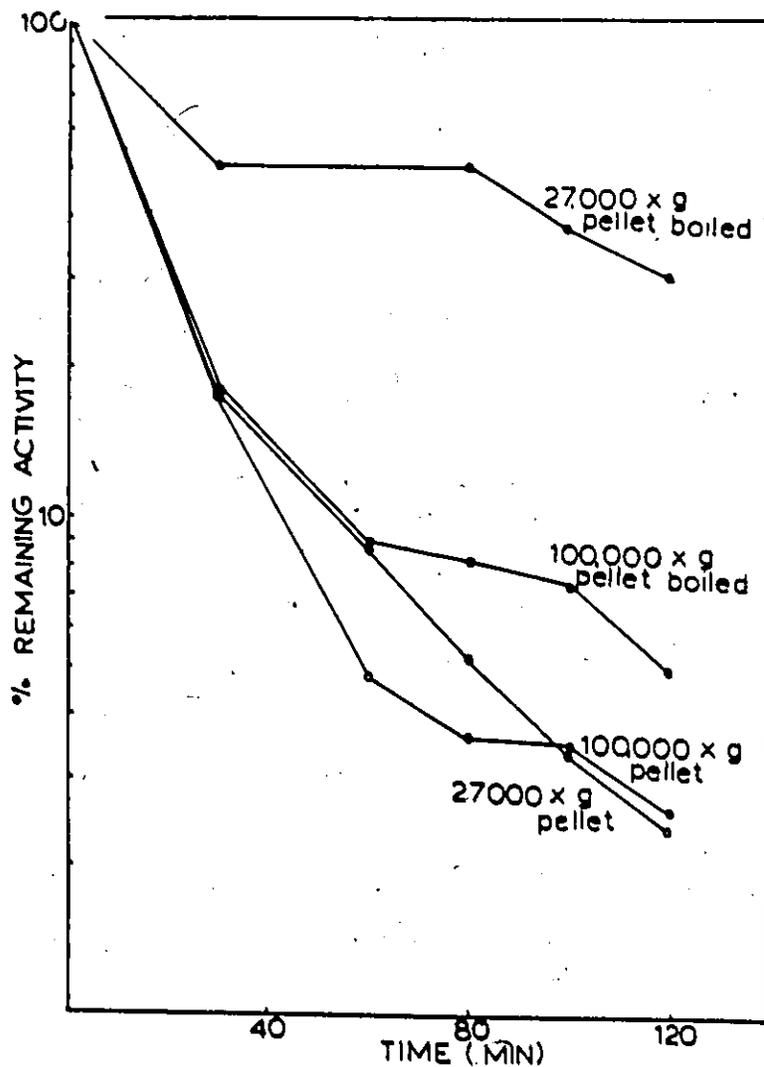


Fig. 8. Inactivation of DHFR by subcellular membrane fractions of LM cells

Incubations contained 0.46 mg of protein from the 27,000 x g or 100,000 x g pellet of LM cells and 6.6 units of DHFR in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4

Similar results were obtained in four different experiments.

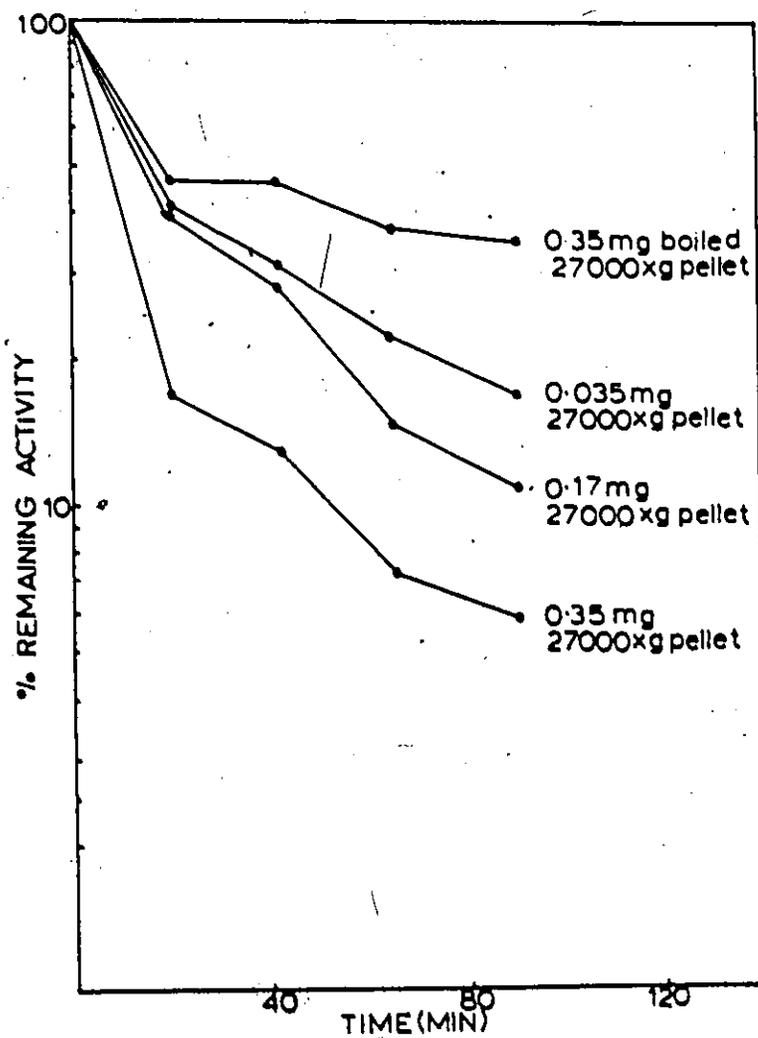


Fig. 9. Inactivation of DHFR by increasing amounts of the 27,000 x g pellet of LM cells

Incubations contained 7.3 units of DHFR and varying amounts of protein of the 27,000 x g pellet in a final volume of 0.39 ml, 0.25M sucrose, 0.02N Tris-HCl, pH 7.4.

Similar results were obtained in six different experiments.

TABLE 7

DISTRIBUTION OF MARKER-ENZYMES IN LM CELLS

Cell Fraction	NADPH cyt c reductase $\Delta OD/\text{min}/\text{mg}$	Cyt c oxidase $\Delta OD/\text{min}/\text{mg}$	Acid phosphatase $\Delta OD/\text{min}/\text{mg}$	Cathepsin D dpm/min/mg
Mitochondria 600-3,000 x g	0	33.7×10^{-2}	1.53×10^{-3}	9,351
Lysosomes 3,000-8,000 x g	0	18.1×10^{-2}	1.44×10^{-3}	17,605
Heavy microsomes 8,000-27,000 x g	28.3×10^{-4}	0	0.74×10^{-3}	7,542
Light microsomes 27,000-100,000 x g	16.7×10^{-4}	0	0.3×10^{-3}	94

Results given represent the mean of duplicate assays. Similar results were obtained in two different experiments.

the rate of inactivation by equivalent amounts of each of these fractions was compared. The loss of activity of DHFR in the boiled mitochondria control was the same as that in the boiled lysosome control (Fig. 10), more activity was lost in the incubation which contained boiled heavy microsome fraction. When the enzyme was incubated with unboiled mitochondria-rich, lysosome-rich or microsome-rich fraction, the rate of inactivation was much greater than in the boiled controls and was comparable in each case (Fig. 10).

Under the conditions used in these incubations, 21% of the lysosomal marker enzymes were soluble after 2 h (3.1.1.). Of the total protein in the 27,000 x g pellet, 26% was soluble after 2 h (Table 6). Inactivation of DHFR by the subcellular membrane fractions of LM cells might, therefore, have been produced by proteins, such as lysosomal proteases, which leaked from the organelles or which were solubilized from the membranes during incubation. Such a possibility was examined in several ways. First, the rate of inactivation of DHFR by a lysosomal protease, partially purified cathepsin D, was measured. At pH 7.4, the pH at which DHFR was inactivated by the 27,000 x g pellet, no increased inactivation could be demonstrated by cathepsin D compared to a control which contained boiled cathepsin D (Fig. 11). Second, the 27,000 x g pellet was sonicated. Such treatment disrupts lysosomes thus increasing the levels of free lysosomal proteases. The rate of inactivation of DHFR by sonicated 27,000 x g pellet was the same as that by untreated pellet (Fig. 12). The third approach was to measure inactivation of the enzyme by the 27,000 x g supernatant obtained after incubation of the 27,000 x g pellet for 2 h. The rate

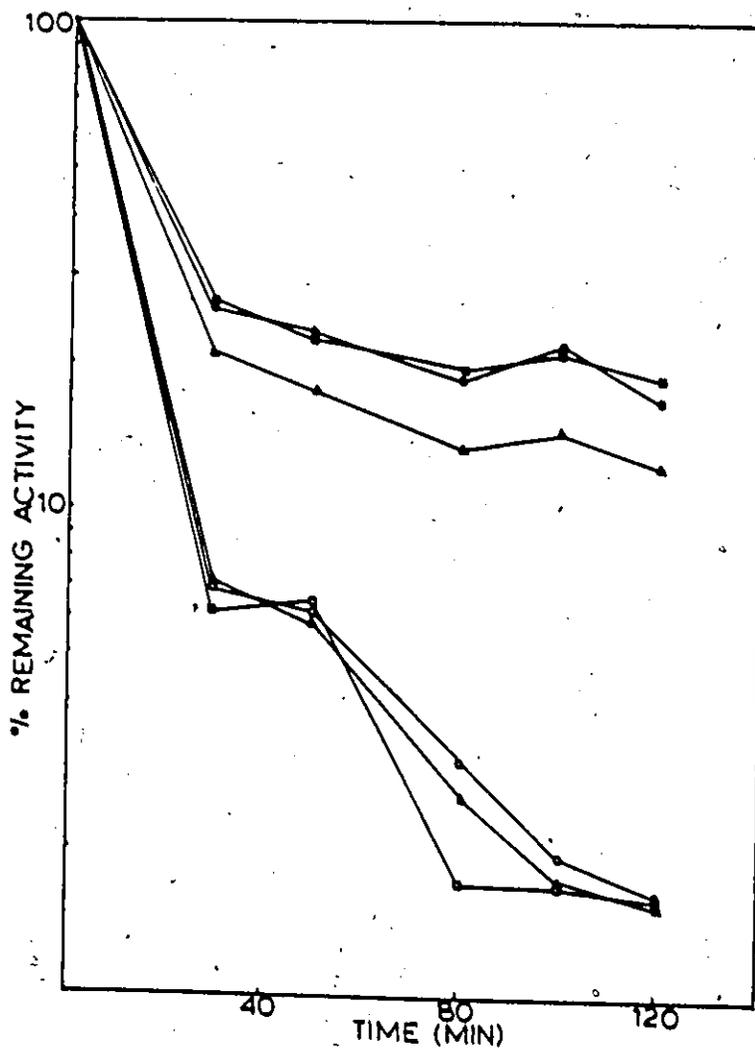


Fig. 10. Inactivation of DHFR by the components of the 27,000 x g pellet of LM cells

Incubations contained 0.70 mg of protein from LM cells and 10.0 units of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Incubation with the mitochondria-rich fraction, \circ — \circ ; incubation with the lysosome-rich fraction, \square — \square ; incubation with the heavy microsome-rich fraction, Δ — Δ . Open symbols show incubations containing unboiled proteins; closed symbols show incubations containing boiled proteins.

Similar results were obtained in three different experiments.

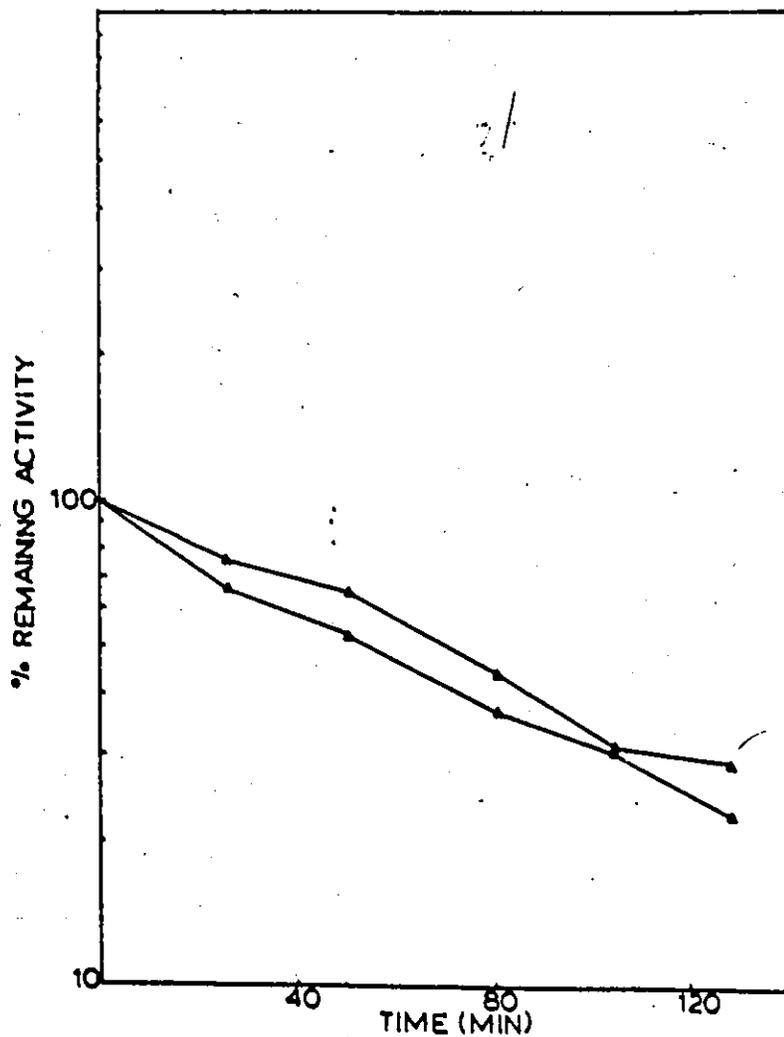


Fig. 11. Inactivation of DHFR by cathepsin D *in vitro*

Incubations contained 3.7 units of DHFR and 0.04 mg of partially purified cathepsin D in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

DHFR + cathepsin D Δ — Δ ; DHFR + boiled cathepsin D, \blacktriangle — \blacktriangle

Similar results were obtained in three different experiments.

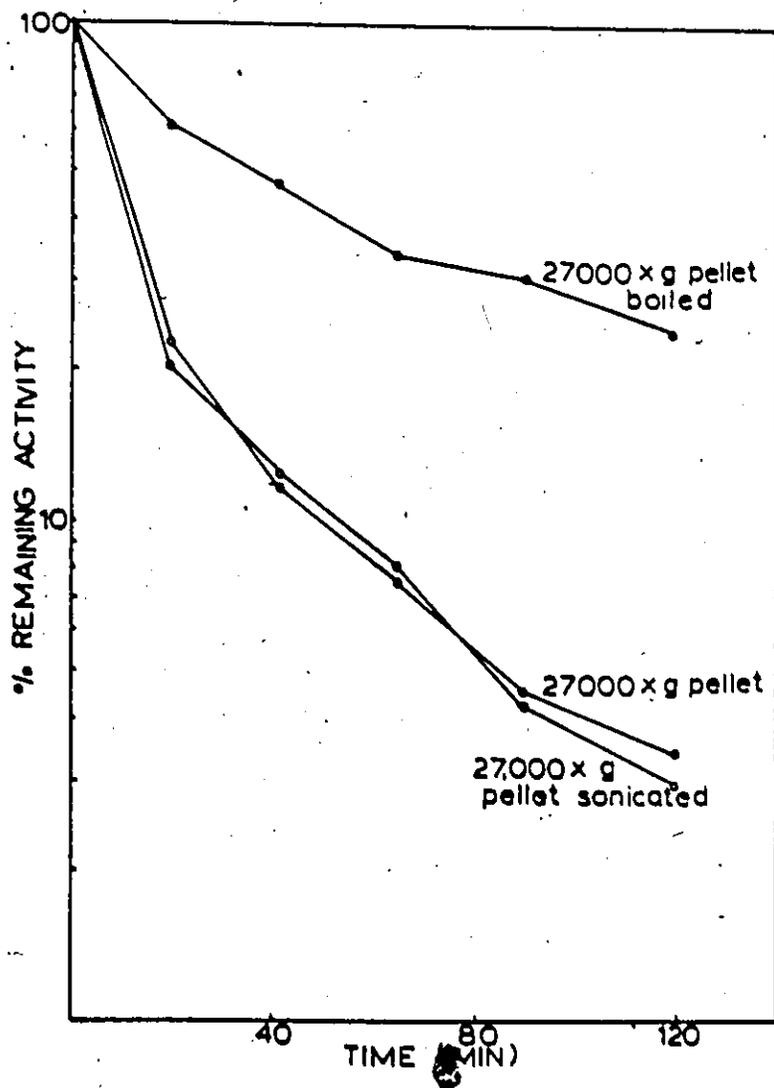


Fig. 12. Inactivation of DHFR by the sonicated 27,000 x g pellet of LM cells

Incubations contained 0.70 mg of protein from the 27,000 x g pellet and 5.1 units of DHFR in a final volume of 0.25M sucrose, 0.02M Tris-HCl, pH 7.4

Similar results were obtained in four different experiments.

of inactivation by this supernatant was similar to that produced by the 27,000 x g pellet (Fig. 13), indicating that inactivation could be produced by proteins which are solubilized from the pellet during incubation. If inactivation was produced entirely by such proteins, however, the rate of inactivation would be expected to be greater by the supernatant than by an equivalent amount of protein from the total pellet.

The 100,000 x g pellet inactivated DHFR by a process that appeared much less heat sensitive than that by which the 27,000 x g pellet inactivated DHFR (Fig. 8). Inactivation may only have been apparent if the 100,000 x g pellet contained factors which were not destroyed by boiling and which simply inhibited the enzyme. The 100,000 x g pellet was dialyzed overnight against 2,000 volumes of 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 and the inactivation of DHFR by dialyzed and undialyzed pellet was compared. Rather than decreasing the rate of inactivation, dialysis of the pellet resulted in an increased rate of inactivation (Fig. 14). Since the rate of inactivation by the pellet, which was dialyzed and then boiled, was greater than that by the boiled pellet, the increased rate seen with the dialyzed pellet was probably due to the removal of ligands which stabilize DHFR. The rate of inactivation of DHFR by the 8,000 x g pellet was unaltered by dialysis (Fig. 15).

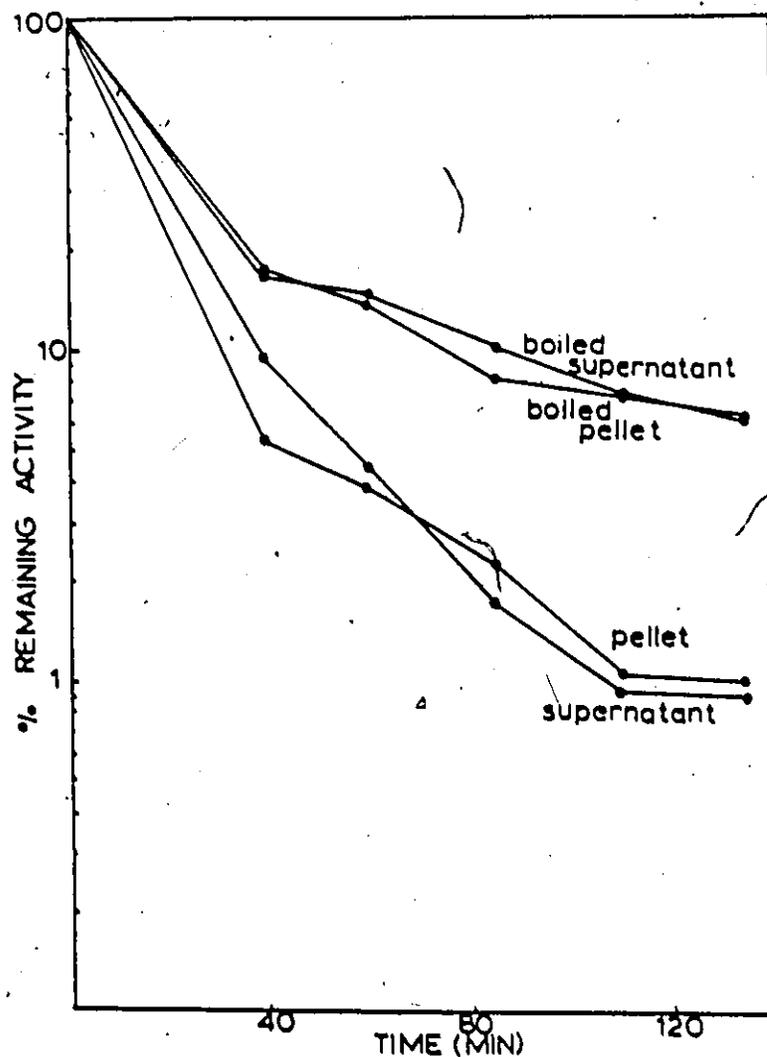


Fig. 13. Inactivation of DHFR by proteins which are solubilized from the 27,000 x g pellet of LM cells during incubation *in vitro*

Incubations contained 13.3 units of DHFR and 0.24 mg of protein from the 27,000 x g pellet of LM cells or from the 27,000 x g supernatant obtained after incubation of the 27,000 x g pellet in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The 27,000 x g supernatant was obtained by incubation of 7.0 mg of protein from the 27,000 x g pellet for 2 h in a final volume of 0.4 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 and centrifugation at 27,000 x g for 20 min.

Similar results were obtained from two different experiments.

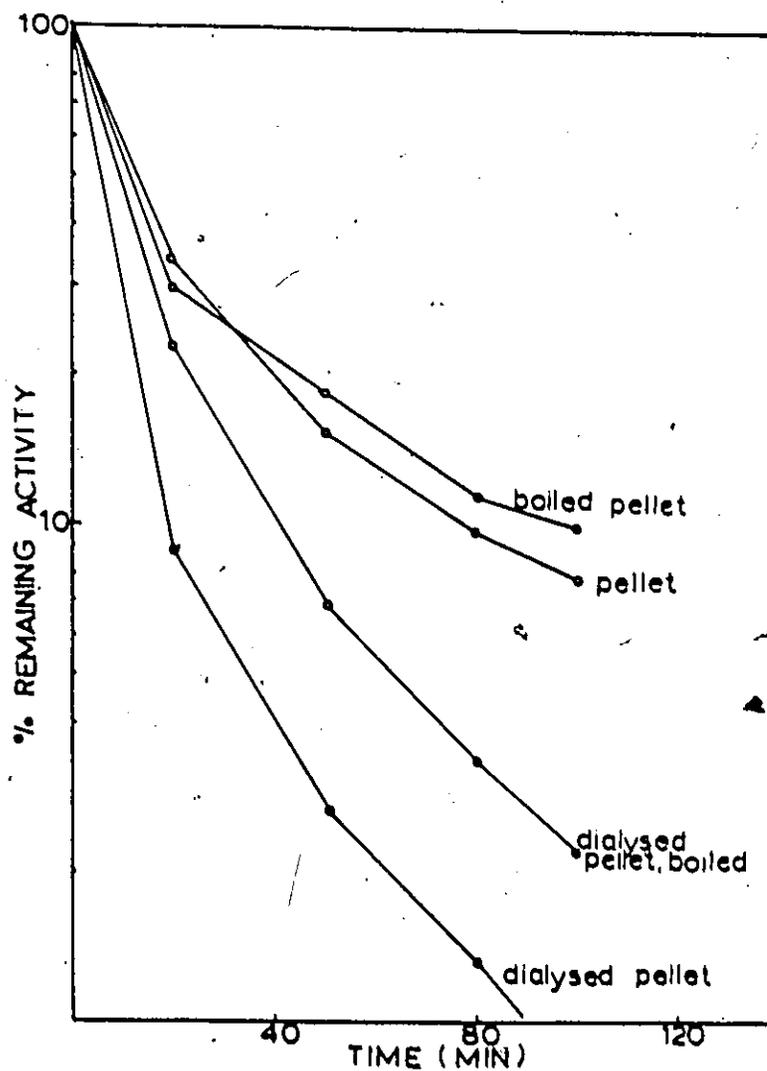


Fig. 14. Inactivation of DHFR by the dialysed 100,000 x g pellet of LM cells

Incubations contained 0.47 mg of protein from the 100,000 x g pellet and 6.0 units of DHFR in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Similar results were obtained in two different experiments.



