THE INFLUENCE OF GAMMA RADIATION ON CATHEPTIC ACTIVITY AND ON ULTRASTRUCTURE OF LYSOSOMES AND POSTMORTEM SKELETAL MUSCLE OF POULTRY (Gallus domesticus)

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We accept this thesis as conforming to the required standard

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ABSTRACT

A three part study is presented dealing with radiation-induced release of cathepsins from isolated lysosomes, irradiation inactivation of cathepsins, and ultrastructural changes in irradiated lysosomes and skeletal muscle. Chicken liver lysosomes were isolated by differential centrifugation and sucrose density gradient technique. Isolated lysosomes were irradiated with doses in the range of 0.10 to 1.0 Mrad of gamma radiation. Irradiation resulted in a decrease in absorbance (540 nm) of lysosomal suspensions incubated at 37°C and a corresponding pronounced increase in free enzyme activity due to release of cathepsins. Rate of release of cathepsin D from lysosomes was considerably slower when incubated at 4°C compared with 37°C.

Cathepsins A, B, C, and D showed a differential release under the influence of gamma radiation. Cathepsins C and D were more readily released as compared with cathepsins A and B. After 72 hours of incubation at 4°C, free activity of cathepsins A, B, C, and D in 1.0 Mrad irradiated lysosomal suspensions reached 80.2, 70.5, 85.5, and 81.6 percent of total enzyme content; free enzyme activity of control samples was 14.0, 16.7, 27.7, and 26.6 percent respectively.
Total activity of cathepsins A, B, C, and D declined as a result of irradiation, due to apparent partial inactivation of the enzymes. Cathepsin A was most radiation resistant and cathepsin B was comparatively sensitive to radiation inactivation. Cathepsins exhibited higher radiation resistance when irradiated in lysosomal suspension compared with soluble enzyme form. Radiation sensitivity of cathepsins was higher at pH 4.0 and 5.5 compared with pH 7.0 and 8.5.

Irradiation induced changes in hemoglobin substrate, rendering it resistant to catheptic digestion. Changes in electrophoretic pattern as well as visible spectra of irradiated hemoglobin were indicative of alterations in the substrate.

Isolated chicken liver lysosomes and chicken pectoralis muscle were subjected to 1.0 Mrad of gamma radiation. Sections of embedded samples were studied by transmission electron microscopy and surface ultrastructure details were examined by scanning electron microscopy. Irradiation enhanced the release of inner dense material from lysosomes. After 48 and 72 hours of incubation most of the irradiated particles appeared as hollow rings of lysosomal membrane. In some irradiated particles, leakage of lysosomal contents from "weak points" in the lysosomal membrane was observed. There were indications that irradiation weakened the membrane structure which caused leakage of material
from lysosomes and eventual disruption of particles on prolonged incubation. Scanning electron microscopy provided further evidence that lysosomal material leaked from "weak points" in the lysosomal membrane causing lysosomes to appear as "empty sacks" rather than totally disrupted particles.

Transmission electron microscopy of cryofractured skeletal muscle revealed that irradiation caused an increase in interfibrillar spaces and some breaks in the myofibres especially at the I band region. Scanning electron micrographs of irradiated muscle showed fissures between the myofibrils. Control samples had a smooth surface at the transverse breaks, while irradiated tissue had spike-like structures at the surface of these breaks.

These studies provide evidence that irradiation caused structural changes in lysosomes resulting in increased leakage of lysosomal contents and release of lysosomal enzymes. Structural changes at the fibre and fibril level of irradiated muscle are also indicated. The effects are likely fundamental to textural alterations of muscle subjected to post-mortem irradiation.
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CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Radiation-sterilization has been successfully applied to obtain microbiologically stable meats. A major problem is presented by radiation resistance of enzymes, as residual proteolytic activity has been shown to cause off-flavors, bitter taste, and textural changes in radiation sterilized meats (Cain et al., 1958; Coleby et al., 1961; Bailey and Rhodes, 1964).

The proteolytic activity in post-mortem muscle has been attributed to lysosomal cathepsins (Suzuki et al., 1967; Okitani et al., 1973). It has been noted that individual enzymes possess greatly differing radiation sensitivity (Vas, 1966); information regarding individual cathepsins is lacking and is highly desirable.

Another important factor influencing the proteolysis in irradiated tissue is the availability of lysosomal enzymes. Increased proteolytic activity after irradiation of muscle tissue has been reported by Klein and Altman (1972b); this could be due to radiation-induced changes in lysosomal structure resulting in release of cathepsins and increasing the availability of the enzymes. The rate and extent of release of lysosomal cathepsins will markedly affect the course of proteolysis. However, as yet detailed information on the release of cathepsins has not been re-
ported and it was the aim of this study to investigate the effect of irradiation on the release of cathepsins A, B, C, and D from lysosomes.

Influence of irradiation on ultrastructure of lysosomes would provide further information regarding their stability as well as release of cathepsins. Ultrastructural changes in irradiated muscle might be partially responsible for its extensive post-irradiation degradation by proteolytic enzymes. Ultrastructural studies of lysosomes and muscle would help greatly in understanding the events associated with irradiation process and post-irradiation behaviour of meats.

This thesis describes a three part study dealing with the effects of gamma radiation on:

1). Release of cathepsins A, B, C, and D from isolated lysosomes.

2). Inactivation of lysosomal cathepsins under various conditions; and changes in hemoglobin substrate.

3). Ultrastructure of isolated lysosomes and chicken pectoralis muscle as studied by transmission electron microscopy and scanning electron microscopy.
1.2. LITERATURE REVIEW

1.2.1. Effect of Ionizing Radiation on Meats

Irradiation has been noticed to exert a tenderization effect in beef and pork (Bailey and Rhodes, 1964) at doses of 4 Mrad. Coleby et al. (1960) reported that irradiation was effective in controlling microbial spoilage in whole eviscerated chicken when stored at 1°C, but the quality of irradiated carcasses tended to deteriorate during the storage, and flavor changes were not masked by roasting. These findings differ from those of Proctor et al. (1956) who, working with chicken meat blanched by steaming before irradiation, reported that flavor was not significantly affected by 2 Mrep. It is possible that enzymes, which otherwise might cause off-flavors, were inactivated by the blanching treatment.

Irradiated fresh meats have been reported to develop a bitter taste, thought to be due to the accumulation of free tyrosine (Cain et al., 1958; Drake et al., 1957b). Drake et al. (1961) reported a decrease in consumer taste-panel preference during unrefrigerated storage of irradiated raw ground beef, which correlated to some extent with the activity of intracellular tissue proteolytic enzymes — cathepsins.

Doty et al. (1958) found an increase in non-protein
nitrogenous compounds in ground beef after irradiation treatment, and it was accompanied by an appreciable decrease in the concentration of soluble proteins. Zender et al. (1958) also reported a decrease in glycine-NaOH-soluble protein content of beef muscle after irradiation.

Bautista et al. (1961) reported an increased rate of release of amino nitrogen, total soluble nitrogen, and TCA-soluble nitrogen after irradiation treatment of beef.

El-Badawi et al. (1964) found a significant increase in glycine-NaOH-soluble proteins contained in the drip fluid from irradiated beef. On the other hand, Anglemier et al. (1964) added the drip fluid back to the sample prior to extraction, and did not find a significant difference in the amount of extractable proteins in irradiated and un-irradiated meat. They also noticed the disappearance of one band in the electrophoretic pattern of the glycine-NaOH-soluble proteins extracted from muscle and concluded that irradiation-sterilization results in a fragmentary action on the structure of meat proteins. These workers indicated that the number of electrophoretic bands was expected to increase rather than decrease or not be affected. They explained that one possibility is that protein fragments are held together by hydrogen and/or the electrostatic bonds. The other possibility is that an irradiation fragmentation effect is exerted mainly on the meat proteins not extracted by the glycine-NaOH buffer. Uzonov et al. (1972) observed that irradiation of beef resulted in a decrease in
5.
total content of soluble proteins, and electrophoretic characteristics of the soluble proteins were changed by a decrease in the cathodic fraction. Klein and Altman (1972a) reported changes in electrophoretic mobility of the soluble proteins from chicken meat; at a 1.0 Mrad dose there was a considerable decrease in high molecular weight bands, and at 3.0 Mrad there was a nearly complete levelling of the high molecular weight bands.