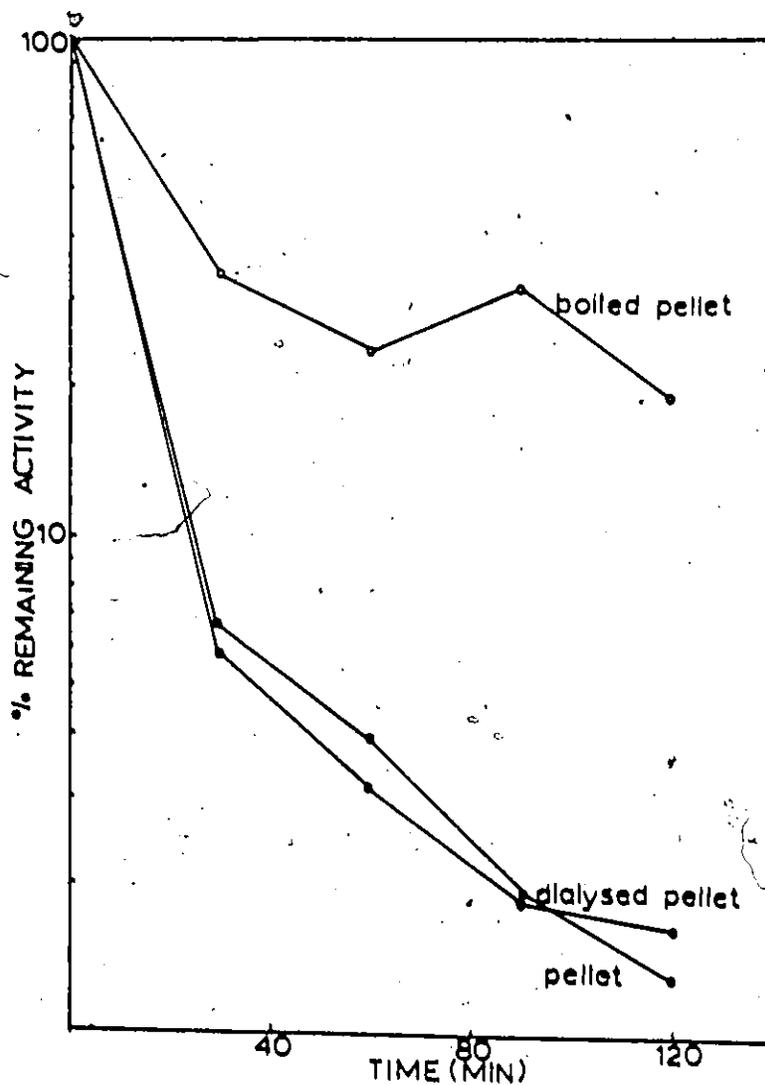


Fig. 15. Inactivation of DHFR by the dialysed 8,000 x g pellet of LM cells

Incubations contained 0.47 mg of protein from the 8,000 x g pellet of LM cells and 5.2 units of DHFR in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Similar results were obtained in two different experiments.

c) Effect of various compounds on the loss of activity of
DHFR *in vitro*

The rate of degradation of proteins *in vivo* can be decreased by ligands such as substrates and cofactors (Schimke et al., 1965, 1965a). The effect of substrate, cofactor and inhibitor on the inactivation of DHFR by the 27,000 x g pellet was therefore investigated.

When 1×10^{-4} M DHF was present in incubations, DHFR was totally protected from inactivation (Fig. 16). At a concentration of 2×10^{-4} M, NADPH almost completely prevented loss of activity of the enzyme (Fig. 16). Methotrexate, 5×10^{-7} M, also stabilized the enzyme (Fig. 16).

There have been reports that protein degradation is an energy requiring process (Simpson, 1953; Poole, 1975; Hershko and Tomkins, 1971). For this reason an ATP regenerating system was used to see whether it increased the rate of inactivation of DHFR by the 27,000 x g pellet of LM cells. Quite the opposite was found. The ATP regenerating system totally prevented the heat sensitive inactivation of DHFR (Fig. 17). When the ATP regenerating system was added after an initial period of inactivation, the activity was not restored but further inactivation was prevented (Fig. 18). Stabilization probably did not involve incorporation of inorganic phosphate into the enzyme since when it was incubated with 27,000 x g pellet, [32 P]-ATP and the other components of the ATP regenerating system and then purified by affinity chromatography on Sepharose-MTX, no [32 P] was detected in the purified enzyme (data not shown). When the effect of the individual components of the ATP regenerating system on the inactivation of DHFR was studied, it was found that ATP alone, magnesium alone and potassium alone

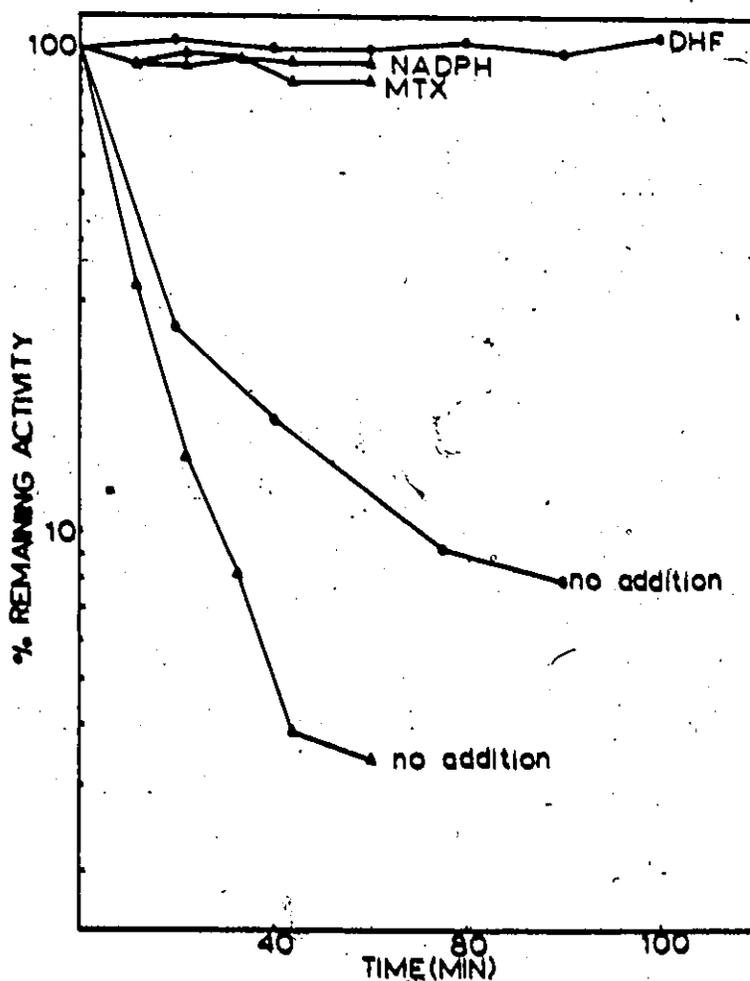


Fig. 16. Stabilization of DHFR by NADPH, MTX and DHF

Incubations shown by triangles, contained 0.76 mg of protein from the 27,000 x g pellet of LM cells and 7.8 units of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. Where present, MTX was at a final concentration of $5 \times 10^{-7}M$ and NADPH at $2 \times 10^{-4}M$. Incubations shown by circles contained 0.41 mg of protein from the 27,000 x g pellet of LM cells and 2.04 units of DHFR in a final volume of 0.26 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. Where present, DHF was at a concentration of $1 \times 10^{-4}M$.

The results obtained with DHF present are representative of three different experiments, those with NADPH of two different experiments.

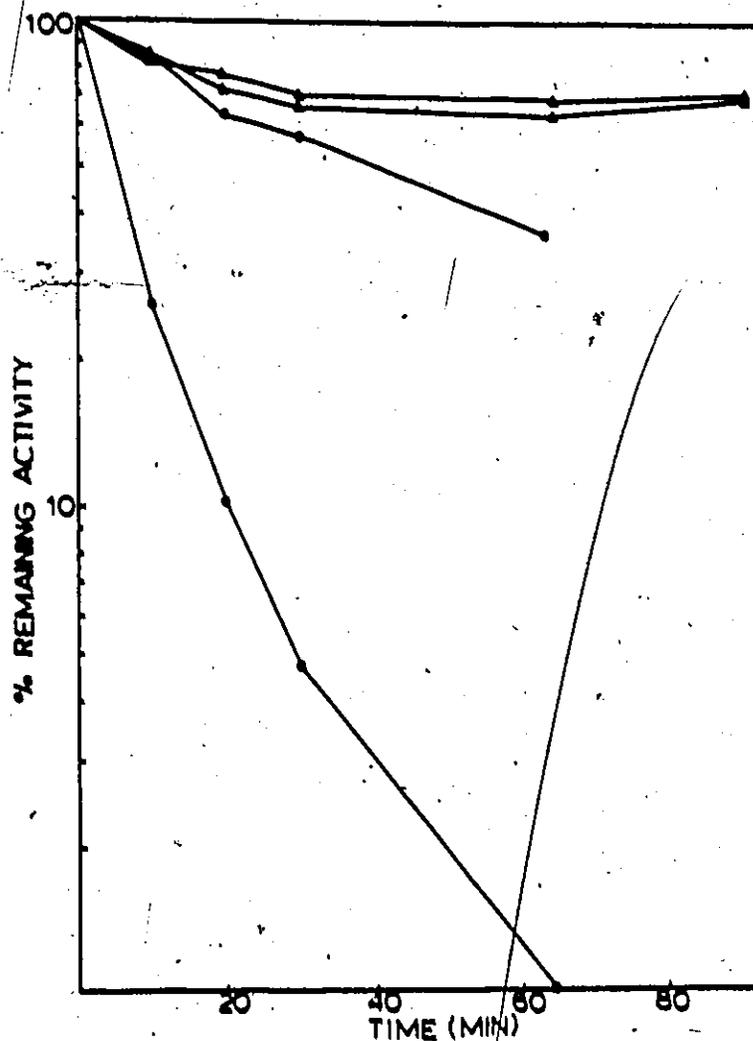


Fig. 17. Inactivation of DHFR by the 27,000 x g pellet of LM cells in the presence of an ATP regenerating system

Incubations contained 0.29 mg of protein from the 27,000 x g pellet and 13.8 units of DHFR in a final volume of 0.8 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Open symbols represent non-boiled 27,000 x g pellet, closed symbols represent boiled 27,000 x g pellet. Incubations without ATP regenerating system, ○—○; incubations with ATP regenerating system, △—△.

Similar results were obtained in two different experiments.

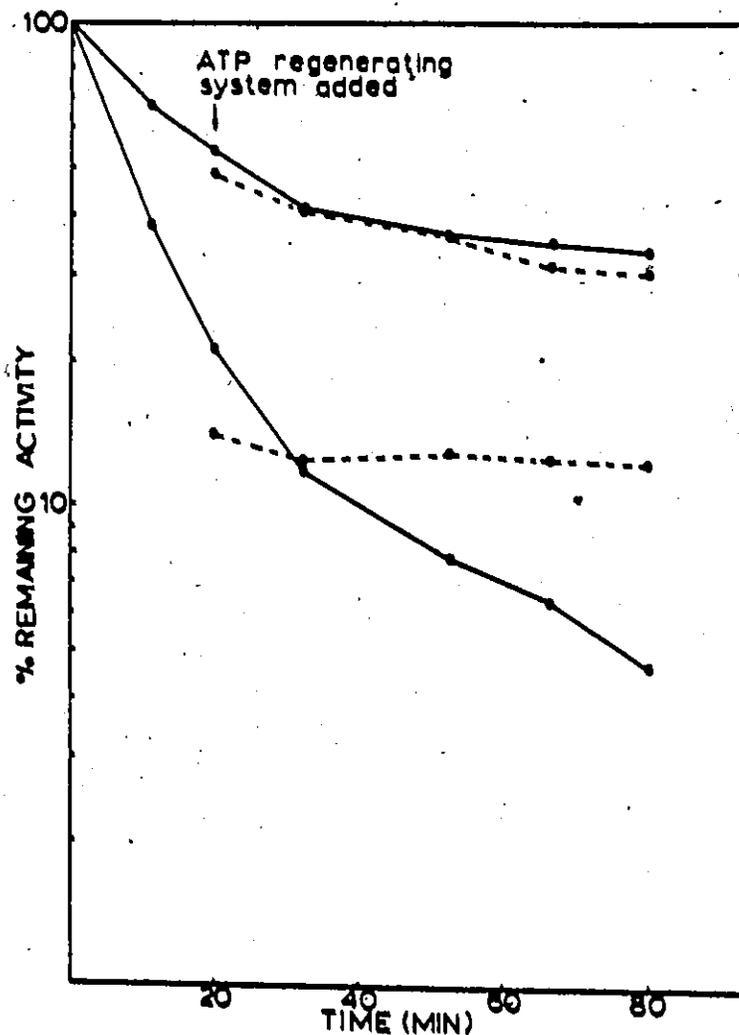


Fig. 18. Effect of an ATP regenerating system on the inactivation of DHFR by the 27,000 x g pellet of LM cells

Incubations contained 0.68 mg of protein from the 27,000 x g pellet, and 20.0 units of DHFR in a final volume of 0.78 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The ATP regenerating system was added in a volume of 0.14 ml.

Open symbols represent 27,000 x g pellet; closed symbols represent boiled pellet.

Similar results were obtained in three different experiments.

decreased the rate of inactivation of DHFR by the 27,000 x g pellet (Fig. 19). Pyruvate kinase also protected DHFR but was less effective than the other components of the ATP regenerating system (Fig. 19). Although ATP and magnesium prevented inactivation by the 27,000 x g pellet, they did not stabilise the enzyme against denaturation when it was incubated alone (Fig. 20).

3.2.2. Mechanism of Inactivation of DHFR *in vitro*

To investigate the mechanism by which DHFR was inactivated by the subcellular membrane fractions of LM cells, radioactive label was incorporated into the molecule. In this way the inactive molecule could be detected and changes in it examined.

a) Production of acid-soluble material from [¹⁴C]-labelled DHFR

A possible mechanism by which DHFR was inactivated by the subcellular membrane fractions of LM cells was by degradation of the protein to its constituent amino acids. Using [¹⁴C]-labelled DHFR, the production of acid soluble material from the enzyme by the 27,000 x g pellet of LM cells was studied. Little acid-soluble material was produced from DHFR when it was incubated with the 27,000 x g pellet (Table 8) under conditions that have been shown to produce extensive inactivation of the enzyme (Fig. 8).

DHFR was inactivated by the 27,000 x g pellet of LM cells and the 27,000 x g supernatant subsequently added. Again little acid-soluble material was produced (Table 9).

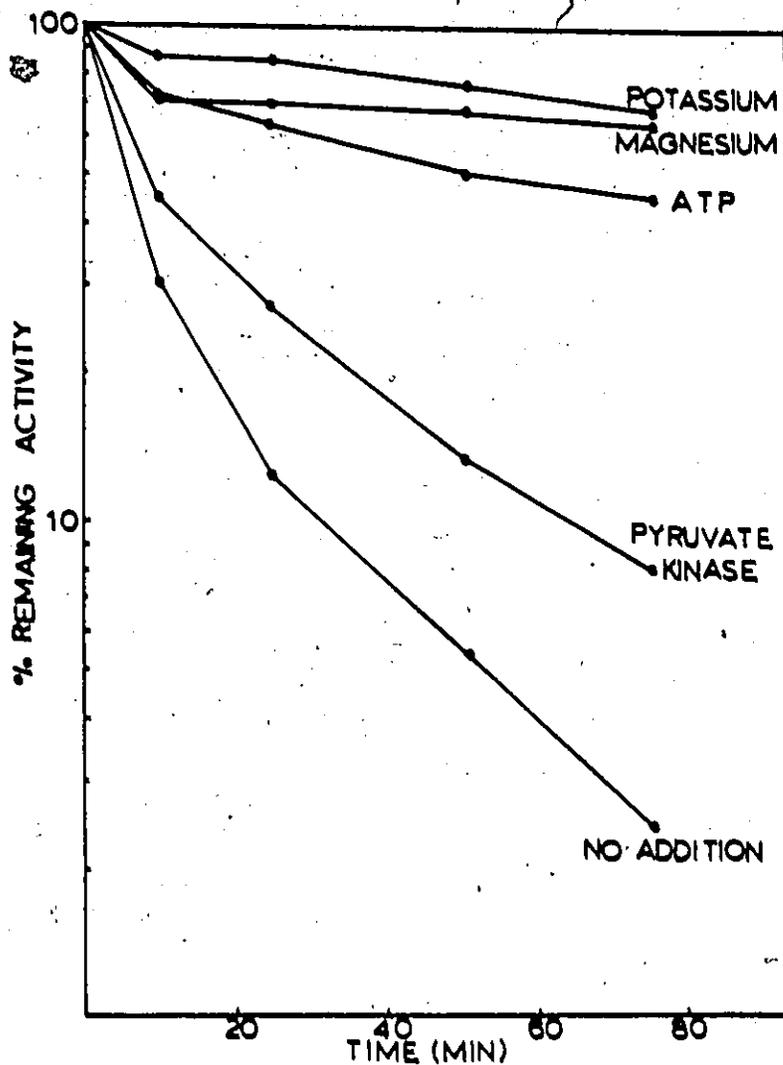


Fig. 19. Inactivation of DHFR by the 27,000 x g pellet of LM cells in the presence of the components of an ATP regenerating system

Incubations contained 0.29 mg of protein from the 27,000 x g pellet and 21.0 units of DHFR in a final volume of 0.84 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.

Components of the ATP regenerating system were present as shown and were at the concentrations described in the Methods Section (2.2.6).

Similar results were obtained in two different experiments.

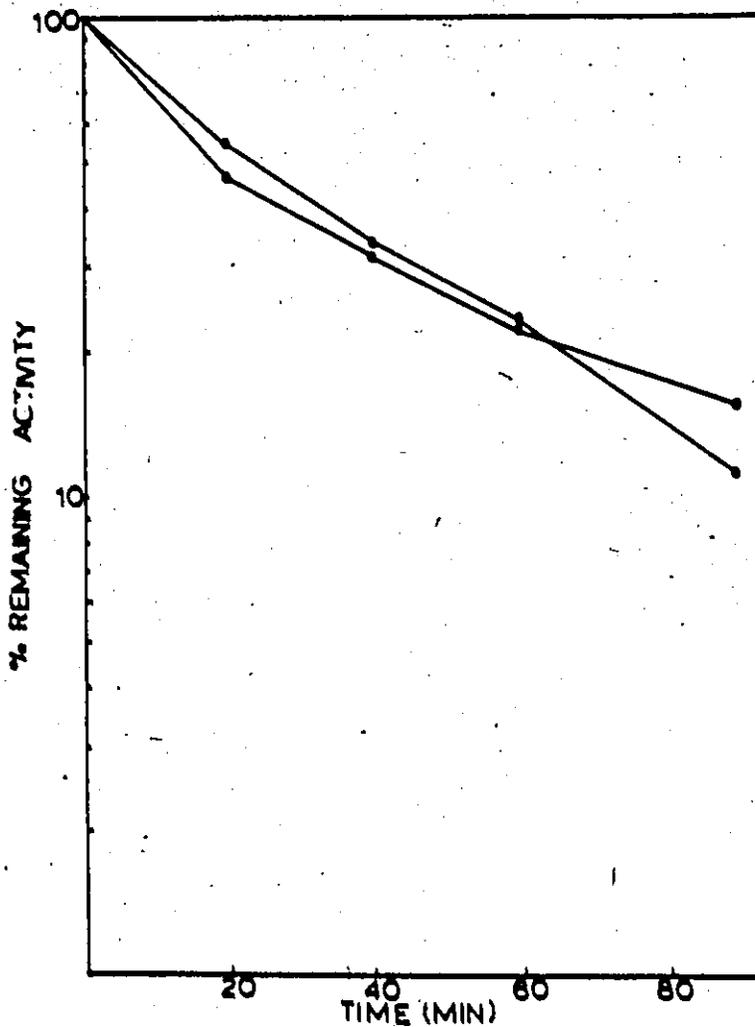


Fig. 20. Heat inactivation of DHFR in the presence of ATP and magnesium
 Incubations contained 10.9 units of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. ATP and magnesium were at the concentrations described in the Methods Section (2.2.6).
 No addition, ○—○; ATP and magnesium, ●—●.

Similar results were obtained in three different experiments.

TABLE 8

PRODUCTION OF ACID-SOLUBLE MATERIAL FROM [^{14}C]-LABELLED DHFR AFTER
INCUBATION WITH THE 27,000 x g PELLET OF LM CELLS

	Acid-soluble dpm	% Acid- soluble
Not incubated	738	5.18
Incubated with 27,000 x g pellet for 90 min	862	6.04
Incubated with boiled 27,000 x g pellet for 90 min	790	5.54

Incubations were for 90 min, they contained 4.1 μg DHFR and 0.39 mg of protein from the 27,000 x g pellet in a final volume of 0.35 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4.

Incubations contained 14,253 dpm. The results given represent duplicate samples; cpm measured ranged from 263 to 314.

Although the amount of acid-soluble material in the sample before incubation varied, similar results were obtained in three different experiments.

TABLE 9

PRODUCTION OF ACID-SOLUBLE MATERIAL FROM [^{14}C]-LABELLED DHFR

Treatment of DHFR	% Acid-soluble
0 time	5.18
90 min + 27,000 x g pellet	6.04
90 min + boiled 27,000 x g pellet	5.54
90 min + 27,000 x g pellet, then 90 min + 27,000 x g supernatant	6.60
90 min + boiled 27,000 x g pellet, then 90 min + 27,000 x g supernatant	6.19
90 min + 27,000 x g pellet, then 90 min + boiled 27,000 x g supernatant	6.18

Initial incubations were at 37°C and contained 0.39 mg of protein from the 27,000 x g pellet and 4.1 µg DHFR in a final volume of 0.35 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.5. After 90 min, 2.07 mg of protein from the 27,000 x g supernatant were added in a volume of 0.30 ml of the same buffer, incubation was continued for another 90 min.

Incubations contained 14,253 dpm. cpm measured ranged from 163 to 314. The results given represent the mean of duplicate samples.

b) Changes in the molecular weight of DHFR during inactivation

in vitro

The molecular weight of DHFR, as determined by polyacrylamide-SDS gel electrophoresis, is 18,000 (Fig. 21). This was unaltered after incubation with the total 600 x g cell homogenate (Fig. 21), or with the 27,000 x g lysosomal pellet (Fig. 22A). Some [14 C]-labelled protein was found in the 27,000 x g pellet at the end of the incubation. The molecular weight of this material was the same as that which remained in the soluble fraction (Fig. 22B).

c) Changes in the charge of DHFR

If inactivation of DHFR is the result of removal of amino acids from the molecule, a change in the charge of the enzyme might occur. Polyacrylamide gel electrophoresis was used to see whether such a change could be demonstrated. The radioactivity which was recovered at the buffer front represents acid-soluble material, that at an RF of 0.2 - 0.3 represents [14 C]-labelled DHFR. No change in the charge of the enzyme which remained in the 27,000 x g supernatant could be detected after incubation with the 27,000 x g pellet as compared to a control which was incubated with boiled pellet (Fig. 23). It was found, however, that some material did not enter the 7% polyacrylamide gels. Radioactivity was recovered on the top slice of the gels. Formation of this material was catalyzed by the presence of unboiled 27,000 x g pellet. No such material was formed when the enzyme was incubated alone (Fig. 24).

After incubation with 8M urea and 1.2M mercaptoethanol, the amount of radioactivity which remained at the origin compared to the amount

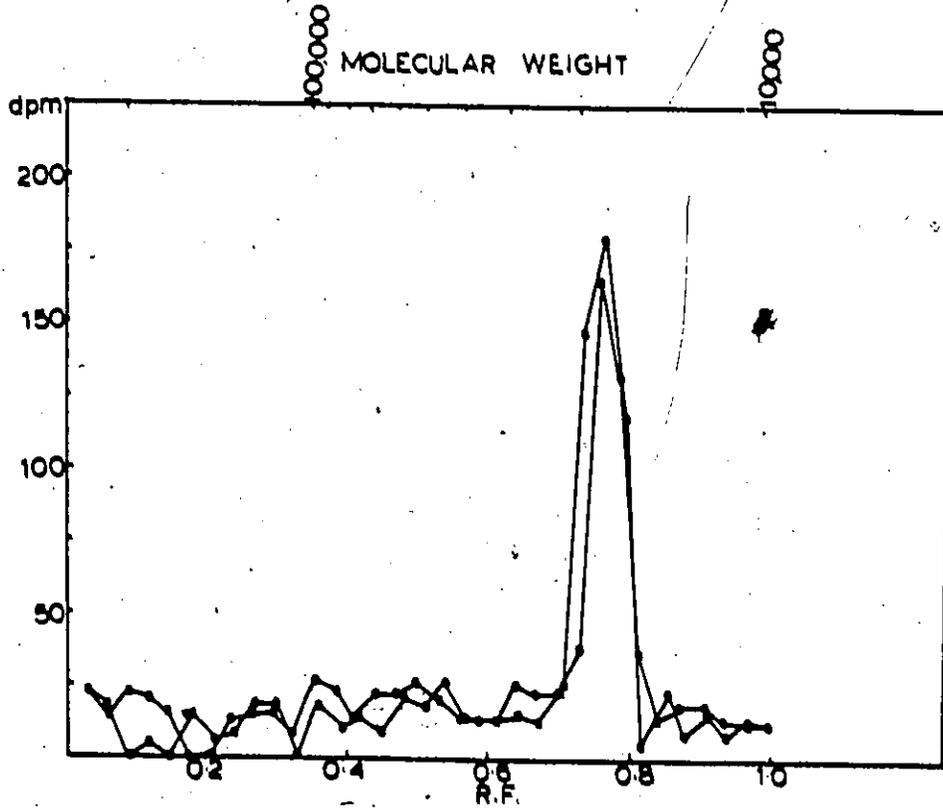


Fig. 21. Polyacrylamide-SDS gel electrophoresis of [14 C]-labelled DHFR after incubation with the 600 x g supernatant of LN cells

Incubations contained 1.8 μ g of protein from the 600 x g supernatant and 0.81 μ g of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h at 37°C samples of the 27,000 x g supernatant were applied to polyacrylamide-SDS gels. The volume of sample which was put on each gel was equivalent to that which contained 0.24 μ g of DHFR at the beginning of the incubation.

After incubation with 600 x g supernatant, ○—○;
after incubation with boiled 600 x g supernatant, ●—●.

Similar results were obtained from two different experiments.

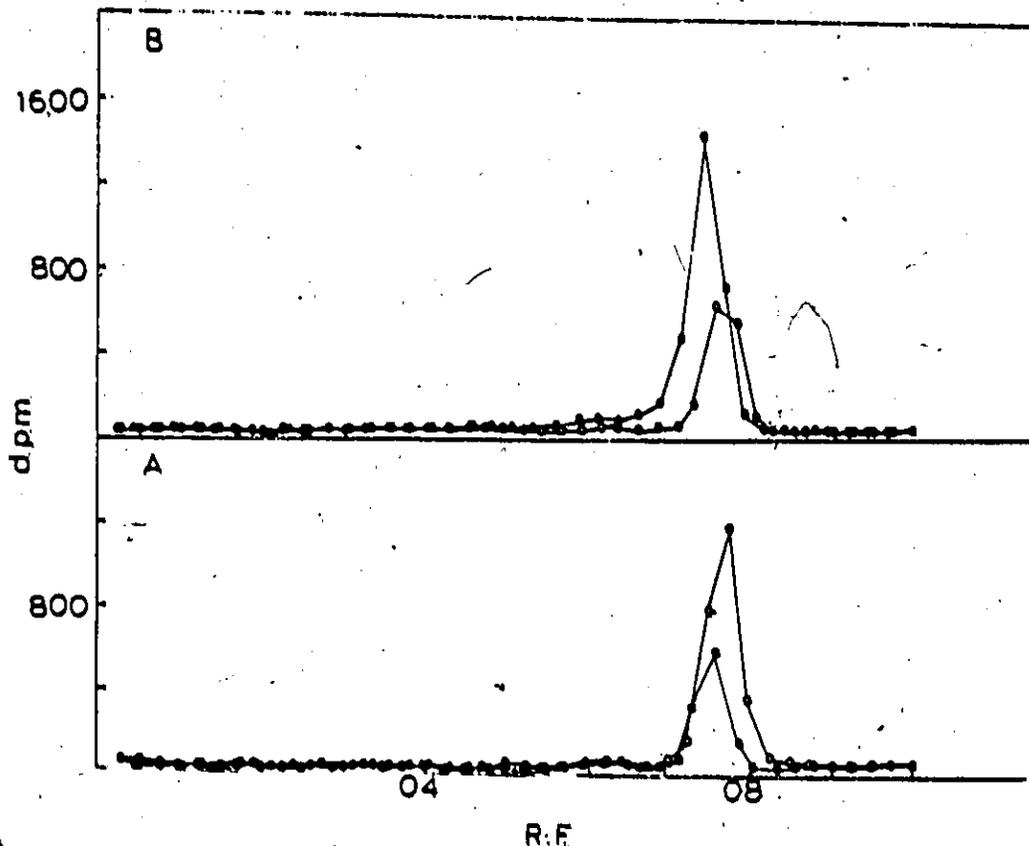


Fig. 22. Polyacrylamide-SDS gel electrophoresis of [14 C]-labelled DHFR after incubation with the 27,000 x g pellet of LM cells
 Incubations were at 37°C and contained 1.9 μ g of DHFR and 0.61 mg of protein from the 27,000 x g pellet at pH 7.4 in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl. After 80 min, the incubation was terminated by centrifugation at 27,000 x g.

A. Material which remained in the supernatant of incubations with the 27,000 x g pellet. A volume of sample equivalent to that which had contained 0.66 μ g of DHFR at the beginning of the incubation was applied to the gels.

B. Material which was recovered in the 27,000 x g pellet: The pellet from a volume of sample equivalent to that which contained 1.41 μ g of DHFR at the beginning of the incubation was applied to the gels.

After incubation with 27,000 x g pellet, ○—○; after incubation with boiled 27,000 x g pellet, ●—●.

These results are representative of seven different experiments in which the supernatant of incubations with the 27,000 x g pellet was applied to gels and three experiments in which the 27,000 x g pellet was applied to gels.

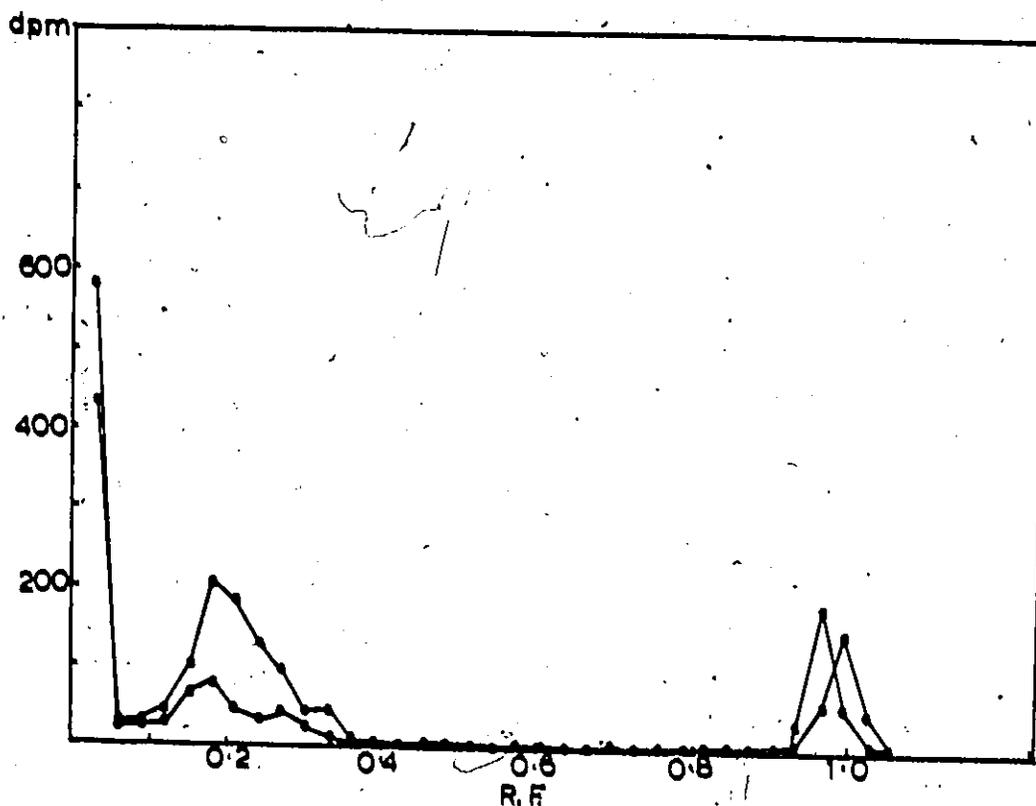


Fig. 23. Polyacrylamide gel electrophoresis of [14 C]-labelled DHFR which remains in the 27,000 x g supernatant after incubation with the 27,000 x g pellet of LM cells

Incubations were at 37°C and contained 0.73 mg of protein from the 27,000 x g pellet and 1.05 μ g of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 90 min a volume of sample equivalent to that which had contained 0.44 μ g of DHFR at the beginning of the incubation was applied to the gels.

After incubation with 27,000 x g pellet, \circ — \circ ; after incubation with boiled 27,000 x g pellet, \bullet — \bullet .

Similar results were obtained in three different experiments.

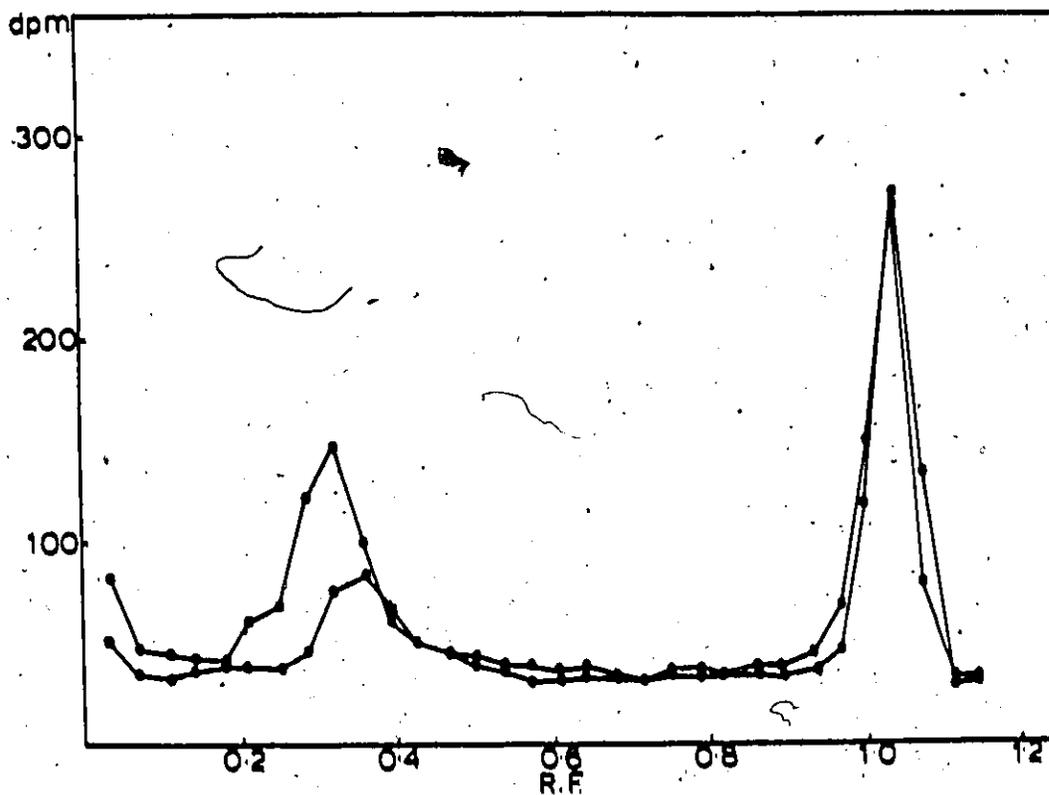


Fig. 24. Polyacrylamide gel electrophoresis of [14 C]-labelled DHFR after incubations in vitro

Incubations contained 1.8 μ g of DHFR in 0.35 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, at 0° or at 37°C. After 80 min, a volume of the 27,000 \times g supernatant equivalent to that which contained 0.8 μ g of DHFR at the beginning of the incubation was applied to polyacrylamide gels.

After incubation at 0°C, ○—○; after incubation at 37°C, ●—●.

which was recovered in the enzyme peak was greatly reduced (Fig. 25). This indicated either that the material found on the top of the gels (Fig. 23) represented denatured material which was solubilized by urea and mercaptoethanol or that it represented material of high molecular weight which was dissociated by incubation with urea and mercaptoethanol. The charge of this [14 C]-labelled material could not be shown to be different from that which entered the polyacrylamide gels, since when it entered the polyacrylamide gels in the presence of urea and mercaptoethanol all the radioactivity was recovered in a single peak which was the same in samples incubated with boiled or unboiled 27,000 x g pellet (Fig. 25).

Treatment with urea and mercaptoethanol resulted in reactivation of the enzyme (Table 10), while the activity in controls which had contained no protein or protein from the boiled 27,000 x g pellet was decreased by this treatment. This suggests that the most likely explanation for the formation of the [14 C]-labelled enzyme which did not enter 7% polyacrylamide gels is that it was inactive high molecular weight enzyme which was dissociated and reactivated by treatment with urea and mercaptoethanol. To eliminate the possibility that the increased DHFR activity was the result of activation of latent endogenous DHFR, controls were performed in which the endogenous activity of the 27,000 x g pellet of LM cells was measured before and after treatment with urea and mercaptoethanol. No DHFR could be detected in the 27,000 x g pellet either before or after treatment with urea and mercaptoethanol (data not shown).

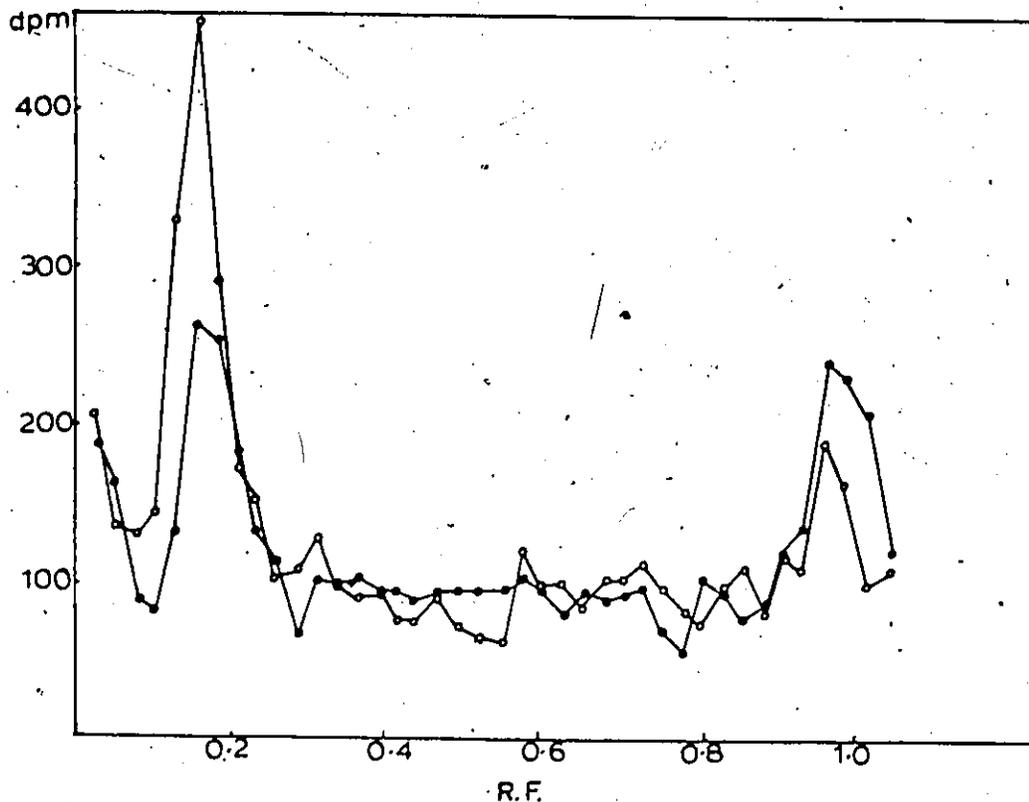


Fig. 25. Polyacrylamide gel electrophoresis in the presence of 8M urea of the [14 C]-labelled DHFR which remained in the 27,000 x g supernatant after incubation with the 27,000 x g pellet of LM cells

Incubations were for 2 h at 37°C and contained 0.5 mg of protein from the 27,000 x g pellet and 1.9 μ g of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4

Aliquots applied to the gels contained 8M urea and 1.2 mM mercaptoethanol. The volume was equivalent to that which contained 0.4 μ g of DHFR at the beginning of the incubation.

After incubation with 27,000 x g pellet, o—o; after incubation with boiled 27,000 x g pellet, ●—●.

Similar results were obtained in two different experiments.

TABLE 10

EFFECT OF UREA AND MERCAPTOETHANOL ON THE ACTIVITY
OF PREVIOUSLY INACTIVATED DHFR

Incubation	% of original activity remaining after 2 h incubation	% of original activity remaining after 2 h incubation and subsequent treatment with urea and mercaptoethanol
DHFR + 27,000 x g pellet	14.0	24.0
DHFR + boiled 27,000 x g pellet	31.8	7.9
DHFR + buffer only	25.9	2.9

Incubations contained 0.5 mg of protein from the 27,000 x g pellet of LM cells and 1.9 μ g DHFR in a final volume of 0.39 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4. After 2 h, the 27,000 x g pellet was removed by centrifugation. An aliquot of the 27,000 x g supernatant was assayed before and after incubation with 8 M urea, 1.2 mM mercaptoethanol.

Results given represent the mean of duplicate incubations.

This experiment is one of a series in which DHFR, initially inactivated by subcellular membrane fractions, was reactivated by urea (Table 10, 12-15).

d) Association of DHFR with the subcellular membranes of LM cells

Most of the high molecular weight [^{14}C]-labelled material which remained in the 27,000 x g supernatant after incubation of DHFR with the 27,000 x g pellet of LM cells, entered a column of Sepharose 2B (Fig. 26). After incubation of DHFR either alone or with boiled 27,000 x g pellet, most of the radioactivity was recovered as a single peak. After incubation with unboiled pellet, radioactivity was found in the single peak but was also recovered in several fractions of higher molecular weight. The material in the single peak was active, that in each of the higher molecular weight fractions was not active (Fig. 27).

The inactive high molecular weight material recovered on Sepharose 2B columns might represent the formation of aggregates of DHFR catalyzed by the 27,000 x g pellet. On the other hand, it might represent enzyme which was bound to components of the 27,000 x g pellet which remain in the 27,000 x g supernatant at the end of the incubations. The presence of the inactive material in several different fractions suggests that the latter explanation is the most likely. In order to detect the positions of the subcellular proteins in the eluate of Sepharose 2B columns [^3H]-labelled proteins of the 27,000 x g pellet were used in the incubations. Each fraction of the column eluate which contained [^{14}C]-labelled material of high molecular weight was also found to contain [^3H]-labelled proteins of the 27,000 x g pellet (Fig. 28).

e) Characterization of the proteins associated with inactive DHFR

The 27,000 x g pellet of LM cells contained mitochondria, lysosomes and heavy microsomes. Fractions rich in each of these organelles were

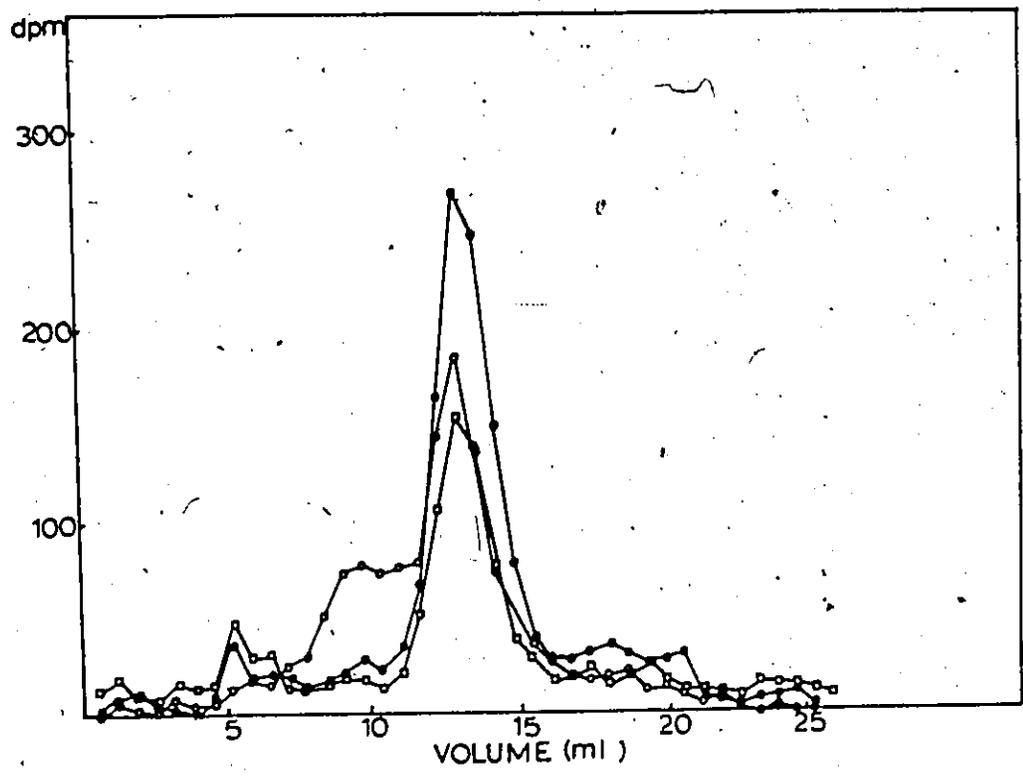


Fig. 26. Column chromatography of [¹⁴C]-labelled DHFR on a Sepharose 2B column after incubation with the 27,000 x g pellet of LM cells
Incubations contained 0.53 mg of protein from the 27,000 x g pellet and 4.75 µg of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4.
After 2 h, 0.25 ml of the 27,000 x g supernatant was applied to a Sepharose 2B column.
After incubation with the 27,000 x g pellet, o—o; after incubation with boiled 27,000 x g pellet, ●—●; after incubation alone, □—□.
Similar results were obtained in three different experiments.