Anglemier et al. (1964) reported that irradiation sterilization decreased the hydration of beef muscle in pH range 3.5 - 7.0 with depression maximum near the isoelectric region. These results are in agreement with Lawrie et al. (1961). Alteration of hydrogen or electrostatic bond of a protein should have maximum effect on hydration around the isoelectric region (Hamm, 1960). Irradiation may have some tendency to tighten the meat protein structure by increasing the number of these weak bonds. This hypothesis is in agreement with information presented by Drake et al. (1957a) on bovine serum albumin. Lawrie et al. (1961) and Batzer et al. (1959) reported that irradiation caused an increase of muscle pH. Bendall and Wismer-Pedersen (1962) observed an increase in pH of washed fibrils after heating. Such shift in pH has been suggested to be due to release of tyrosine hydroxyl groups on denaturation of proteins (Bendall, 1964).

Studies have shown that proteolytic enzymes are not fully inactivated at a radiation dosage required for sterilization (Drake et al., 1957b; Doty and Wachter, 1955).
Landmann (1963) found that one of three active proteinase fractions in beef retained its activity after irradiation of meat at sterilizing doses. Rhodes and Meegungwan (1962) reported a considerable proteinase activity in liver remaining after irradiation with 4 Mrad. They also indicated that at higher doses proteases were radiation resistant. Proctor and Goldblith (1951) stated that much higher doses might be required to inactivate the enzymes or reduce their activities to a level acceptable for long-term storage of meat.

Accumulation of free tyrosine in irradiated meat has been noted, and it was thought by some workers to be liberated by proteolytic enzymes (Cain et al., 1958; Drake et al., 1957b). Losty et al. (1973) reported that 2 - 6 Mrad gamma radiation destroyed up to 75% of the proteolytic activity in ground beef. Shults et al. (1975) observed that gamma irradiation with 2 Mrad at -80°C resulted in only a 1% reduction of proteolytic enzymes in beef, an 11% reduction in chicken and a 26% reduction in pork, while an 8 Mrad dose at 21°C resulted in 79% - 82% reduction in proteolytic enzymes in the three kinds of meats. Klein and Altman (1972b) found that proteolytic enzymes in chicken breast muscle were more radiation resistant than the enzymes in leg muscle. There was 50% inactivation of proteases at 2 Mrad and 85% at 5 Mrad in the breast muscle, while the leg muscle showed almost complete inhibition of the enzymes.

1.2.2. Effect of Ionizing Radiation on Proteins and Amino Acids

Because of the high percentage of water in meats, the
reactions which occur when water is irradiated are of primary importance. By radiolysis of water H, OH, H₂O₂ and H₂ are the final major products, but free radicals are formed along the path of the primary electron and react with each other as diffusion occurs. Also, some of the products formed along the track escape and can then react with solute molecules (Kuzin, 1964).

Adhikari and Tappel (1975) reported gamma irradiation of glutamic acid, phenylalanine, serine, arginine, and methionine in aqueous solutions resulted in the formation of malonaldehyde, which reacted with other amino acid molecules to form Schiff's bases. Free arginine, tyrosine, and methionine have been shown to be destroyed more readily than other amino acids by gamma radiation (Fujimaki et al., 1961). In cysteine, however, the SH group is so efficient in trapping free radicals, the rest of the molecule is almost completely protected from attack, and cystine is the major product formed (Swallow, 1962).

Products from the irradiation of proteins resemble those of amino acids, except that there is a greater attack on amino acid side chains when amino acids are combined in peptide chains. There is a general decrease in the amounts of unchanged amino acids with almost total loss of methionine and cysteine, and 25% loss of histidine (Drake et al., 1957a; Hedin et al., 1960). When protein solutions are irradiated in the presence of oxygen, the break in the peptide chain is not the usual hydrolytic break, but occurs between the N-C
bonds to form amides and carbonyls (Garrison et al., 1962). Oxidative damage to amino acids, peptides and proteins as a result of irradiation, have been observed by Ambe and Tappel (1961).