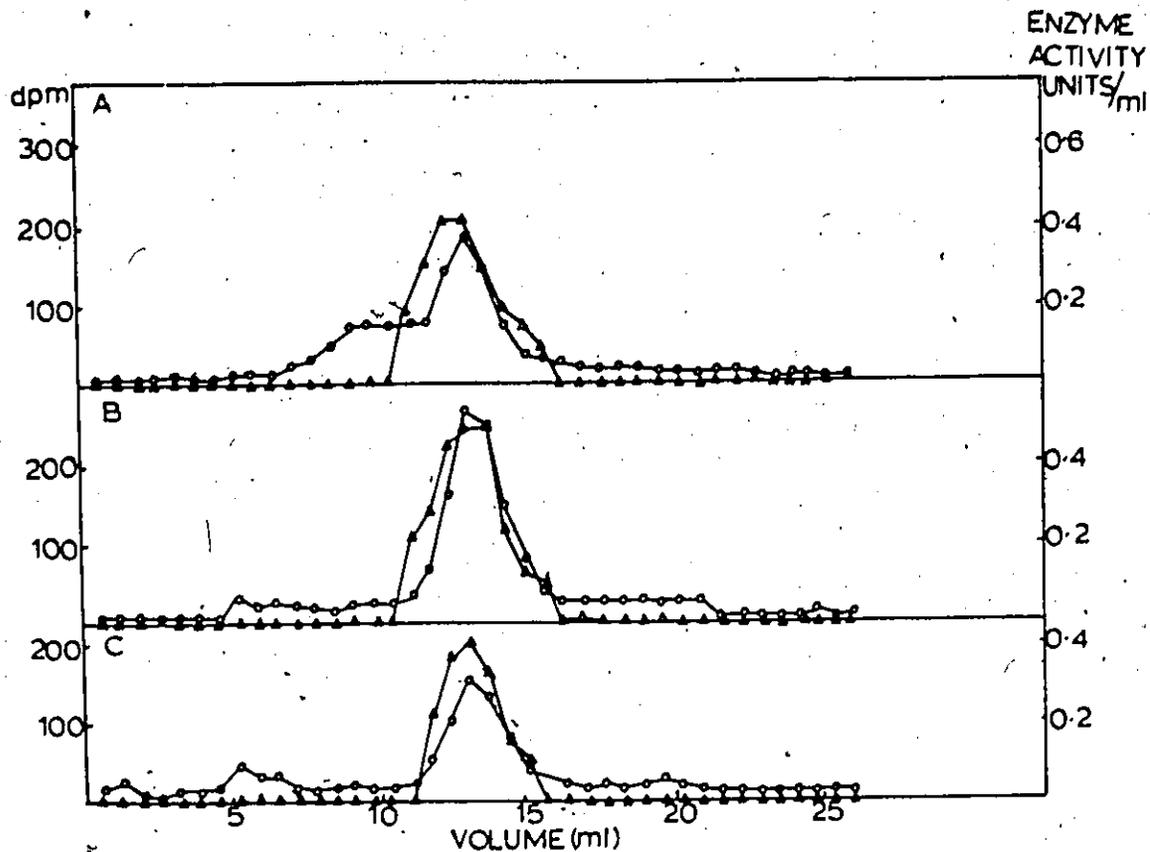


Fig. 27. Enzyme activity and [14 C]-labelled material in the fractions eluting from a column of Sepharose 2B

After incubation of 0.53 mg of protein from the 27,000 x g pellet of LM cells and 4.75 μ g of DHFR for 2 h in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, 0.25 ml of the 27,000 x g supernatant was applied to a column of Sepharose 2B.

Enzyme activity is shown by triangles, [14 C]-dpm are shown by circles. (A), after incubation with the 27,000 x g pellet; (B), after incubation with boiled 27,000 x g pellet; (C), after incubation alone.

Similar results were obtained in three different experiments.

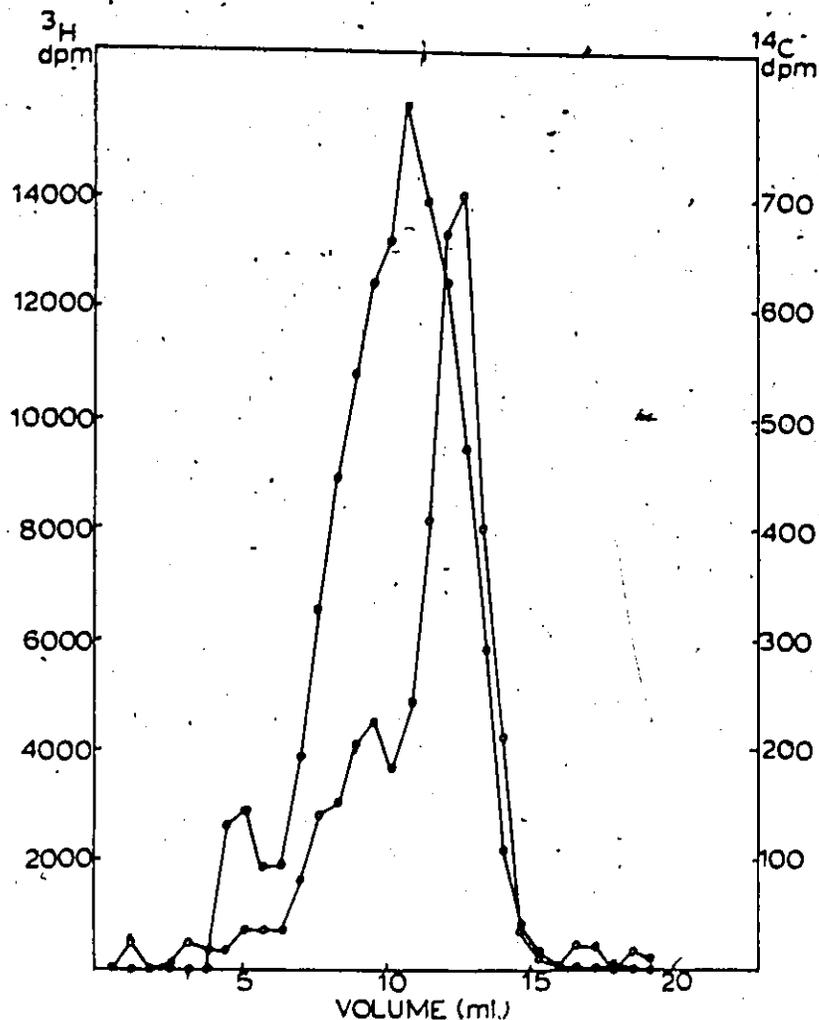


Fig. 28. Column chromatography of [¹⁴C]-labelled DHFR and [³H]-labelled protein from the 27,000 x g pellet of LM cells on a column of Sepharose 2B

Incubations contained 2.64 mg of protein from the 27,000 x g pellet and 14.14 μg of DHFR in a final volume of 0.40 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h the 27,000 x g pellet was removed by centrifugation and 0.25 ml of the 27,000 x g supernatant applied to a column of Sepharose 2B.

[¹⁴C]-labelled DHFR, ○—○; [³H]-labelled cellular protein, ●—●.

This experiment is one of a series in which the [¹⁴C]-labelled material of high molecular weight eluted at a constant volume (7.1 - 9.7) only when cellular protein was also present in that volume (Fig. 28-32).

prepared from cells labelled with [^3H]-leucine and were incubated with [^{14}C]-labelled DHFR. When a sample containing DHFR and the pellet rich in mitochondria was applied to a Sepharose 2B column, a small amount of [^{14}C]-labelled material eluted in the void volume with the bulk of the [^3H]-labelled protein (Fig. 29A). When a sample containing DHFR and the pellet rich in lysosomes was applied to a column of Sepharose 2B, some [^{14}C]-labelled material again eluted in the void volume with the bulk of the [^3H]-labelled protein. Some [^{14}C]-labelled material of high molecular weight entered the column and eluted between 7.1 and 9.1 ml (Fig. 29B, Table 11). When a sample containing DHFR and the pellet rich in heavy microsomes was applied to a column of Sepharose 2B the [^{14}C]-labelled material of high molecular weight which entered the column increased (Fig. 29C, Table 11). The elution volumes of the [^{14}C] and [^3H]-labelled proteins corresponded (Fig. 29C).

The total amount of protein applied to each of the Sepharose columns was the same, yet the [^3H]-dpm eluting from each was different (Fig. 29), indicating differential labelling of the proteins of the three subcellular pellets. To see how much cellular protein was in each of the fractions of the Sepharose 2B column, samples of the labelled pellets were applied to columns and the total protein eluting in each fraction, rather than the radioactivity, was measured (Fig. 30). Comparison of the amount of cellular protein (Fig. 30) and of [^{14}C]-labelled DHFR (Fig. 29) in each fraction shows that the ratio of [^{14}C]-dpm to μg of protein in the void volume was very low in incubations which contained proteins from the pellet rich in mitochondria or lysosomes as compared to one which contained proteins from

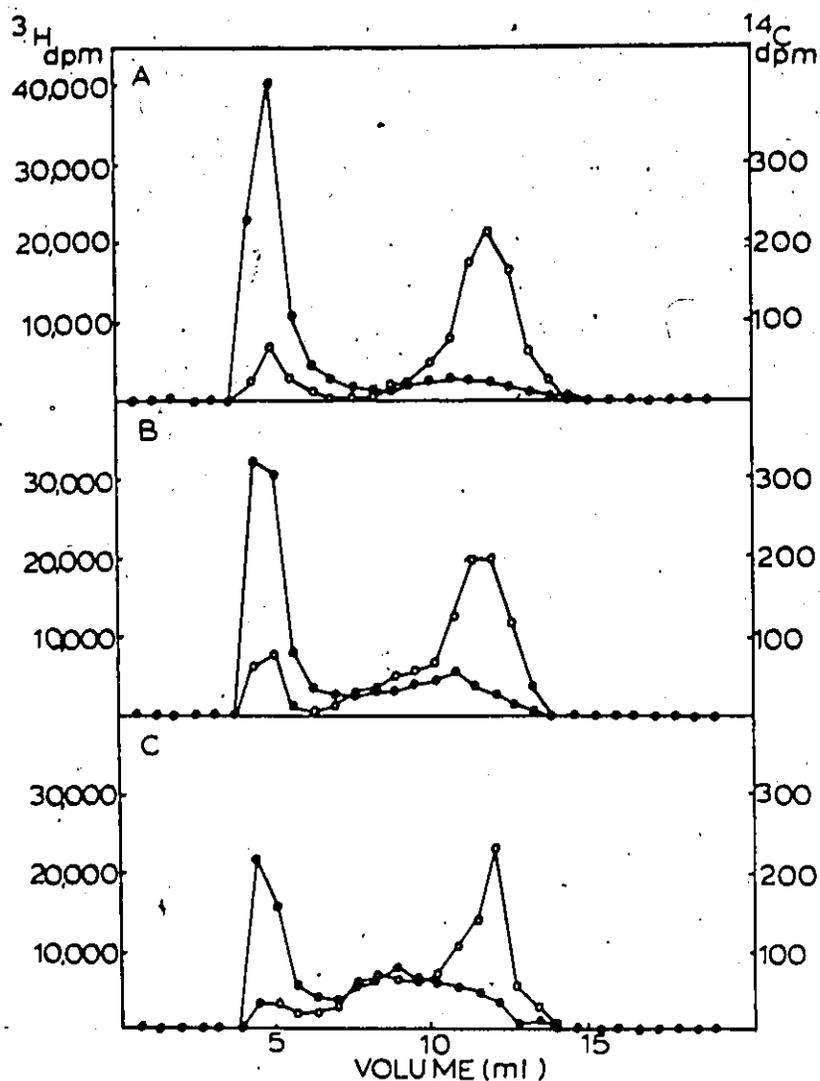


Fig. 29. Column chromatography of [^3H]-labelled subcellular fractions of LM cells and [^{14}C]-labelled DHFR on columns of Sepharose 2B

Incubations contained 2.7 μg of DHFR and 0.47 mg of protein from the subcellular fraction in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml of the incubation mixture was applied to a column of Sepharose 2B.

Closed circles represent [^3H]-dpm and open circles represent [^{14}C]-dpm.

(A), after incubation with the fraction rich in mitochondria; (B), after incubation with the fraction rich in lysosomes; (C), after incubation with the fraction rich in heavy microsomes.

This experiment is one of a series in which the [^{14}C]-labelled material of high molecular weight eluted at a constant volume (7.1 - 9.7) only when cellular protein was also present in that volume (Figs. 28-32).

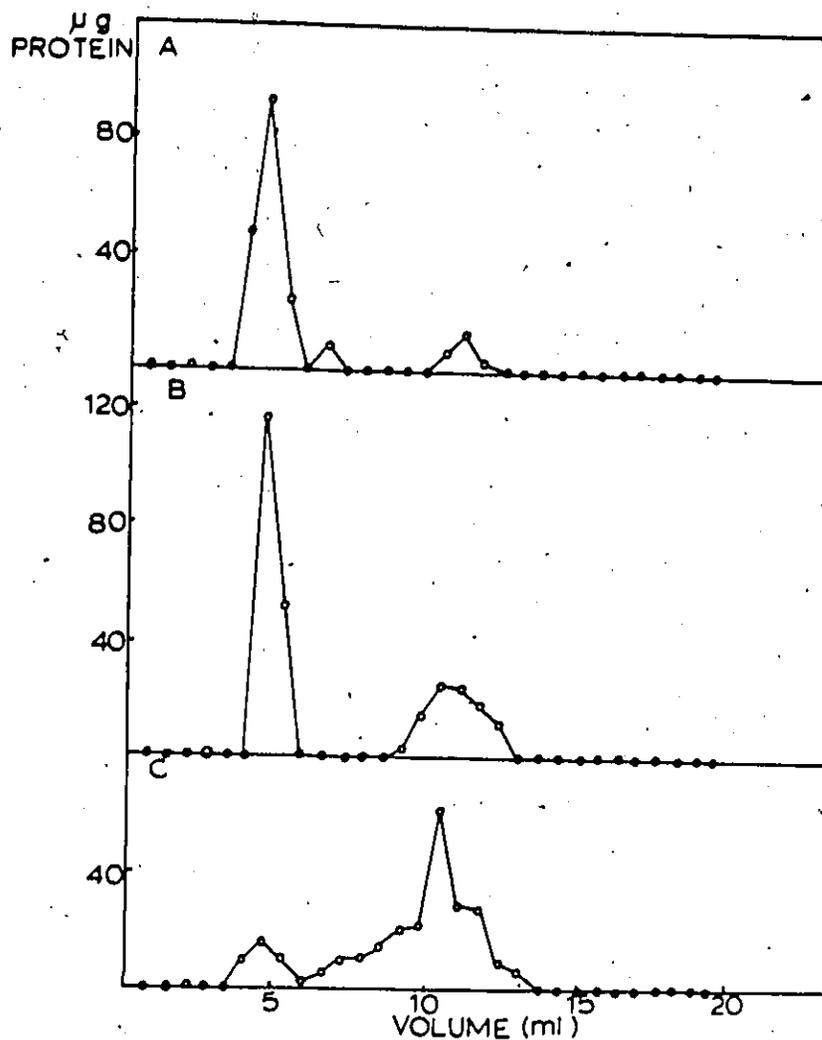


Fig. 30. Protein content of the eluate of columns of Sepharose 2B after application of various subcellular pellets of LM cells

Each pellet, 0.47 mg was incubated in 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml of the sample was applied to a column of Sepharose 2B. Each eluate fraction was concentrated to dryness by lyophilization and the protein content determined.

(A), mitochondria-rich fraction; (B), lysosome-rich fraction; (C), heavy microsomes-rich fraction.

This experiment is one of a series in which the [^{14}C]-labelled material of high molecular weight eluted at a constant volume (7.1 - 9.7) only when cellular protein was also present in that volume (Figs. 28-32).

TABLE 11
 $[^{14}\text{C}]$ -LABELLED DHFR AND μg OF CELLULAR PROTEIN
 IN THE ELUATE OF A SEPHAROSE 2B COLUMN

Eluate Volume	Sample	μg protein	$[^{14}\text{C}]$ -dpm	$[^{14}\text{C}]$ -dpm/ μg protein
3.8-4.5 ml	Mitochondria-rich	94	24	0.26
	Lysosome-rich	118	66	0.60
	Heavy Microsome-rich	16	34	2.12
7.1-7.8 ml	Mitochondria-rich	-	6	- ^a
	Lysosome-rich	-	28	- ^a
	Heavy microsome-rich	10	66	6.60
7.8-8.5 ml	Mitochondria-rich	-	11	- ^a
	Lysosome-rich	-	36	- ^a
	Heavy microsome-rich	14	71	5.07
8.5-9.1 ml	Mitochondria-rich	-	21	- ^a
	Lysosome-rich	4	52	13.00 ^b
	Heavy microsome rich	20	66	3.30

Incubations were performed as described in the legends to Fig. 29 and 30. The $[^{14}\text{C}]$ -dpm and μg of protein in each fraction of a Sepharose 2B column were calculated.

a, ratio could not be calculated since the protein was too low to measure

b, ratio is probably not correct since the protein was too low to be accurately determined.

the pellet rich in heavy microsomes (Table 11). The ratio of [^{14}C]-dpm to microsomal protein was much higher in the fractions eluting between 7.1 and 9.1 ml. The protein concentration was too low for a ratio to be calculated for these fractions in incubations which contained the mitochondria-rich or lysosome-rich pellets, but the ratio was probably also high. (Table 11).

The most likely explanation for these results is that inactive enzyme was associated to a small extent to a large amount of lysosomal and mitochondrial protein which was excluded from the gel, while there was much greater specific binding of enzyme to the microsomal fraction or the microsomal proteins which contaminated the lysosome-rich and mitochondria-rich pellets. Further support for such an explanation was obtained by incubation of DHFR with [^3H]-labelled 100,000 x g light microsomal pellet. There was a good correlation between the position of [^{14}C]-labelled material of high molecular weight and the [^3H]-labelled proteins (Fig. 31).

f) Relationship between the inactivation of [^{14}C]-labelled DHFR and the formation of [^{14}C]-labelled material of high molecular weight

If formation of the inactive [^{14}C]-labelled DHFR of high molecular weight was the result of binding of the enzyme to the subcellular membranes, then it would be expected that none of this material would be formed during incubation with proteins which did not inactivate the enzyme.

When [^{14}C]-labelled DHFR was incubated with [^3H]-labelled proteins of the 100,000 x g supernatant, a fraction which did not

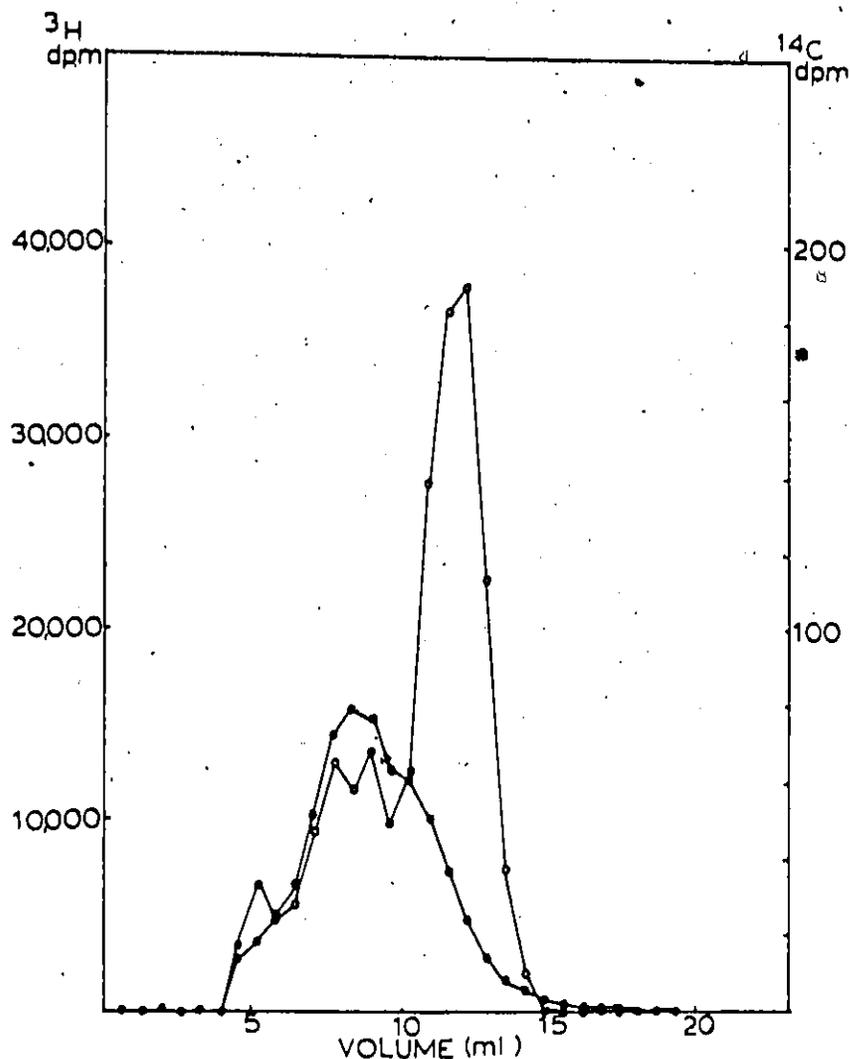


Fig. 31. Column chromatography of [^{14}C]-labelled DHFR and [^3H]-labelled proteins from the 100,000 x g pellet of LM cells on Sepharose 2B

Incubations contained 0.47 mg of protein from the 100,000 x g microsomal pellet and 2.7 μg of DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml of the incubation mixture was applied to a column of Sepharose 2B.

Open circles represent [^{14}C]-dpm; closed circles represent [^3H]-dpm.

This experiment is one of a series in which the [^{14}C]-labelled material of high molecular weight eluted at a constant volume (7.1 - 9.7) only when cellular protein was also present in that volume (Figs. 28-32).

inactivate DHFR *in vitro* (Fig. 7), little material of high molecular weight was formed (Fig. 32). The effective fractionation range of Sepharose 2B is such that the DHFR monomer was not separated from the [³H]-labelled proteins of the 100,000 x g supernatant on this column. Although it can therefore be concluded that in the presence of the 100,000 x g supernatant, enzyme of molecular weight as high as that formed in incubations containing the 27,000 x g or 100,000 x g pellets was not formed, this experiment does not show whether or not DHFR was bound to components of the 100,000 x g supernatant. If such binding did occur, then the 100,000 x g supernatant might compete with the 27,000 x g or the 100,000 x g pellets for enzyme molecules. To test this, dialyzed 100,000 x g supernatant was incubated with [¹⁴C]-labelled DHFR and 100,000 x g pellet. No decrease in the amount of [¹⁴C]-labelled material eluting in the fractions of high molecular weight could be demonstrated (Fig. 33). The supernatant used in this experiment was dialyzed in order to remove any ligands which might stabilize DHFR against inactivation. The 100,000 x g supernatant contained endogenous DHFR which might compete with [¹⁴C]-labelled enzyme for the inactivating system. The activity added with the 100,000 x g supernatant, however, was only 1.0% of the total activity in the incubation.

The effect of BSA on the formation of [¹⁴C]-labelled material of high molecular weight in the presence of 100,000 x g pellet was also investigated. BSA is another protein which did not inactivate DHFR (Fig. 6). It did not alter the amount of high molecular weight material that was formed (Fig. 33).

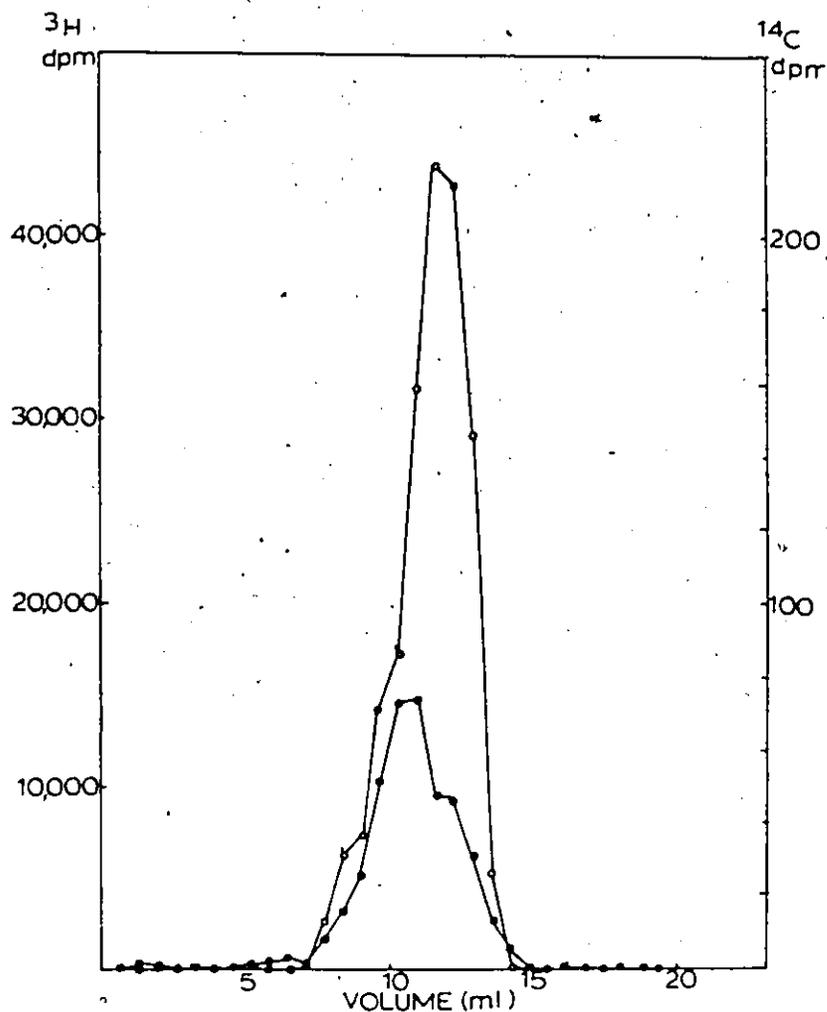


Fig. 32. Column chromatography of [³H]-labelled proteins from the 100,000 x g supernatant of LM cells and [¹⁴C]-labelled DHFR on Sepharose 2B

Incubations contained 0.47 mg of 100,000 x g supernatant protein and 2.7 μ g of DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml of the incubation mixture was applied to a column of Sepharose 2B.

Open circles represent [¹⁴C]-dpm; closed circles represent [³H]-dpm.

This experiment is one of a series in which the [¹⁴C]-labelled material of high molecular weight eluted at a constant volume (7.1 - 9.7) only when cellular protein was also present in that volume (Figs. 28-32).

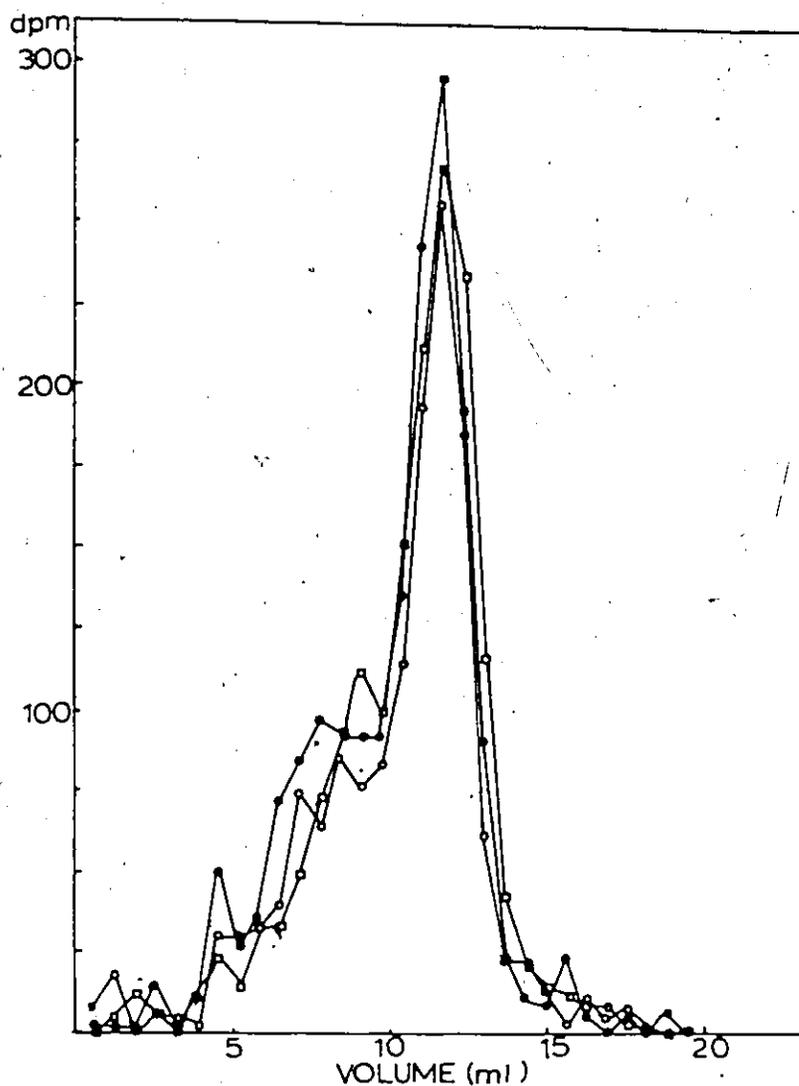


Fig. 33. Effect of BSA and dialyzed 100,000 x g supernatant protein on the amount of high molecular weight [^{14}C]-labelled material which is formed during incubation of DHFR with the 100,000 x g pellet of LM cells

Incubations contained 0.53 mg of protein from the 100,000 x g pellet of LM cells and 1.03 μg of DHFR in a final volume of 0.34 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml aliquots was passed through a column of Sepharose 2B.

No addition, \circ — \circ ; 0.53 mg of BSA present in incubation, \bullet — \bullet ; 0.49 mg of dialyzed 100,000 x g supernatant from LM cells present in incubation, \square — \square .

Similar results were obtained in two different experiments.

By increasing the amount of DHFR in the incubations, while keeping the amount of 100,000 x g pellet constant, it was shown that a greater loss of enzyme activity in absolute amounts was accompanied by an increase in the total radioactivity which eluted in the fractions of high molecular weight of a Sepharose 2B column (Fig. 34). Almost twice as much enzyme activity was lost in the incubation which contained 2.3 μ g as in the incubation which contained 1.4 μ g of DHFR. Almost twice as much radioactivity was recovered in fractions of high molecular weight in the incubation which contained 2.3 μ g of enzyme.

It has been shown that the rate of inactivation of DHFR in incubations containing the 27,000 x g pellet of LM was decreased by MTX at a concentration of 5.0×10^{-7} M (Fig. 16). MTX at a concentration of 1.4×10^{-7} M did not prevent the formation of [14 C]-labelled material of high molecular weight (Fig. 35). The effect of MTX appears to be due to stabilization of the enzyme which was recovered in the peak at an elution volume of 11 - 14 ml. While the amount of radioactivity in this volume was the same in samples incubated with and without MTX, the enzyme activity was much higher in the sample which was incubated with MTX (Fig. 35). When [14 C]-labelled DHFR was incubated in buffer alone until 86% of the activity was lost by heat inactivation all the radioactivity eluted at this volume on a Sepharose column (Fig. 36). The enzyme eluting at this volume, therefore, represents active enzyme and heat inactivated enzyme so that the effect of MTX on the rate of inactivation of DHFR may be due to stabilization of the enzyme against heat denaturation. Alternatively, stabilization of the enzyme by MTX could be due to a decreased rate of formation of some other inactive form of the enzyme which eluted in this volume.

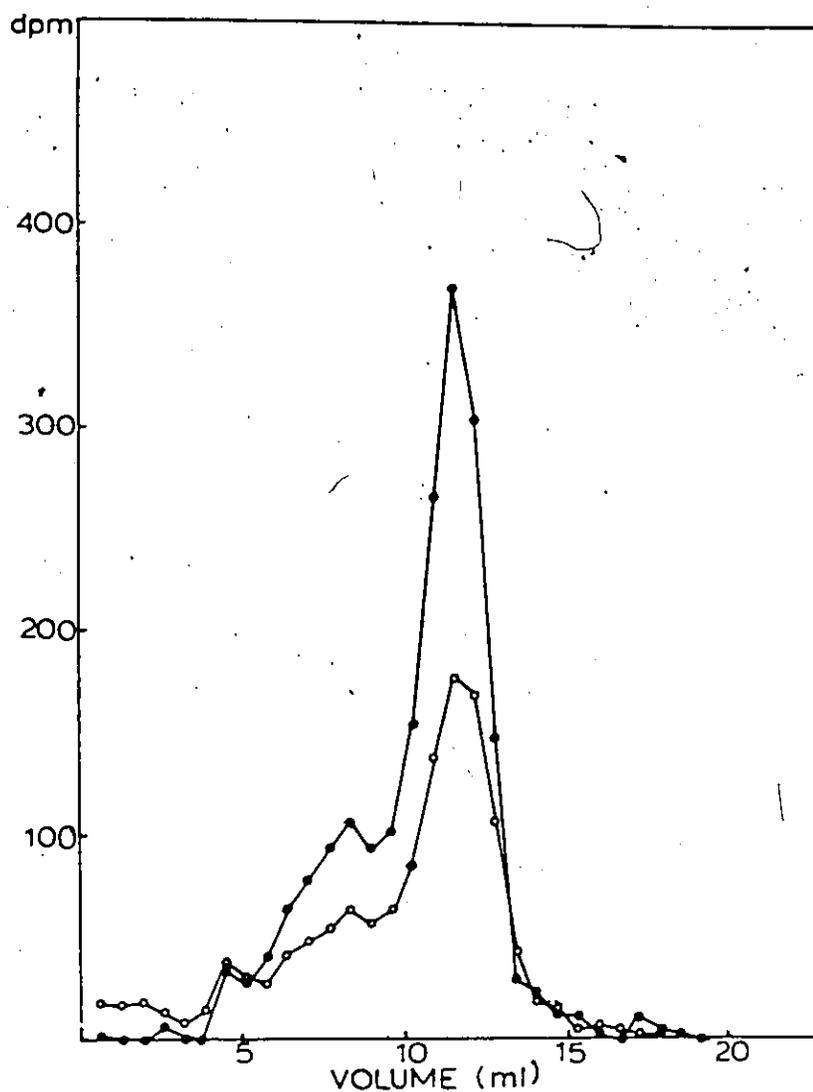


Fig. 34. Effect of increasing [^{14}C]-labelled DHFR concentration on the formation of inactive DHFR of high molecular weight

Incubations contained 0.53 mg of protein from the 100,000 x g pellet of LM cells and [^{14}C]-labelled DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml aliquots were applied to a column of Sepharose 2B.

Incubation containing 1.4 μg DHFR, \circ — \circ ; incubation containing 2.3 μg DHFR, \bullet — \bullet .

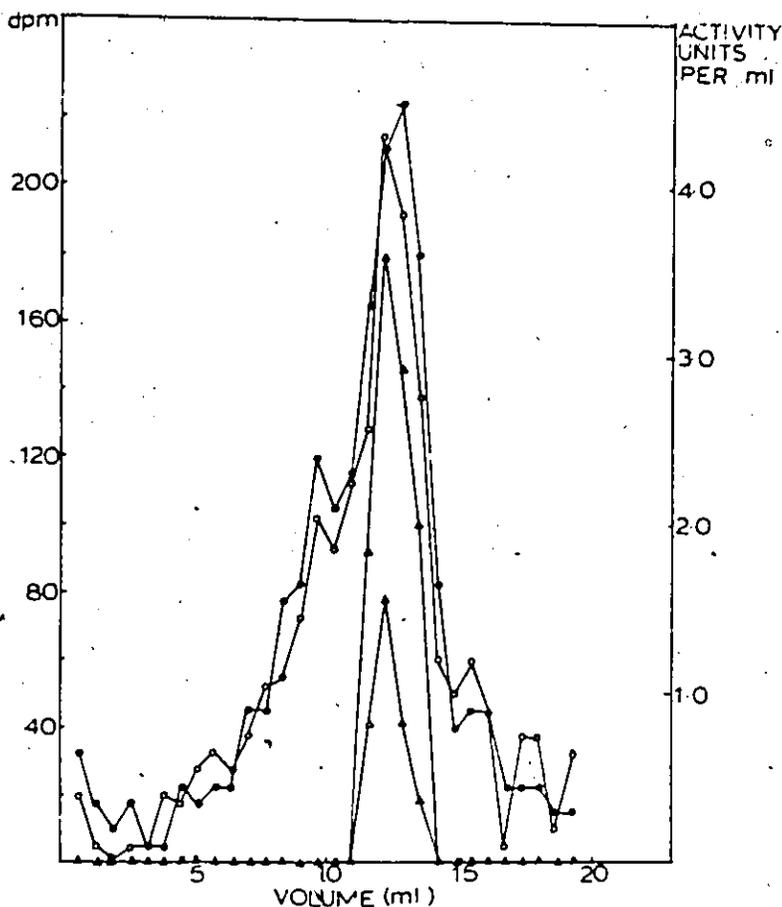


Fig. 35. Effect of MTX on the formation of [¹⁴C]-labelled DHFR of high molecular weight and on the activity of the enzyme which is recovered on a column of Sepharose 2B

Incubations contained 0.53 mg of protein from the 27,000 x g pellet of LM cells and 5.7 μ g of DHFR in a final volume of 0.39 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. MTX, where present was at a concentration of 1.4×10^{-7} M. After 2 h, 0.25 ml aliquots were applied to a column of Sepharose 2B. Enzyme activities were measured at pH 8.5.

[¹⁴C]-labelled DHFR, \circ — \circ ; DHFR activity, Δ — Δ . Open symbols represent incubations in the absence of MTX; closed symbols represent incubations in the presence of MTX.

Similar results were obtained in four different experiments.

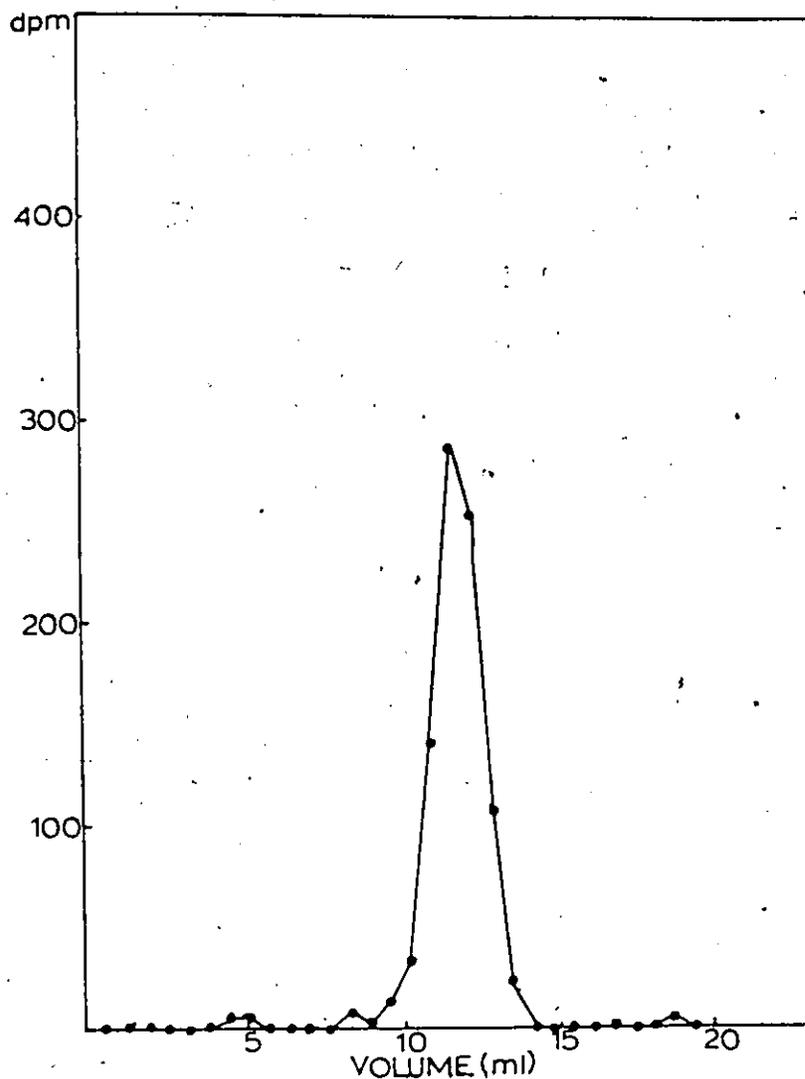


Fig. 36. Column chromatography of heat inactivated DHFR on Sepharose 2B
Incubations contained 1.03 μg of [^{14}C]-labelled DHFR in 0.28 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml was applied to a column of Sepharose 2B.

g) Inactivation of DHFR prior to the formation of
material of high molecular weight

It has been suggested that denaturation is an early step in protein degradation (Coffey and deDuve, 1968, Li and Knox, 1972; Knowles and Ballard, 1976). It was therefore of interest to see whether more [^{14}C]-labelled material of high molecular weight was formed during incubation of denatured DHFR with the 100,000 x g pellet than during incubation of active enzyme with the pellet. Dihydrofolate reductase labelled with [^{14}C] was incubated at 37°C until 86% of the activity was lost. The 100,000 x g pellet was then added and a sample of the incubation immediately passed through a column of Sepharose 2B. Little DHFR of high molecular weight was formed (Fig. 37). Since binding may be heat dependent, incubation at 37°C was continued for 1 h after addition of the 100,000 x g pellet, but the amount of material of high molecular weight in the column eluate did not increase (Fig. 37).

The formation of [^{14}C]-labelled material of high molecular weight may represent enzyme which was inactivated by some mechanism other than binding to microsomal membranes. When DHFR was incubated with boiled 100,000 x g pellet, activity was lost rapidly (Fig. 8). Elution of the incubation mixture on a column of Sepharose 2B showed that much of the [^{14}C]-labelled enzyme was present in the void volume (Fig. 38) where the proteins of the boiled pellet would be expected to elute. This material was inactive, but activity was restored by treatment with urea and mercaptoethanol (Table 12). This enzyme activity per dpm was compared with enzyme activity per dpm after the same

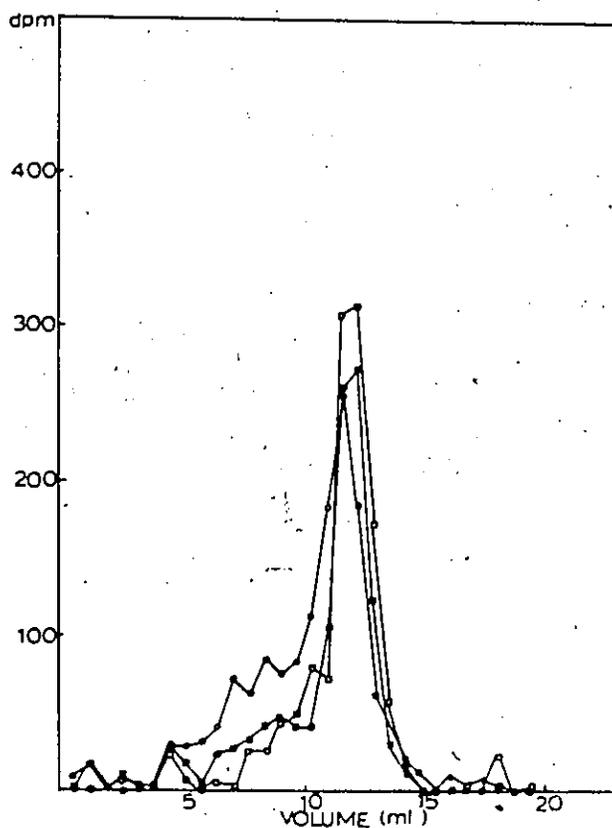


Fig. 37. Effect of previous inactivation on the formation of [^{14}C]-labelled material of high molecular weight in the presence of protein from the 100,000 x g pellet of LM cells

Incubations contained 1.03 μg of [^{14}C]-labelled DHFR in 0.28 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h at 37°C, 0.02 ml of the same buffer containing 0.53 mg of protein from the 100,000 x g pellet was added. An aliquot of 0.25 ml of one sample was immediately applied to a column of Sepharose 2B while another sample was incubated at 37°C for a further 1 h before being applied to a Sepharose 2B column.

A control incubation contained 1.03 μg of [^{14}C]-labelled DHFR and 0.53 mg of protein from the 100,000 x g pellet in 0.28 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.02 ml of the same buffer was added and an aliquot of 0.25 ml passed through a column of Sepharose 2B.

[^{14}C]-labelled DHFR incubated with 100,000 x g pellet protein, \circ — \circ ; [^{14}C]-labelled DHFR incubated alone, 100,000 x g pellet protein added and sample applied immediately to Sepharose 2B column, \square — \square ; [^{14}C]-labelled DHFR incubated alone, 100,000 x g pellet protein added and sample incubated for a further 1 h, \blacksquare — \blacksquare .