Tsaien and Johnson (1959) reported that the soluble proteins of beef, when exposed to a dose of 5.6 Mrads, suffered severe destruction of certain amino acids as measured by the Moore and Stein method after acid hydrolysis. Their results indicated that about three-quarters of serine and glutamic acid, about one-half of threonine and aspartic acid and about one-fifth of glycine, methionine, lysine, histidine, and arginine could not be recovered from irradiated protein as ninhydrin reacting amino acids. The total of the amino acids destroyed amounted to 28% of the initial content. In contrast to this observation Rhodes (1966), using ion-exchange chromatography, could not detect any significant change in the proportion of ninhydrin-reacting components (except ammonia) when meat was exposed at 0°C to gamma radiation at dose levels of 5 or 20 Mrads.

Increased solubility of the collagen in intact beef muscle after irradiation has been reported (Bailey and Rhodes, 1964), but this effect diminished when collagen was irradiated in meat juice or in saline, which indicates that the effect is due to the indirect action of radiation and that these changes may not affect the tenderness of meat. Grant et al. (1970) reported an altered reactivity of both native and cross-linked collagen when irradiated in a dry or wet condition with 100 Mrads of electron irradiation. It was observed that fibres irradiated dry
showed greater damage when examined in the electron microscope. Similar results were obtained using 100 Mrad gamma radiation by Grant et al. (1973), who concluded that irradiation of collagen in the dry state resulted in scission of the polypeptide chains but in the presence of water, this was accompanied by the formation of intermolecular bonds, thus changing the configuration of polypeptide chains. Jelénska and Dancewicz (1972) noted an apparent decrease in the content of free ε-amino groups in irradiated tropocollagen, indicating that there were conformational changes due to formation of new interchain cross-linking bonds. Coelho (1969) reported fragmentation as well as reticulation through intermolecular cross-linking of gamma irradiated actomyosin.

Alteration in solubility characteristics of irradiated collagen (Grant et al., 1970, 1973; Bowes and Moss, 1962) and actomyosin (Coelho, 1969) have been reported. Braams (1961, 1963) found reduced tensile strength of bovine tendon due to irradiation treatment. Friedberg (1969) observed that gamma irradiation caused chain breaks resulting in a decrease in molecular weight of dry collagen. This effect decreased when metal ions were present in the system (Friedberg et al., 1975).

Paul and Kumta (1973) observed increased tryptic hydrolysis of irradiated horse heart myoglobin. McArdle and Desrosier (1955) reported an increased hydrolysis of irradiated casein and egg albumin by trypsin, and concluded that ionization of the bonds by radiation caused an opening of the molecules,
rendering them more susceptible to tryptic digestion. Grant et al. (1970, 1973) observed an altered reactivity of both native and cross-linked collagen with collagenase and elastase -- wet (but not dry) irradiated native collagen became resistant to collagenase; cross-linked collagen, normally resistant to enzyme attack, was found to be more sensitive after irradiation.

McArdle and Desrosier (1955) reported a change in electrophoretic mobilities and patterns of irradiated casein and egg albumin. Both fragmentation (Carrol et al., 1962) and polymerization (McArdle and Desrossier, 1955; Zakrzewski et al., 1973) of proteins have been reported. The predominant effect of ionizing radiation on proteins in aqueous solution consists of aggregation, leading ultimately to precipitation of insoluble material. Aggregation of human serum albumin (Hay and Zakrzewski, 1968), myoglobins (Brown and Akoynoglou, 1964; Satterlee et al., 1971; Paul and Kumta, 1973), ribonuclease (Mee et al., 1972), and egg-white lysozyme (Stevens, 1967) are a few examples.

Studies on hemoproteins have shown spectral changes due to irradiation. Such changes have been attributed to alteration in heme group (Brown and Akoynoglou, 1964; Giddings and Markakis, 1972; Satterlee et al., 1972) as well as the globin group of the protein (Clarke and Richards, 1971; Lycometros and Brown, 1973). Brown and Akoynoglou (1964) noted that gamma irradiation of metmyoglobin split small peptides from globin. Satterlee et al. (1971) suggested
that alteration in irradiated metmyoglobin might be due to loss of amide nitrogen from the protein.