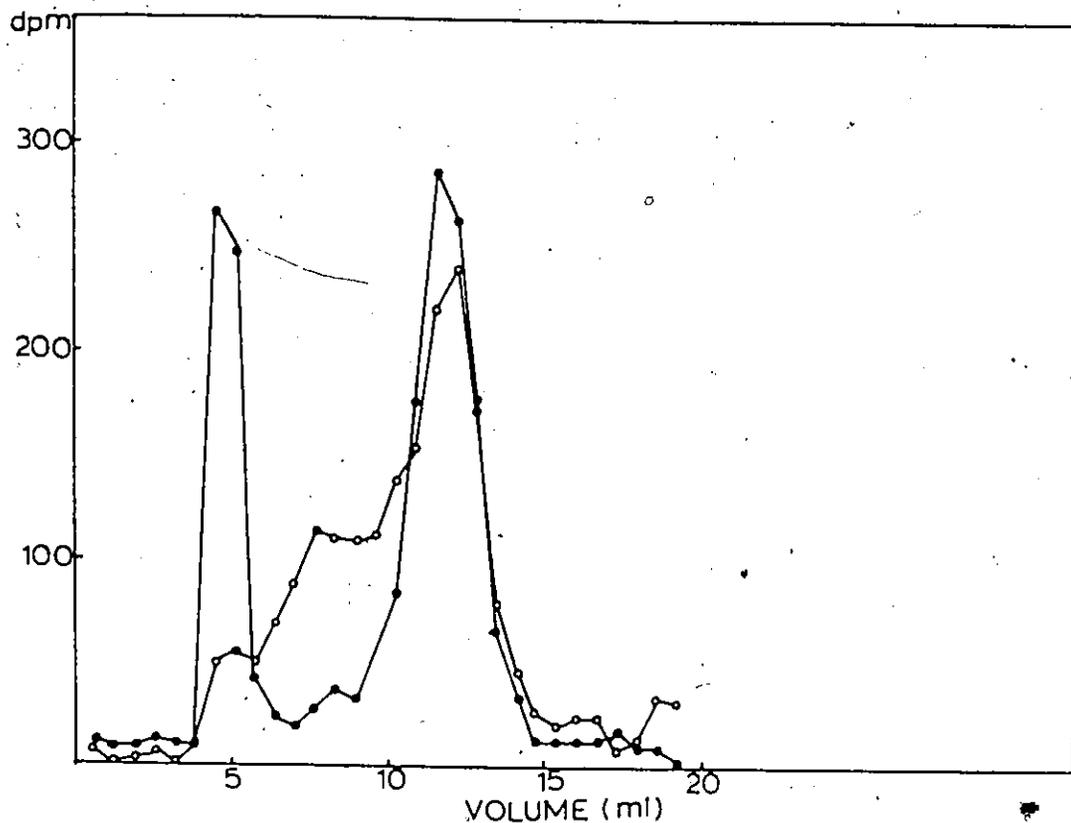


Fig. 38. Formation of [^{14}C]-labelled material of high molecular weight in incubations containing boiled or unboiled protein from the 100,000 \times g pellet

Incubations contained 0.47 mg of protein from the 100,000 \times g pellet of LM cells and 5.0 μg of DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h aliquots of 0.25 ml were applied to columns of Sepharose 2B.

After incubation with 100,000 \times g pellet, o—o; after incubation with boiled 100,000 \times g pellet, ●—●.

TABLE 12

REACTIVATION OF [¹⁴C]-LABELLED DHFR IN FRACTIONS OF HIGH MOLECULAR WEIGHT AFTER INCUBATION OF DHFR WITH BOILED OR UNBOILED PROTEINS FROM THE 100,000 x g PELLET OF LM CELLS

Elution volume	Subcellular fraction in incubation	Initial activity (units/ml)	Activity after treatment with urea and mercaptoethanol (units/ml)
3.9 - 4.5 ml	100,000 x g pellet	0.00	0.25
3.9 - 4.5 ml	boiled 100,000 x g pellet	0.00	1.74
8.5 - 9.1 ml	100,000 x g pellet	0.00	0.65
8.5 - 9.1 ml	boiled 100,000 x g pellet	0.00	0.11

Incubations contained 0.47 mg of protein from the 100,000 x g pellet of LM cells and 5.0 µg of DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, aliquots of 0.25 ml were applied to columns of Sepharose 2B. Fractions eluting from the column were assayed for enzyme activity before and after incubation with 8M urea, 1.2 mM mercaptoethanol at room temperature for 60 min.

Results given represent the mean of duplicate incubations.

This experiment is one of a series in which DHFR, initially inactivated by subcellular membrane fractions, was reactivated by urea (Table 10, 12-15).

treatment of enzyme present in the fractions containing proteins of the unboiled pellet. The enzyme associated with membranes of the boiled and unboiled fractions was reactivated to the same extent. This result suggests that previous alteration of DHFR, by a heat sensitive process, was not necessary for association of the enzyme with microsomal membranes since inactivation and association also occurred with boiled microsomal membranes.

h) Dissociation of the [^{14}C]-labelled material of high molecular weight

Inactive [^{14}C]-labelled DHFR in fractions of high molecular weight was not dissociated by dilution. There was no difference in the distribution of radioactivity in the fractions of Sepharose 2B columns between a sample which was incubated with 27,000 x g pellet and applied to Sepharose 2B immediately and one which was diluted three times after incubation, left at 4°C overnight and then applied to Sepharose 2B (Fig. 39).

MTX and NADPH did not cause dissociation of the material of high molecular weight. After incubation of DHFR with the 100,000 x g pellet, MTX and NADPH were added and the sample left at 4°C overnight before being applied to a column of Sepharose 2B. The amount of radioactivity eluting in the high molecular weight fractions was not reduced as compared to a control which had no MTX or NADPH (Fig. 40).

Experiments have already been presented which suggest that the formation of [^{14}C]-labelled material of high molecular weight was reversed and the enzyme reactivated by urea and mercaptoethanol (Fig. 25,

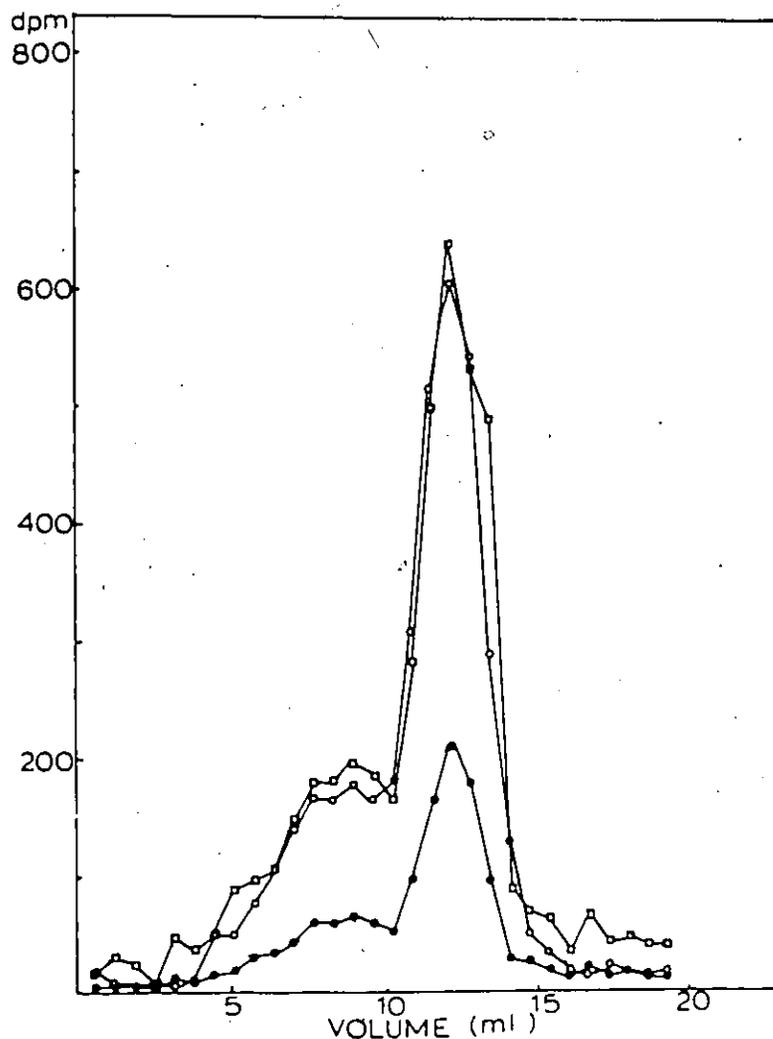


Fig. 39. Effect of dilution on the [^{14}C]-labelled material of high molecular weight

Incubations contained 7.75 mg of 27,000 x g pellet protein of LM cells and 16.44 μg of [^{14}C]-labelled DHFR in a final volume of 0.6 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 at 37°C. After 2 h, the sample was centrifuged at 27,000 x g for 20 min and a 0.25 ml aliquot of the 27,000 x g supernatant applied to a column of Sepharose 2B. Another portion of the 27,000 x g supernatant was diluted three times in the same buffer and left overnight at 4°C. A 0.25 ml aliquot of the diluted sample was then applied to a Sepharose 2B column.

Undiluted sample, \circ — \circ ; diluted sample, \bullet — \bullet ; [^{14}C]-dpm of the diluted sample corrected for the dilution factor, \square — \square .

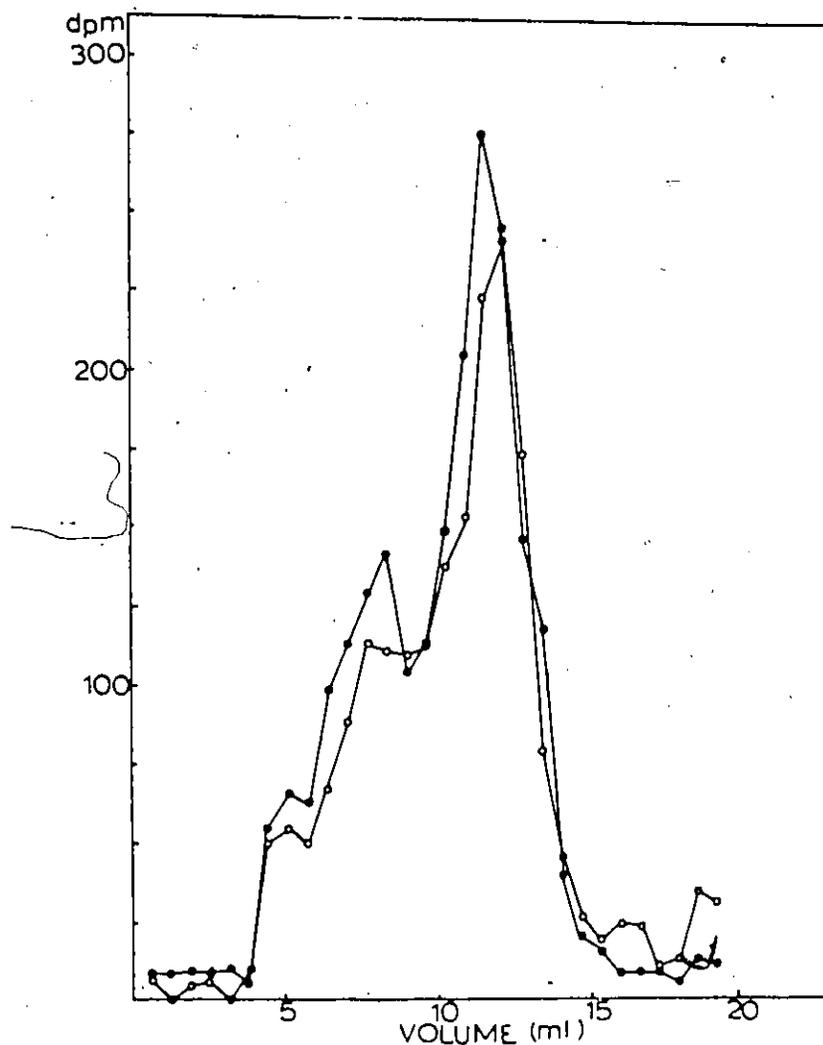


Fig. 40. Effect of MTX and NADPH on the formation of [^{14}C]-labelled material of high molecular weight

Incubations contained 0.47 mg of protein from the 100,000 x g pellet of LM cells and 5.0 μg of DHFR in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 17 μl of the same buffer or 17 μl of the buffer containing NADPH and MTX, to give a final concentration of 10^{-6}M NADPH and $3 \times 10^{-6}\text{M}$ MTX, were added to the incubations. The control sample without NADPH and MTX was immediately passed through a column of Sepharose 2B, previously equilibrated with 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The sample containing NADPH and MTX was left at 4°C overnight and then passed through a column of Sepharose 2B, previously equilibrated with 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 containing 10^{-6}M NADPH and $3 \times 10^{-6}\text{M}$ MTX.

Control, \circ — \circ ; sample containing NADPH and $3 \times 10^{-6}\text{M}$ MTX, \bullet — \bullet .

Table 10). When the inactive high molecular weight material was separated from the enzyme of low molecular weight by column chromatography on Sepharose 2B and urea and mercaptoethanol added, the previously inactive material of high molecular weight became active. In contrast, the previously active enzyme of lower molecular weight was inactivated (Table 13).

To determine whether urea or mercaptoethanol was responsible for the reactivation observed, DHFR labelled with [^{14}C] was incubated with the 100,000 x g pellet and after 2 h aliquots of the incubations treated with urea or with mercaptoethanol. Enzyme activity was not restored by treatment with mercaptoethanol. Treatment with urea re-activated the enzyme (Table 14).

Since urea and mercaptoethanol activate only the inactive material of high molecular weight (Table 13), treatment of incubations with urea and mercaptoethanol should provide a method for detecting the production of such inactive material without using [^{14}C]-labelled enzyme. A comparison of the inactivation and reactivation of unlabelled and [^{14}C]-labelled DHFR suggested that both forms of the enzyme are inactivated by the formation of high molecular weight material (Table 15).

1) Binding of MTX to the enzyme in fractions of high molecular weight

It has been shown that at a concentration of $1.4 \times 10^{-7}\text{M}$ MTX did not prevent the formation of inactive material of high molecular weight (Fig. 35). Since it was of interest to see whether MTX binds to this form of the enzyme, [^3H]-labelled MTX was used and the distribution of this isotope in the eluate of Sepharose 2B columns determined. A

TABLE 13

REACTIVATION OF DHFR WITH UREA AND MERCAPTOETHANOL

Elution volume	Subcellular fraction in incubation	Initial activity (units/ml)	Activity after treatment with urea and mercaptoethanol (units/ml)
3.9 - 4.5 ml	mitochondria-rich	0.12	0.24
3.9 - 4.5 ml	lysosome-rich	0.20	0.22
3.9 - 4.5 ml	microsome-rich	0.00	0.32
3.9 - 4.5 ml	light microsome-rich	0.00	0.07
8.5 - 9.1 ml	mitochondria-rich	0.11	0.07
8.5 - 9.1 ml	lysosome-rich	0.14	0.20
8.5 - 9.1 ml	microsome-rich	0.15	0.45
8.5 - 9.1 ml	light microsome-rich	0.03	0.34
11.7 - 12.4 ml	mitochondria-rich	4.54	0.64
11.7 - 12.4 ml	lysosome-rich	3.99	0.61
11.7 - 12.4 ml	microsome-rich	2.78	0.44
11.7 - 12.4 ml	light microsome-rich	1.60	0.71

Incubations were carried out, the products passed through Sepharose 2B columns as described in the legends to Fig. 29 and 31. Samples eluting between 3.9 ml and 4.5 ml, 8.5 and 9.1 ml, 11.7 and 12.4 ml were brought to 8M urea and 1.2 mM mercaptoethanol and after 60 min at room temperature DHFR activity was assayed.

Results given represent the mean of duplicate incubations.

This experiment is one of a series in which DHFR, initially inactivated by subcellular membrane fractions, was reactivated by urea (Table 10, 12-15).

TABLE 14

REACTIVATION OF DHFR BY UREA OR BY MERCAPTOETHANOL

Incubation	% of original activity remaining after 2 h incubation	% of original activity remaining after 2 h incubation and subsequent treatment with:		
		urea + mercaptoethanol	urea only	mercaptoethanol only
[¹⁴ C]-labelled DHFR + 100,000 x g pellet	6.98	41.00	36.76	9.20
[¹⁴ C]-labelled DHFR in buffer only	11.51	5.16	2.10	7.04

Incubations contained 0.53 mg of protein from the 100,000 x g pellet of LM cells and 1.8 μ g of [¹⁴C]-labelled DHFR in a final volume of 0.39 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4.

After 2 h, aliquots were incubated with 8 M urea or 1.2 mM mercaptoethanol for 60 min at room temperature. Incubations containing mercaptoethanol without urea were diluted with water to compensate for the increased volume produced by urea in the other incubations.

Results given represent the mean of duplicate incubations.

This experiment is one of a series in which DHFR, initially inactivated by subcellular membrane fractions, was reactivated by urea (Table 10, 12-15).

TABLE 15

REACTIVATION OF UNLABELLED DHFR WITH UREA AND MERCAPTOETHANOL

Incubation	% of original activity remaining after 2 h incubation	% of original activity remaining after 2 h incubation and subsequent treatment with urea and mercaptoethanol
[¹⁴ C]-labelled DHFR + 100,000 x g pellet	6.98	41.00
[¹⁴ C]-labelled DHFR in buffer	11.51	5.16
Unlabelled DHFR + 100,000 x g pellet	1.23	65.15
Unlabelled DHFR + buffer	9.82	2.94

Incubations contained 0.53 mg of protein from the 100,000 x g pellet of LM cells and 1.44 µg of unlabelled DHFR or 1.8 µg of [¹⁴C]-labelled DHFR in a final volume of 0.39 ml, 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4. After 2 h, aliquots were incubated with 8 M urea, 1.2 mM mercaptoethanol for 60 min at room temperature.

Results given represent the mean of duplicate incubations.

This experiment is one of a series in which DHFR, initially inactivated by subcellular membrane fractions, was reactivated by urea (Table 10, 12-15).

single peak of [³H]-MTX was found (Fig. 41), indicating that MTX, at a concentration of 1.4×10^{-7} M did not bind to the high molecular form of DHFR. No information could be obtained concerning the binding of MTX to the heat inactivated enzyme since free MTX eluted in the same volume as this form of the enzyme. Since binding to the enzyme of high molecular weight may have occurred but the MTX displaced during the column chromatography, another method to see whether MTX bound to the inactive material of high molecular weight was used. After incubation of [¹⁴C]-labelled DHFR with the 100,000 x g pellet, a sample was applied to a column of Sepharose-MTX. The column was eluted by the method used in the preparation of DHFR by affinity chromatography (Gauldie and Hillcoat, 1972). Some active enzyme was recovered in the third buffer but most of the [¹⁴C]-labelled material passed straight through the column and was recovered as inactive enzyme (Fig. 42), showing that the inactive enzyme did not bind to MTX on the Sepharose-MTX column.

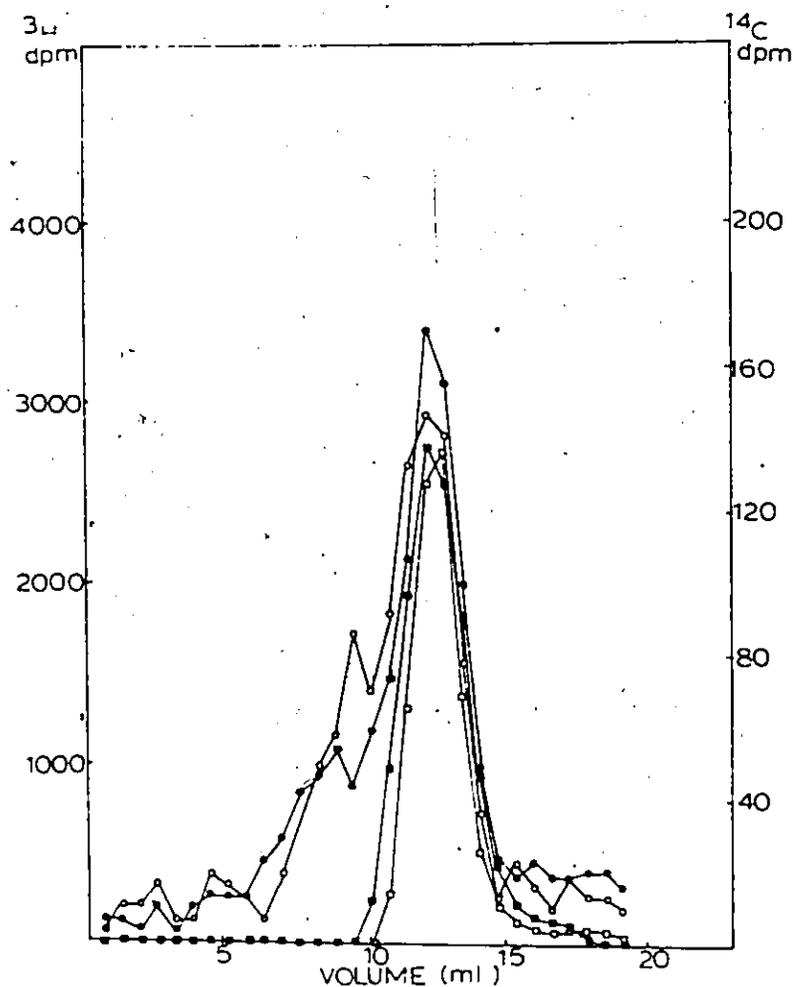


Fig. 41. Column chromatography of [¹⁴C]-labelled DHFR and [³H]-labelled MTX after incubation of DhFR, MTX and the 27,000 x g pellet of LM cells

Incubations contained 0.53 mg of protein from the 27,000 x g pellet and 4.8 μ g of [¹⁴C]-labelled DHFR in a final volume of 0.33 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, for 2 h at 37°C. Where present, [³H]-labelled MTX was at a concentration of 1.4×10^{-8} M. The incubations were terminated by centrifuging at 27,000 x g for 20 min and 0.25 ml of the 27,000 x g supernatant applied to a column of Sepharose 2B. The elution volume of free MTX was determined with 0.25 ml of 1.4×10^{-8} M MTX.

Open circles show [¹⁴C]-labelled DHFR in a sample incubated without MTX. Closed circles show the [¹⁴C]-labelled DHFR in a sample incubated with MTX.

[³H]-labelled MTX in incubation containing 27,000 x g pellet and DHFR, \square — \square ; [³H]-labelled MTX only, \blacksquare — \blacksquare .

Similar results were obtained in four different experiments.

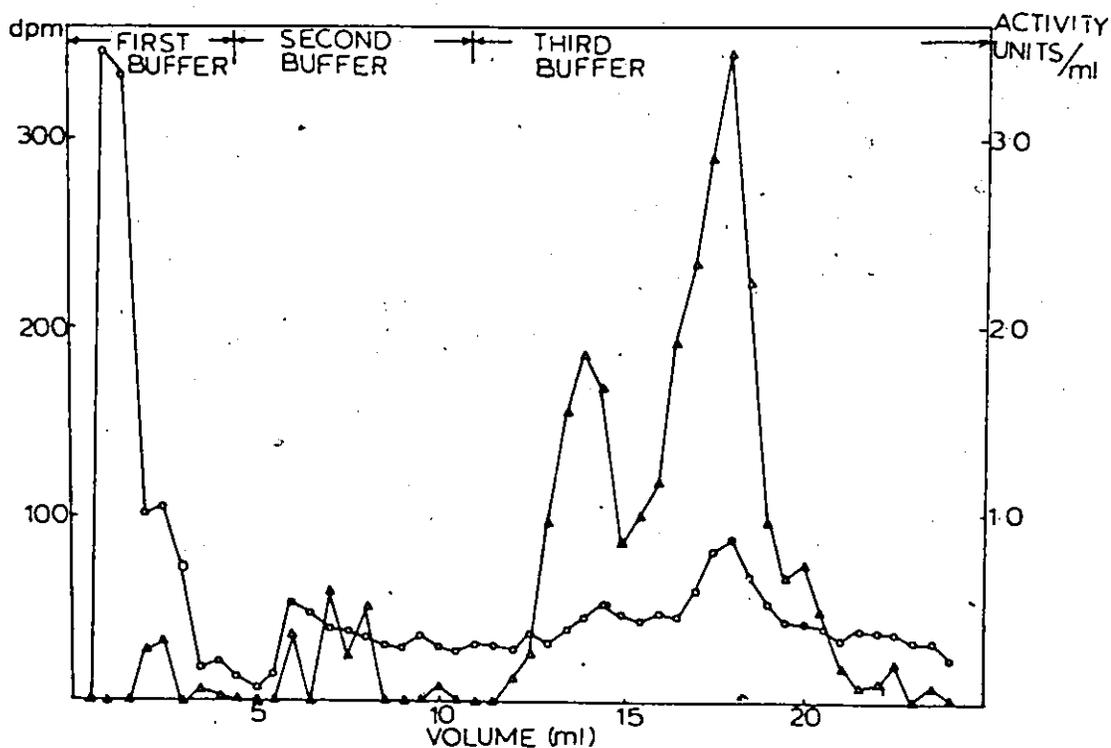


Fig. 42. Affinity chromatography of inactivated [^{14}C]-labelled DHFR on Sepharose-MTX

Incubations contained 0.47 mg of protein from the 100,000 x g pellet of LM cells and 5.0 μg of DHFR in 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 2 h, 0.25 ml was applied to a column of Sepharose-MTX (1 x 1.5 cm) previously equilibrated with 0.1M Tris-HCl, pH 7.5 containing $1 \times 10^{-5}\text{M}$ NADPH. The column was eluted with the buffers used in the purification of DHFR by affinity chromatography (2.2.4).

[^{14}C]-labelled DHFR, \circ — \circ ; enzyme activity Δ — Δ .

Similar results were obtained in two different experiments.

4. DISCUSSION

4.1. PROTEOLYSIS IN CELL-FREE SYSTEMS

Intracellular proteins are degraded to their constituent amino acids in intact cells (Swick, 1958), yet no significant loss of amino acids either from proteins of HTC cells or from purified DHFR during incubations *in vitro* at neutral pH could be demonstrated in this study.

4.1.1. HTC cell proteins

The production of acid-soluble material by the total homogenate of HTC cells was very low, less than 0.3% an hour (Table 4,5) and of this only 0.05% of the proteins were degraded, per hour, by a heat sensitive process (Table 5). This was much lower than the rate of 3% an hour which was observed in intact HTC cells (Hershko and Tomkins, 1971; Gelehrter and Emanuel, 1974; Hillcoat, personal communication). The homogenate in these incubations was 12 times more dilute than in intact cells. When this dilution factor was taken into account, assuming the process to be concentration dependent, the rate was still much lower in the total homogenate than in intact cells.

The rate of production of acid-soluble material by homogenates of HTC cells fell to less than 0.2% an hour when intact cells and nuclei were removed by centrifugation at 600 x g. When the 27,000 x g pellet was removed, negligible acid-soluble material was produced from the remaining supernatant. This suggests either that membrane proteins were

degraded more rapidly than soluble proteins or that the proteases which produced the acid-soluble material were in the 27,000 x g pellet. Since the 27,000 x g pellet contains lysosomes, mitochondria and microsomes, each of which contain proteases (deDuve and Wattiaux, 1966; Løvaas, 1974; Bohley, 1971), the latter explanation seems the most likely. The possibility that proteins of different fractions compete for degradation with labelled proteins to different extents is unlikely since each fraction was labelled to a similar extent (Table 4). The results presented in Table 3 indicate that this pellet does indeed contain proteases which degrade the proteins from the 27,000 x g supernatant at a slow rate. The 27,000 x g pellet in these incubations was four times more dilute than in intact cells. Allowing for this factor the rate of production of acid-soluble material in these incubations was lower than in the intact cell.

When incubations were performed at acidic pH, degradation was much more rapid (Table 2). Although the 27,000 x g pellet in this experiment was from a different cell line than in the experiments just discussed, the production of acid-soluble material appeared to depend on the low pH rather than the different source of the pellet, since in incubations containing the 27,000 x g pellet from LM cells at neutral pH, little acid-soluble material was produced (Table 1).

The [³H]-labelled proteins from HTC cells which were used in these experiments represented mainly those proteins which are rapidly degraded in intact cells (Arias et al., 1969). Other proteins were labelled with [¹⁴C]-leucine in such a way that they represented mainly those proteins which are degraded more slowly in intact cells (Arias et al., 1969). The rate of production of acid-soluble material by the 27,000 x g pellet of LM cells at pH 5.0 was greater from the [³H]-labelled proteins which have short half-lives in intact cells than from

the [^{14}C]-labelled proteins which have longer half-lives in intact cells (Table 2). As discussed in the Introduction, a correlation between the rate of degradation of proteins in the presence of lysosomes and their rate of degradation *in vivo* has been shown by other groups also (e.g. Segal et al., 1974; Dean, 1975). These authors interpreted their results as showing that lysosomes are responsible for degrading proteins *in vivo* and that the rate of degradation *in vivo* depends on the susceptibility of proteins to lysosomal enzymes (Segal et al.; 1974; Dean, 1975). Such a correlation does not necessarily mean that the intralysosomal proteases are the ones which degrade proteins in intact cells, however, since Goldberg has shown a similar correlation between the rate of degradation of proteins from *E. coli* by trypsin or pronase and their rate of degradation *in vivo* (Goldberg, 1972). These enzymes are not known to occur in lysosomes. A correlation between the rate of degradation of proteins of rat liver *in vivo* and their rate of degradation by trypsin and chymotrypsin (Bond, 1971) and by pronase (Dice et al., 1973) has also been shown. The incubations used by Segal et al., (1974) and by Dean (1975) contained Triton X-100, so the lysosomes were disrupted. The pH was in the acidic range. Similarly, in the experiments described above, the pH was acidic. At this pH, lysosomes were less intact than at neutral pH, since 37% of the cathepsin D was soluble at the beginning of the incubations. Thus, at acidic pH [^3H]-labelled proteins from HTC cells were most likely degraded by proteases which leaked from the lysosomes. Evidence for this was that degradation increased after sonication of the 27,000 x g pellet (Table 2). While these results do not, therefore, confirm that lysosomes are responsible for degrading intracellular proteins in intact cells, they

do show that lysosomes contain proteases which can degrade cellular proteins if they come into contact with them at a suitable pH. Since the internal pH of lysosomes is acidic (deDuve and Wattiaux, 1966) it is therefore probable that lysosomal proteases degrade intracellular proteins in intact cells if these proteins enter lysosomes.

More convincing evidence that lysosomes are involved in degradation *in vivo* would be the demonstration that lysosomes degrade intracellular proteins during incubation *in vitro*, at neutral pH. As already mentioned, however, the rate of degradation at this pH was very much lower than in the intact cells (Table 3). The small amount of degradation which was produced in the presence of the 27,000 x g pellet could represent the *in vivo* degrading system which does not function as rapidly under the incubation conditions used in these experiments. A possible reason is that proteins are normally degraded inside lysosomes but under these conditions they cannot enter the lysosomes. Since the isotonic sucrose-tris buffer is a solution of low ionic strength in which interactions, necessary for proteins to enter lysosomes, may not occur, PBS, a buffer of greater ionic strength was used in incubations at neutral pH. The production of acid-soluble material from labelled proteins of HTC cells did not increase (Table 1). Auricchio et al., (1972) have reported that tyrosine transaminase is inactivated in homogenates of rat liver at neutral pH, only when special care was taken to maintain the lysosomes intact. The reason proteins were not degraded in broken cells, in the studies reported here, may be that lysosomes were disrupted during homogenization. The cells, however, were broken gently in a Dounce

homogenizer, using a loose fitted pestle, and the addition of EDTA, which stabilizes lysosomes *in vitro* (Davidson, 1975; Chvapil, 1972), did not increase the production of acid-soluble material (Table 5).

As discussed in the Introduction, lysosomes could be involved in degrading proteins in the intact cell either by uptake of proteins into lysosomes or by leakage of lysosomal proteases which have some activity at neutral pH. If degradation were the result of proteolysis by enzymes which leaked from lysosomes, it would be expected that sonication of the 27,000 x g pellet would increase the rate of degradation *in vitro*. Since little degradation was produced by sonicated pellet at neutral pH (Table 1), it seems unlikely that leakage of lysosomal proteases would play a role in degradation *in vivo*.

Since sonication of the 27,000 x g pellet neither increased nor decreased the rate of degradation which was observed *in vitro*, it is possible that this small amount of degradation was not produced by intralysosomal proteases. This degradation could have been produced by proteases on the outer surfaces of lysosomal or mitochondrial membranes or by microsomal proteases. If this were so then it could represent limited cleavage of proteins such as that described by Katunuma's group (1.3.2.). Katunuma suggests that cleavage by non-lysosomal enzymes occurs before a protein is totally degraded to its constituent amino acids by other proteases. When polyacrylamide-SDS gel electrophoresis was used to measure the molecular weights of proteins, no changes could be shown. This technique is probably not sensitive enough to detect the changes in molecular weight which would result from the very low rate of production of acid-soluble material by the 27,000 x g pellet if these amino acids

were removed from the ends of the molecules. If internal peptide bonds were broken, however, so that a protein was cleaved into more than one protein of lower molecular weights, such changes would probably have been detected.

These results show that while the 27,000 x g pellet contains proteases which can degrade intracellular proteins at acidic pH, the pH inside lysosomes, very little degradation occurred at neutral pH. The small amount that did may have been produced by membrane bound enzymes rather than by intralysosomal enzymes. Whether or not it represents a protein degrading system that functions *in vivo* is not known. Whatever the mechanism of the degradation observed in these incubations, at neutral pH, it was much lower than the rate observed in intact cells. This indicates that the incubation medium was not suitable, that component(s) of the degrading system were very unstable or that intact cells are required for protein degradation to continue. The observation that most of the degrading activity was lost simply by breaking the cells is consistent with reports that acid-soluble material was produced much more rapidly in rat liver slices than in homogenate (Simpson, 1953).

4.1.2. Dihydrofolate Reductase

DHFR was rapidly inactivated by the 27,000 x g pellet of LM cells. This pellet consists of mitochondria, lysosomes and microsomes, each of which contain proteases (deDuve and Wattiaux, 1966; Løvaas, 1974; Bohley, 1971). It was also inactivated by the 100,000 x g pellet which contained mainly microsomes (endoplasmic reticulum and Golgi apparatus) but which

was also contaminated with some lysosomes and mitochondria as judged by marker enzymes (Table 7). Evidence in the literature suggests that lysosomes are a likely site of degradation of intracellular proteins (1.3.1.), so inactivation of DHFR could have been caused by leakage of proteases from these organelles. Cathepsin D, a lysosomal protease which has been implicated in protein degradation (Dean, 1975b) has an optimum pH of 3.0 - 3.5 (Barrett, 1972). If this protease were responsible for degrading DHFR outside the lysosomes, it would have to act at pH 7.4. However, it did not inactivate DHFR at this pH (Fig. 11). Moreover, if inactivation of DHFR was due to proteolysis by enzymes which leaked from organelles during incubation, then the inactivation in the presence of a fixed amount of protein would increase when DHFR was incubated with the 27,000 x g supernatant obtained after incubation of the 27,000 x g pellet or when DHFR was incubated with sonicated 27,000 x g pellet. Similarly, if inactivation was the result of proteolysis only by enzymes confined to organelles, sonication of the pellet, or incubation with only those proteins which were solubilized from the pellet, would decrease the rate of inactivation. Neither sonication nor incubation with only those components which were solubilized from the 27,000 x g pellet altered the rate of inactivation of DHFR (Fig. 12, 13). This suggests that inactivation was not produced by a limited number of proteases which leaked from the pellet nor was it produced by proteolysis inside lysosomes or mitochondria.

Direct evidence that inactivation did not result from extensive proteolysis was provided by using [¹⁴C]-labelled DHFR. When labelled enzyme was incubated with the 27,000 x g pellet there was no increased production of acid-soluble material compared to incubations which contained

boiled 27,000 x g pellet (Table 8), nor was there any change in the molecular weight of the enzyme as detected by polyacrylamide-SDS gel electrophoresis (Fig. 22). No change in molecular weight of the enzyme could be detected after inactivation in the presence of the total 600 x g homogenate (Fig. 21). While showing that inactivation was not the result of extensive proteolysis, these results do not show whether or not more limited proteolysis occurred. Kominami et al., (1975) have shown that the N-terminal amino acid of ornithine transaminase was different after inactivation, although no change in the molecular weight of the enzyme could be detected. Inactivation of DHFR may also result from cleavage of peptide bond(s) close to the end of the molecule since such small changes in molecular weight would not be detected by polyacrylamide-SDS gel electrophoresis. This type of limited cleavage might produce a change in the charge of the enzyme. No such change could be shown by polyacrylamide gel electrophoresis (Fig. 23).

Better evidence that proteolysis had not occurred may be obtained if the amino acid composition of the inactive enzyme could be analyzed. Inactive DHFR could not be easily purified, however, since it did not bind to MTX on a column of Sepharose to which MTX had been bound (Fig. 42). The observation that inactive DHFR could be separated from active DHFR by gel filtration through Sepharose 2B (Fig. 26) and that the inactive material was reactivated by treatment with urea (Table 13, 14) suggests that it may be possible to purify the inactivated enzyme by separating it from active enzyme on a column of Sepharose 2B and then applying it to a column of Sepharose-MTX in the presence of urea. Comparison of the amino acid composition and of the N-terminal and C-terminal amino acids of

active and inactive DHFR would provide better evidence that inactivation was not accompanied by cleavage of peptide bonds.

It has been suggested that inactivation of enzymes is an initial step in degradation and proteins are only recognized by the degrading system when they are inactive (Katunuma et al., 1972; Katunuma, 1973, Katunuma, 1975). If this is so, then inactivation and degradation may occur in separate parts of the cell. The absence of the degrading system could explain why inactivation of the enzyme by the 27,000 x g pellet was not accompanied by degradation in the experiments presented here. To test this, enzyme was inactivated by incubation with the 27,000 x g pellet and the 27,000 x g supernatant was then added. In this way, inactive enzyme was present in incubations which contained all the cellular components except those which had been removed by centrifugation at 600 x g. No acid-soluble material was produced (Table 9).

The results obtained with this enzyme were, therefore, similar to those obtained when proteins from HTC cells were used as the protein substrate. Under no conditions could significant degradation be demonstrated in cell-free incubations, at neutral pH.

4.2. BINDING OF PROTEINS TO SUBCELLULAR MEMBRANES

4.2.1 Association of inactive DHFR with high molecular weight material

Two lines of evidence suggest that inactivation of DHFR was accompanied by the formation of material of high molecular weight.

(i) Polyacrylamide gel electrophoresis showed that the 27,000 x g pellet catalyzed the formation of material which included [^{14}C]-labelled enzyme and which did not enter 7% polyacrylamide gels (Fig. 23, 24). Reactivation of the enzyme with urea and mercaptoethanol (Table 10) and the conversion of the enzyme into a form which entered gels in the presence of urea (Fig. 25) suggested that the material which was recovered on top of the gels in the absence of urea was an inactive form of enzyme of high molecular weight.

(ii) Column chromatography on Sepharose 2B showed that the 27,000 x g pellet catalyzed the formation of [^{14}C]-labelled enzyme which eluted in fractions of high molecular weight (Fig. 26). Evidence that the formation of this material was related to inactivation of the enzyme was that the [^{14}C]-labelled enzyme in these fractions increased when the amount of inactivation of enzyme increased (Fig. 34). Measurement of the activity of DHFR in each fraction showed directly that the high molecular weight form of the enzyme was inactive (Fig. 27).

In *E. coli*, abnormal proteins accumulate in aggregates before they are degraded to their constituent amino acids (Prouty and Goldberg, 1972; Prouty et al., 1975). The formation of inactive DHFR of high molecular weight in these experiments could represent the production of

aggregates of DHFR. The appearance of the enzyme in several different fractions of high molecular weight which corresponded to the positions of proteins from the subcellular membranes (Fig. 28, 29, 31) suggests that binding of enzyme to subcellular membranes is a more likely explanation for the production of this inactive enzyme.

Comparison of the [^{14}C]-labelled DHFR/ μg of membrane protein in the eluate of Sepharose 2B columns showed that while some enzyme eluted in the void volume with proteins of the mitochondria-rich and lysosome-rich pellets, the dpm/ μg was very much lower than in fractions which contained proteins from the heavy microsome-rich pellet (Fig. 29, 30, Table 11). While urea and mercaptoethanol reactivated the enzyme which was associated with microsomal membranes, it had little effect on the enzyme in fractions containing lysosomal and mitochondrial proteins (Table 13). This suggests that greatest binding of DHFR occurred with membranes of the microsomal fraction and that the low recovery of enzyme, which was not reactivated by urea, in the void volume with mitochondrial and lysosomal membranes represented non-specific association perhaps due to trapping of heat denatured enzyme in the suspension of high molecular weight organelles. Some [^{14}C]-labelled enzyme of high molecular weight entered the Sepharose 2B column in samples which contained the pellet rich in lysosomes (Fig. 29B, Table 11). The ratio of [^{14}C]-dpm to μg of protein in these fractions was probably high (Table 11). This suggests either that high specific binding of DHFR occurred only to a very small percentage of lysosomal proteins which have a lower molecular weight than the bulk of lysosomal proteins (Fig. 29B), or that formation of this material was due

to contamination by microsomal membranes. Since the molecular weight of the material was the same as that of microsomal proteins (Fig. 29C), the latter explanation seems the most likely. While no microsomal marker enzyme was demonstrated in the lysosome-rich fraction (Table 7), the sensitivity of the assay may have been too low to detect contaminating enzyme. If greater amounts of cellular protein were used in these incubations, it may be possible to assay for marker enzymes in each fraction of eluate of the Sepharose 2B columns. This would be valuable in determining whether the binding to proteins of the lysosome-rich fraction was the result of contamination by microsomes.

These results show that greatest association of DHFR with sub-cellular membranes occurred with microsomal membranes. If inactivation of DHFR was due only to binding of the enzyme to membranes, then such inactivation would be produced only by those membranes to which the enzyme bound. Thus, greatest inactivation should be by the microsomal fraction. A comparison of the rate of inactivation by the lysosome-rich fraction, mitochondria-rich fraction and heavy microsome-rich fraction, however, showed that the rate of inactivation by each of these fractions was comparable (Fig. 10). A possible explanation for this result is that inactivation by heat denaturation varied between incubations as discussed later (4.2.3.). Although the same amount of protein was present in each fraction incubated, in an attempt to keep the protection from heat denaturation constant, such protection depended not only on the amount but also on the type and possibly also on the solubility of the protein (Fig. 6, Table 6). Greater inactivation of the enzyme by heat

denaturation may, therefore, have occurred in the incubations which contained fractions rich in mitochondria and lysosomes. Although the factors protecting the enzyme against heat denaturation are not understood, it seems likely that more of the heavy microsome proteins may have remained in suspension and been available to protect the DHFR during incubation than did the proteins of the intact lysosomes and mitochondria of higher molecular weight which would have a greater tendency to settle out of the suspension.

While inactivation of the enzyme was produced by membrane fractions which did not appear to bind the enzyme, in no case was binding detected by proteins which did not inactivate the enzyme. Although on columns of Sepharose 2B, it could not be shown directly that BSA and proteins of the 100,000 x g supernatant did not bind the enzyme, these proteins did not decrease the association of enzyme with microsomal membranes (Fig. 33), suggesting that they did not bind the enzyme.

4.2.2. Nature of the inactive material of high molecular weight

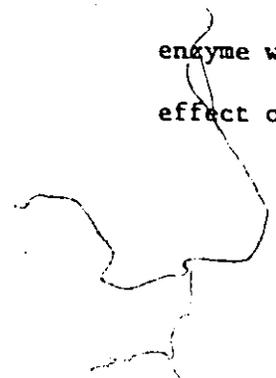
When the inactive [^{14}C]-labelled material of high molecular weight was eluted on a column of Sepharose 2B and treated with 8 M urea and 1.2 mM mercaptoethanol, enzyme activity was restored (Table 13). In contrast, the active enzyme eluting in fractions of lower molecular weight was inactivated by this treatment. This suggests that DHFR was dissociated from microsomal membranes by this treatment or that the conformation of inactive enzyme, bound to microsomal membranes, was altered so that the bound enzyme became active. The observation that this treatment converted

[¹⁴C]-labelled material which did not enter 7% polyacrylamide gels into a form which entered gels and which had the same charge as native enzyme (Fig. 23, 25), suggests the former explanation is the more likely. Thus, binding to the microsomal membranes may be by hydrophobic or hydrogen bonds or by the formation of disulphide bonds. Treatment of incubations containing inactive enzyme and the 100,000 x g pellet with urea or mercaptoethanol showed that reactivation of the enzyme was produced only by urea. This indicates that the enzyme was probably bound to microsomal membranes by hydrophobic or hydrogen bonds, to microsomal proteins, lipids, or, less likely, to nucleic acids.

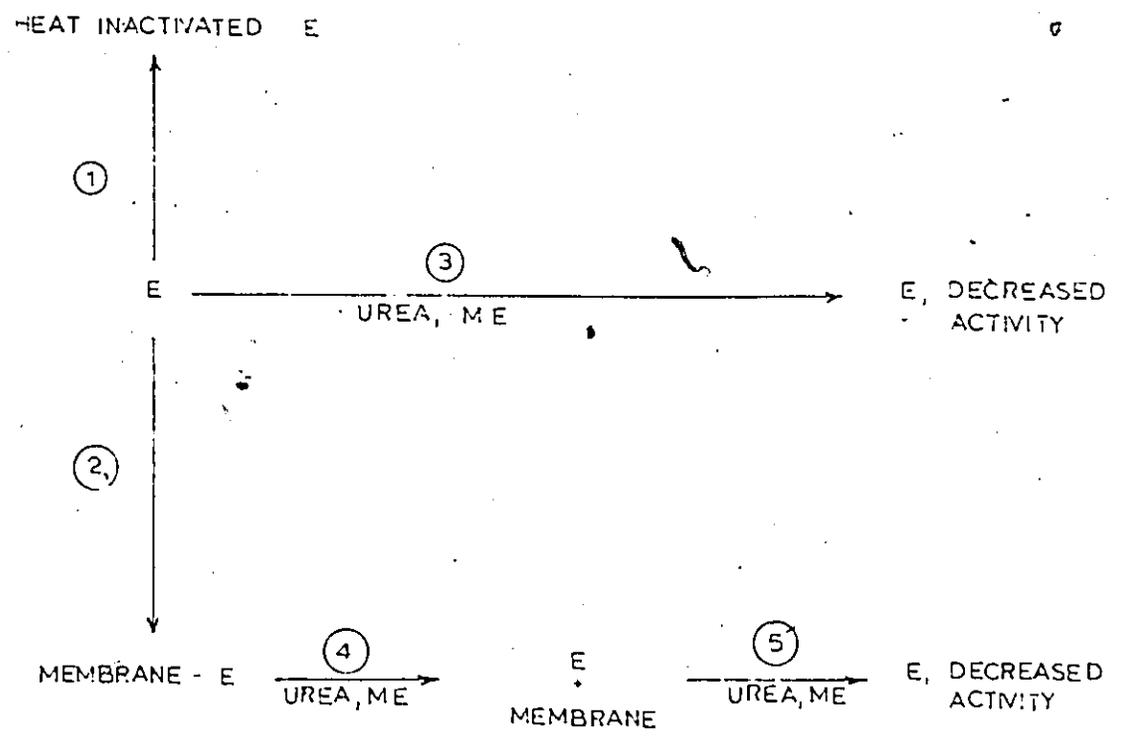
4.2.3. Nature of the inactivation process

The results presented here do not show whether inactivation was the result of binding to microsomal membranes or whether the enzyme was inactivated first and then bound.