Radiation damage to sulfur-containing amino acids has often been reported. In proteins irradiated in the dry state the number of sulfhydryl groups may decrease, as in serum albumin (Alexander and Hamilton, 1960) and ribonuclease (Hunt and Williams, 1964), or increase, as in lysozyme (Stevens et al., 1967), accompanied by migration of disulfide. In proteins irradiated in solution, the number of sulfhydryls decreases rapidly and mixed disulfides are formed (Augenstine, 1962). Another important effect, observed in proteins irradiated in solution in the presence of oxygen, is the formation of carbonyl groups, attributed to oxidation scission of peptide bonds (Jayko and Garrison, 1958).

1.2.3. Effect of Ionizing Radiation on Enzymes and Enzyme Activity

The absorption of radiation energy by the water phase of food materials leads to the formation of reactive water radicals which very quickly interact with cellular constituents. Damage caused by water radicals is usually referred to as an indirect action of ionizing radiation. Radiation changes caused by energy absorption in organic molecules are referred to as a direct action. The observed destruction of intracellular enzymes is due to the combined effect of direct and indirect action. The relative contribution of these mechanisms to radiation damage may vary for different proteins,
depending on their localization and microenvironments (Pihl and Sanner, 1963).

The inactivation dose for enzymes decreased with increasing temperature (Proctor and Goldblith, 1951). This effect was also reported by Bellamy and Lawton (1955) for electron irradiation of aqueous pepsin, tyrosinase, and polyphenol oxidase; and neutron irradiation of dry catalase (Setlow and Doyle, 1953). It has been suggested that hydrogen bonds in the enzyme molecule are weakened by the higher temperatures (Scheraga, 1963), making the molecule subject to inactivation by excitation as well as ionization. Low sensitivity has been suggested to result from changes in the water sheath surrounding an enzyme (Klotz, 1958), modification in enzyme conformation or changes in the distribution of radiation products in preparations irradiated in the frozen state (Augenstein, 1962).

The effect of pH on radiation sensitivity of enzymes has been found to be considerable but unpredictable both in solution (Pihl and Sanner, 1963; Brustad, 1966; Robins and Butler, 1962) and in the dry state (Wilson, 1959). This is largely due to the fact that pH influences the change in dissociable protein groups. This would influence the radiation sensitivity by altering protein conformation (Sanner and Pihl, 1968), and distribution of radical species produced by irradiation (Robins and Butler, 1962; Okada, 1957).

The inactivation dose for enzymes increases with concentration as revealed by the studies on carboxypeptidase
(Dale, 1952), trypsin (Sanner and Pihl, 1967), and α-chymotrypsin (Butler et al., 1960). A similar effect has been reported by Giovannozzi-Sermanni (1969) for cathepsin C. These results are supported by the findings of Vas (1969) for pectin methylesterase and cellulase preparations.

Regarding effects of oxygen on radiation resistance of enzymes, a number of published works indicate an increase in radiosensitivity in the presence of oxygen, while others do not (Marples and Glew, 1958). Alexander (1957) reported a marked sensitizing effect of oxygen on trypsin when irradiated in dry state. Hunt and Williams (1964) showed a sensitizing effect of oxygen on ribonuclease. Lynn and Skinner (1974) reported equal rates of loss of alkaline phosphatase activity in aqueous solution under O₂, N₂, and N₂O; however, composition of the radiolysed enzyme proteins varied depending on gaseous atmosphere used. It has been suggested that oxygen concentration will influence the course of radiolysis of water. When the irradiated solutions are in equilibrium with air, the $\tilde{e}_{aq}$ will be effectively trapped by oxygen to give O₂ radicals (Sanner and Pihl, 1967). Experiments on other enzymes (Sanner and Pihl, 1967; Bustard, 1966) indicated that O₂ radicals possess low activity and in general do not participate in enzyme inactivations.