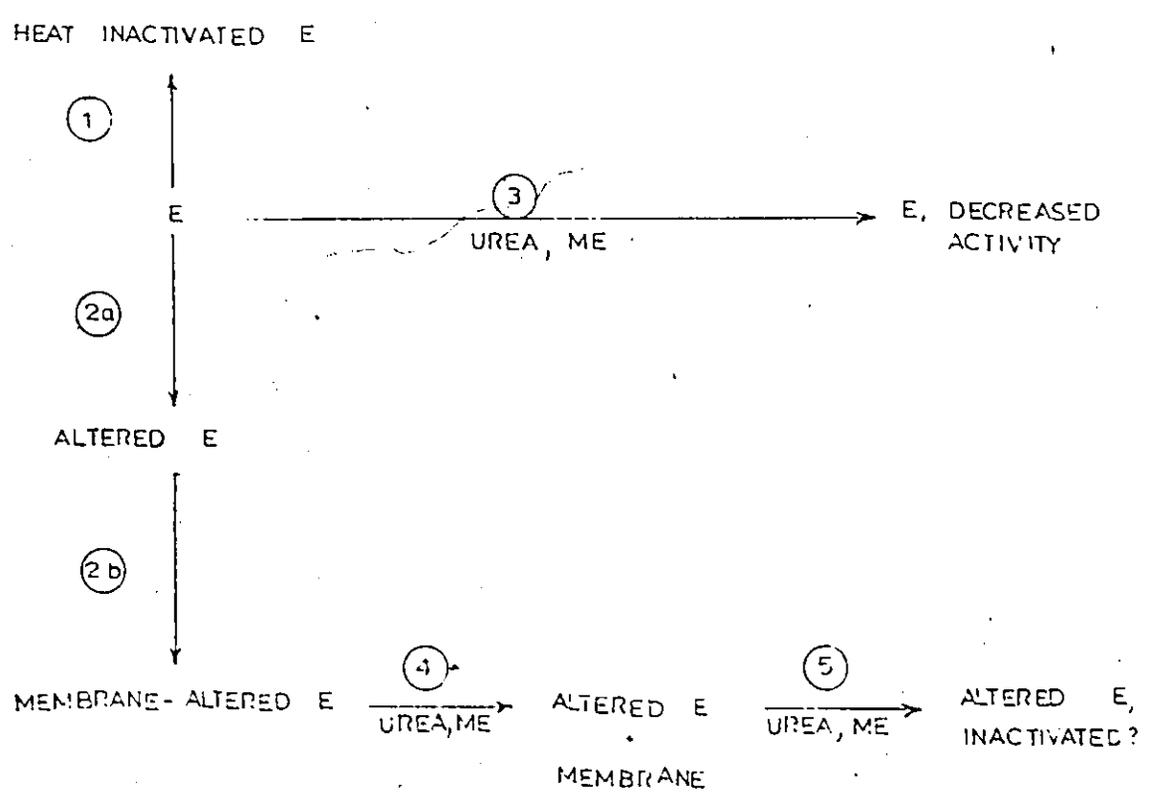
After treatment with mercaptoethanol and urea, the inactive high molecular weight material eluted on a column of Spharose 2B was reactivated (Table 13). Although this suggests that the enzyme activity was restored by dissociation of the enzyme from the high molecular weight material, it does not give any information as to whether or not the enzyme was altered before binding to the membrane. Two schemes for the effect of urea are shown below.



SCHEME I



SCHEME II



According to these schemes, DHFR(E) can be inactivated by heat, (1). It can also be inactivated by binding to microsomal membranes (2), Scheme I. According to Scheme II it is altered by some unknown mechanisms (2a), followed by binding of the altered enzyme to microsomal membranes (2b). When the active enzyme which remains is treated with urea and mercaptoethanol (ME), its activity is decreased, presumably as a result of a conformational change (3).

When the membrane-enzyme complex is treated with urea and mercaptoethanol, it is assumed that the enzyme is dissociated from the membranes (4). Free enzyme is formed. According to Scheme I, the enzyme is the same as the original enzyme and will be inactivated to the same extent as the enzyme of low molecular weight (5). The effect of urea on the activity of DHFR varies between species (Blakley, 1969). If the bound enzyme were altered in some way, as in Scheme II, then when dissociated from the membranes it may be inactivated by urea to a different extent than would unaltered enzyme (5). If binding of DHFR to microsomal membranes occurred according to Scheme I, it might be expected that after treatment with urea, the activity/dpm of the enzyme in the fractions of high molecular weight would be the same as the activity/dpm of the low molecular weight enzyme. Since this ratio is in fact much higher (Table 13, Fig. 29) it could be interpreted as evidence that binding occurred according to Scheme II.

Several complicating factors must be considered, however. First the enzyme of low molecular weight which eluted from the column of Sepharose 2B represented heat inactivated enzyme as well as active enzyme

(Fig. 36). The activity/dpm of this enzyme would, therefore, not be as high as the original ratio of the enzyme before incubation. When DHFR was incubated with the 100,000 x g pellet, 66% of the enzyme activity was lost, while only about 35% of the radioactively labelled enzyme was recovered as material of high molecular weight (Fig. 34). This result suggests that as much as half of the enzyme in the peak of lower molecular weight in this experiment may have been inactive. If the same were true in the experiment shown in Fig. 29, then the activity/dpm of the material of low molecular weight, after treatment with urea and mercaptoethanol, would be much less than that of the enzyme in fractions of high molecular weight. Since this result was obtained (Table 13, Fig. 29) it could be taken as evidence for the mechanism which is illustrated in Scheme I as well as for that illustrated in Scheme II.

Secondly, microsomal membranes may affect the inactivation of DHFR by urea since 4M urea inactivates DHFR of chicken liver in the absence of substrate but the presence of DHF completely stabilized the enzyme (Kaufman, 1963). As well, DHFR was protected from heat inactivation by the presence of protein (Fig. 5), so inactivation of DHFR by urea could also be influenced by other proteins.

While these results do not show whether the enzyme of high molecular weight was altered before it was bound to microsomal membranes, the degree of reactivation of the enzyme with urea suggests that if the enzyme was modified before binding to the membranes, such modification was not extensive. This is consistent with the earlier observation that the molecular weight of the enzyme was not markedly altered. Microsomal

membranes contain proteases (Bohley, 1971), so if the enzyme were modified before binding to the microsomal membranes, proteolysis would be a likely method. Although it has not been possible to demonstrate cleavage of peptide bonds, the limitations of the methods used are discussed in section 4.1.

Another mechanism by which enzymes can be inactivated is by dephosphorylation (Brand and Soling, 1975; Seubert and Hamm, 1975). Tyrosine transaminase is inactivated by subcellular membrane fractions of rat liver and kidney. Inactivation is greatest by the microsomal fraction and is prevented by an ATP regenerating system, possibly due to phosphorylation of the enzyme (Seubert and Hamm, 1975). Although inactivation of DHFR by subcellular membranes was decreased by an ATP regenerating system (Fig. 17), it is unlikely that inactivation of the enzyme was the result of dephosphorylation. Inactive enzyme was not reactivated by the ATP regenerating system (Fig. 18), nor has phosphorylation of DHFR been reported. Further evidence that inactivation was not due to this mechanism was that components of the ATP regenerating system stabilized DHFR in the absence of ATP (Fig. 19) and the lack of incorporation of radioactivity into DHFR when 32 [P]-labelled ATP was used in the regenerating system (data not shown).

When DHFR was incubated with the 100,000 x g pellet, inactivation was produced by a mechanism which appeared less heat sensitive than the mechanism by which the enzyme was inactivated by the other subcellular pellets (Fig. 8). The reason for this seems to be that DHFR bound to microsomal membranes even when they were boiled (Fig. 38). This result

provides evidence that previous alteration of the enzyme, by a heat sensitive process, was not needed for the enzyme to bind to these membranes. Although only modified enzyme may have bound to unboiled membranes while unaltered enzyme bound to boiled membranes, treatment of the bound enzyme in both cases with urea and mercaptoethanol resulted in equal recovery of enzyme (Table 12), suggesting that there was no difference in the enzyme which was bound to boiled and unboiled microsomal membranes.

It has been suggested that heat denaturation is an early step in protein degradation (Coffey and deDuve, 1968; Li and Knox, 1972; Knowles and Ballard, 1976). When a sample of the 100,000 x g pellet was added to enzyme which had been previously inactivated by heat denaturation, little high molecular weight material was formed (Fig. 37). Further evidence that inactivation of the enzyme by denaturation was not required for binding to microsomal membranes was provided by the use of MTX in incubations. When MTX was present in incubations during inactivation of DHFR by the 27,000 x g pellet, the enzyme which was recovered in fractions of low molecular weight on a Sepharose 2B column had a much higher activity/dpm than the enzyme from incubations which had not contained MTX (Fig. 35). This increase was observed even though MTX, which partially inhibits the enzyme at the pH used in the measurement of its activity (Hillcoat et al., 1971), was also present in these fractions (Fig. 41). Since these fractions of the Sepharose 2B column contained enzyme inactivated by heat denaturation and MTX is known to stabilize the enzyme against heat denaturation (Hillcoat et al., 1971), it is probable that

there was less denatured enzyme in the incubation which contained MTX. The amount of enzyme of high molecular weight, however, was unaltered by the presence of MTX (Fig. 35).

These results suggest that neither heat denaturation nor a heat sensitive alteration of DHFR was required for the association of the enzyme with membranes of the 100,000 x g pellet. Such inactivation was, therefore, the result of binding to these membranes.

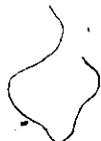
4.2.4. Factors affecting the rate of inactivation of DHFR

As discussed in the introduction, interaction with ligands can stabilize proteins against degradation, presumably by causing conformational changes which decrease the sensitivity of the proteins to degrading enzymes. Such interactions are also thought to be involved in decreasing the rate of degradation of proteins *in vivo* (Schimke et al., 1965; 1965a). Because of this effect of ligands in altering the rate of degradation of proteins, the effect of various compounds on the rate of inactivation of DHFR by the 27,000 x g pellet was examined.

a) Substrate and cofactor

Stabilization of DHFR by DHF and NADPH in crude extracts of human cells has been described (Hillcoat et al., 1971). These ligands were also found to stabilize DHFR in the *in vitro* incubations reported here. Dihydrofolate completely prevented loss of activity while NADPH provided almost complete protection, suggesting that both inactivation by heat denaturation and by binding to subcellular membranes was prevented. The

role of these ligands in controlling the rate of degradation *in vivo* is not known.



b) ATP regenerating system

An ATP regenerating system prevented the heat sensitive inactivation of DHFR by the 27,000 x g pellet (Fig. 17,18). Although the mechanism of this stabilization is not known, several possible mechanisms can be considered:

(i) Since the rate of inactivation of DHFR in buffer alone (Fig. 20) or in incubations containing boiled pellet (Fig. 17) was not affected by these molecules, one explanation for the decreased loss of activity was that the components of the regenerating system caused a conformational change in the enzyme that did not protect it from heat inactivation but prevented its binding to the subcellular membranes. Activation of DHFR from L1210 cells by sodium chloride and by potassium chloride has been described (Ryes and Huennekens, 1967). The effect of the salts is considered to be due to an alteration in the conformation of the enzyme caused by interference with intramolecular bonds. It is possible that magnesium sulphate, potassium sulphate and ATP caused conformational changes in this way. Alternatively, ATP is a nucleotide, so the effect of ATP could have occurred through a conformational change induced by binding of the molecule to the cofactor binding site.

(ii) Reactivation of the inactive enzyme of high molecular weight by urea (Table 13, 14) suggests that binding of the enzyme to subcellular membranes was by hydrophobic or by hydrogen bonds. It is

possible that the increased ionic strength caused by the components of the ATP regenerating system resulted in decreased hydrogen bonding between DHFR and the subcellular membranes. This would explain why DHFR was stabilized only in the presence of the 27,000 x g pellet.

(iii) Another explanation for the protection of DHFR only in the presence of the nonboiled pellet could be that ATP, magnesium sulphate and potassium sulphate each stimulated a heat sensitive reaction of the subcellular membranes. For example, stimulation of an enzymic reaction may result in the production of a metabolite which prevented binding of the enzyme to microsomal membranes.

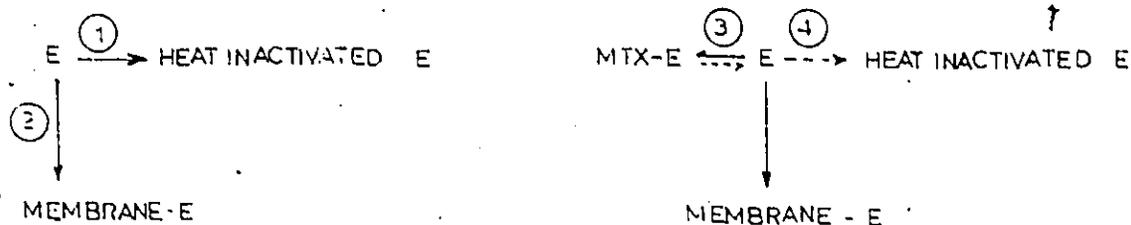
Until the mechanism by which the components of the ATP regenerating system protected DHFR from inactivation by the 27,000 x g pellet is further elucidated, it is difficult to know whether these molecules are involved in controlling the degradation of DHFR in intact cells.

c) Methotrexate

Methotrexate stabilizes DHFR from proteolysis by trypsin and chymotrypsin and from heat denaturation (Hillcoat, et al., 1971). Protection of DHFR by MTX *in vivo* is considered to be the most likely explanation for the increased levels of enzyme in cultured cells and in cells of patients after treatment with MTX (Hillcoat et al., 1971; Hillcoat et al., 1967). Evidence for this has been provided by Jackson and Huennkens (1973) who have shown that the half-life of DHFR increased from 18 h to 39 h in L1210 cells during treatment with MTX.

If binding of DHFR to subcellular membranes is involved in degradation of the enzyme *in vivo*, it might be expected that MTX would prevent the binding observed *in vitro*. When this was tested, it was shown that although MTX stabilizes DHFR against inactivation by the 27,000 x g pellet (Fig. 16), 1.4×10^{-7} M MTX did not prevent the formation of [14 C]-labelled enzyme of high molecular weight (Fig. 35).

The scheme below shows the probable interactions between the various forms of the enzyme:



According to this scheme, DHFR(E) is inactivated by heat denaturation (1), and by binding to microsomal membranes (2). When MTX is present it binds strongly to the enzyme (3), thus markedly reducing the amount of enzyme which is inactivated by heat denaturation (4).

Since heat denatured enzyme is reduced in incubations containing MTX, the concentration of DHFR which is available, either in the free form or as enzyme-inhibitor complex, is much higher in incubations which contain MTX than in those which do not. It was shown that increasing amounts of DHFR resulted in increased formation of high molecular weight material (Fig. 34), so if the enzyme-MTX complex binds as readily as free enzyme, more enzyme of high molecular weight would be formed in

incubations which contained MTX than in those which did not. This result was not obtained (Fig. 35). Rather, the amount formed was no higher than that in a control which contained much less native enzyme. This indicates that enzyme must bind less efficiently to membranes in the presence of MTX.

4.2.5. The significance of the formation of material of high molecular weight

a) Problems involved in extrapolating from *in vitro* incubations to the situation in intact cells

Although these results suggest that DHFR is inactivated by binding to microsomal membranes *in vitro*, it is not known whether such binding occurs in intact cells. The ratio of the amount of DHFR to the amount of subcellular membrane used in these incubations was 1.03 - 5.7 μg of DHFR/0.53 mg of membrane protein. In intact cells, the ratio was approximately 2 - 3 μg of active DHFR to 0.53 mg of protein from the 100,000 x g pellet. The ratio was, therefore, in the same range as in the intact cell, so artifacts due to a great excess of either enzyme or membranes *in vitro* were not involved. The concentration of these proteins in the cell-free incubations was approximately half of that in the intact cells. The effect of this dilution factor on the interaction between enzyme and membranes is not known. Similarly, the total protein concentration in these incubations was approximately one hundred times lower than in intact cells, this could influence interactions between the enzyme and membranes.

Another possible complicating factor in this work was the use of [^{14}C]-labelled enzyme to demonstrate binding of the enzyme to microsomal membranes. Labelled enzyme had to be used since the enzyme of high molecular weight was inactive and the radioactive label provided a means of detecting the molecule. The use of labelled enzyme could have produced misleading results if methylation of the ϵ -amino group of lysine residues resulted in different interactions between the modified enzyme and microsomal membranes than that between unlabelled enzyme and microsomal membranes. The demonstration that urea and mercaptoethanol reactivated the enzyme of high molecular weight (Table 13) suggested that the enzyme was dissociated from the membranes by this treatment. Assuming this to be so, such treatment provided a method of detecting inactive enzyme which was not radioactively labelled. The results in Table 15 show that both labelled and unlabelled enzyme, after inactivation by the 100,000 x g pellet, were reactivated by treatment with urea and mercaptoethanol and indicate that binding of enzyme to the 100,000 x g pellet was not an artifact resulting from the use of modified enzyme.

When the amount of DHFR in *in vitro* incubations was increased while the amount of microsomal protein was kept constant, the formation of high molecular weight enzyme increased (Fig. 34). It would be desirable to use a wide range of enzyme concentrations so that a Scatchard plot could be obtained. The value of the binding constant would be useful in evaluating whether binding of DHFR to microsomal membranes may occur in intact cells.

b) Possible roles for binding of DHFR to microsomal membranes

If DHFR is inactivated by binding to subcellular membranes *in vivo*, this could be important in regulating the activity of the enzyme in two different ways:

(i) Stabilization of DHFR by protein-protein interactions

The activity of DHFR is inhibited by its interaction with thymidylate synthetase *in vitro*. Binding is prevented and reversed by MTX, or by substrates of either enzyme (Kawai and Hillcoat, 1974). Interactions such as these may be important in regulating the activity of enzymes *in vivo*. Enzymes could be present in the cell in a latent form until such time as they were needed. Binding of DHFR to subcellular membranes could represent this type of interaction. If binding did have such a role, however, it would be expected that it would be readily reversible. This could not be demonstrated. At 4°C, the presence of NADPH and MTX did not dissociate the enzyme from the high molecular weight material (Fig. 40) nor did dilution of the material cause dissociation (Fig. 39).

(ii) Binding to membranes as the initial step in protein degradation

Proteins *in vivo* are degraded to their constituent amino acids (Swick, 1958) and the presence of proteases in the lysosomes makes it likely that this is where degradation takes place. Although lysosomes produce acid-soluble material from proteins *in vitro* at acid pH (Table 2) (Segal et al., 1974; Dean, 1975), intact lysosomes at neutral pH do not (Table 1, 3) (Huisman et al., 1973; Hayashi et al., 1973). The reason proteins are not degraded by lysosomes *in vitro* might be that they are

unable to enter intact lysosomes. Binding of proteins to microsomal membranes could represent the initial step in degradation. Lysosomes are formed from vesicles of the Golgi apparatus (deDuve and Wattiaux, 1966) and those intracellular proteins to be degraded could be incorporated into lysosomes at the time of formation of these organelles by binding to microsomal membranes. Alternatively, proteins which were bound to microsomal membranes could be incorporated into autophagic vacuoles which then fuse with primary lysosomes (Holzmann, 1976).

Formation of autophagic vacuoles is known to increase under conditions in which protein degradation increases (Deter and deDuve, 1967; Ashford and Porter, 1962). As discussed in the Introduction, it has been considered that incorporation of intracellular proteins into lysosomes by autophagy could not explain the selective nature of protein degradation. If binding to microsomal membranes were determined by properties of the protein, the heterogeneity of half-lives and the way in which the rate of degradation of individual proteins is altered could be explained.

Reactivation of DHFR by urea (Table 13 and 14) suggests that binding of this enzyme to microsomal membranes may be by hydrophobic bonds. Interestingly, proteins which have short half-lives *in vivo* have a relatively high proportion of hydrophobic amino acids (Bohley et al., 1975).

The results obtained during incubation of HTC proteins could also be explained by binding of proteins to subcellular membranes. Some proteins from the 27,000 x g supernatant of HTC cells were lost from the supernatant during incubation with the 27,000 x g pellet (Fig. 2). These proteins sedimented with the 27,000 x g pellet at the end of incubations (Fig. 3). Although proteins were lost from the supernatant when no pellet

protein was present (Fig. 2), a comparison of the radioactively labelled proteins which remained soluble in five different experiments, as determined by the radioactivity recovered on polyacrylamide-SDS gels, showed that there was an increase in the loss of soluble protein from incubations which contained proteins from the 27,000 x g pellet as compared to controls which did not (Hillcoat, personal communication). Proteins were also lost from the 27,000 x g supernatant during incubation of the intact cells. There was a general loss of proteins without an increase in material of lower molecular weight. This result can be explained as removal of proteins from the supernatant by binding to membranes which sediment at 27,000 x g.

Dean has reported that cytosolic proteins from rat liver bind to lysosomes during incubations *in vitro*. By using labelled proteins he has shown a correlation between the rate of degradation *in vivo* and the amount of binding to lysosomal membranes (Dean, 1975a). He suggests that the rate of degradation of intracellular proteins depends on the rate at which they are bound to lysosomal membranes and proposes that once bound, proteins enter lysosomes by pinocytosis.

While some binding to lysosomal membranes was observed in the results reported here, binding was greatest with the microsomal membranes. As discussed in the Introduction, there could be more than one mechanism of degradation of proteins (1.3.3.). Poole (1975) has provided evidence that some steps in the degradation of proteins may be shared by all proteins while other steps appear to differ. Holzmann (1976) suggests that differences may exist at the level of entry into lysosomes. Binding

to lysosomes could therefore represent one mechanism of entrance while binding to microsomes could represent a second mechanism. There is little evidence, however, that extensive pinocytosis of lysosomal membranes occurs (1.3.1.). The lysosomal preparations used by Dean may have been contaminated by microsomes (Dean, 1975c; Beaufay, 1972), so the binding observed in his experiments could have represented binding to microsomal membranes.

The suggestion that binding to microsomal membranes may represent the preliminary step in the *in vivo* degradation of intracellular proteins is supported by a recent report of Ballard and Hopgood (1976). They have reported that phosphoenolpyruvate carboxykinase (guanosine triphosphate) was inactivated *in vitro* by the microsomal membranes. Their results are also similar to those reported here in that they have been unable to show any proteolysis prior to binding.

If binding of proteins to microsomal membranes occurs in intact cells it would explain how proteins could enter lysosomes in a selective manner. It would also explain why lysosomes do not degrade intracellular proteins *in vitro*, at neutral pH. The work of Ballard and Hopgood and the observation that DHFR was inactivated by binding to microsomal membranes without any detectable production of acid-soluble material, change in molecular weight or change in the charge of the enzyme suggests that binding to these membranes in intact cells could represent the initial step in degradation.

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