Presence of other compounds in the medium plays an important role in inactivation of enzymes by radiation. Concentration and nature of these compounds influences greatly the interaction of radicals with enzymes. The efficiency
of a compound to protect an enzyme by a radical scavenger mechanism in solution depends on its rate of interaction with the water radicals responsible for the inactivation of enzymes. Since the interaction of water radicals with an organic molecule can lead to the formation of a new radical, the result will depend on the reactivity of the new radical. Protection will be observed when the organic radical is less capable of interacting with the enzyme molecule than the parent water radical (Sanner and Pihl, 1969). Radio-protective effects of glycyl-glycine on trypsin in solution have been reported by Sanner and Pihl (1967). Jung (1967) observed similar protection of RNase by cystamine in the dry as well as the aqueous state. Schuessler (1973) observed that inactivation of RNase was reduced in the presence of EDTA. Many other substances, when present in enzyme solutions, exert a radio-protective effect (Dale, 1962; Kuzin, 1964). It has been demonstrated that two solutes could compete for the same kind of radical and thus "protect" each other (Dale, 1962). In addition to such "competitive protection", some protectors might react with enzyme molecules to alter the critical surface site or make it unavailable to inactivating radicals. Such "reactive protection" could be provided by mixed disulfide formation (Eldjahrn and Pihl, 1955; Eldjahrn et al., 1960) or by masking of crucial groups with other chemicals (Barron, 1954; Sutton, 1956).

The reactive protection resulting from an enzymes-substrate complex was predicted (Augenstine, 1959), but
Robinson and Phillips (1960) could not demonstrate it with liver alcohol dehydrogenase. However, other studies suggested that enzymes complexed with their substrates can thus be protected (Okada, 1957; Sutton, 1956). Recently this has been supported by Lynn's studies (1972) on trypsin. It has been demonstrated that enzyme activity was protected at least fifty-fold by complexing it with silica. Further, the relative extent and nature of the radiation damage to amino acid residues of the suspended enzyme were different from those found with dissolved trypsin. Trypsin in a matrix of agar (Holladay et al., 1966) and RNase mixed with glycyglycine (Copeland et al., 1967) showed similar protection due to complexing. Lynn (1974) observed a marked protection of trypsin, chymotrypsin, and chymotrypsinogen when irradiated complexed with DNA. It was concluded that active sites were protected either by the geometry of the DNA/protein complex or by nucleic acid acting to divert the free radical attack.

Dry enzymes are generally more stable to irradiation than those in solution. For example, the electron dose required for complete inactivation of dry trypsin was 170 times that for trypsin in solution (Bier and Nord, 1952). Vas (1969) found that pectin methylesterase and cellulase were highly radiation resistant in the dry state.

The loss of biological activity, when enzymes are irradiated in the dry state is associated with different types of radiation damage, compared with irradiation in the
soluble state. Rupture of hydrogen bonds occurs with subsequent unfolding of molecules. Furthermore, the molecules may aggregate, then may disassociate into subunits, or breakages of peptide bonds may cause fragmentation. In addition, alteration in the amino acid side chains is a possibility (Sanner and Pihl, 1969). Radical reactions play an important role in the radiation effects observed in the dry state (Braams, 1963). This view has been supported by Copeland et al. (1968), who studied the formation and reactions of radiation-induced radicals in the dry RNase.

Relatively little is known about the specific damage responsible for the inactivation of enzymes in the dry state. The available data indicate that no general rules exist concerning the type of damage responsible for the inactivation. This is well illustrated with the two enzymes RNase and papain. In the case of RNase, Jung and Schussler (1966) have succeeded in separating the active RNase molecules from inactivated ones, and have shown that the inactivation is associated with molecular aggregates, indicating that loss of activity was not due to destruction of any particular group within the enzyme. Copeland (1975) suggested that irradiation inactivation of RNase may be due to formation of sulfur radicals at ruptured disulfide bonds. Papain has a single essential SH group, and it has been shown that specific protection of this group, e.g. by mixed disulfide formation, provides significant protection of the enzyme against inactivation in dry state (Pihl and Sanner, 1963).
Enzymes in solution are more radiation sensitive than in the dry state. In the dry state, the effects are mostly mediated by direct action of radiation, but in solution, indirect action is more important. The inactivation of enzymes irradiated in dilute solutions is due to the action of the radiation products of water, the most important being the OH\(^{-}\) and H\(^{+}\) radicals and \(\bar{e}_{aq}\).

For certain enzymes, it appears that the radiation inactivation is due predominantly to destruction of the active site, while for other enzymes, the inactivation seems to be due to general denaturation. In many cases both mechanisms contribute to the inactivation. Sulphur-containing and aromatic amino acid residues have both been separately implicated in the radiation damage of enzymes. Thus, in lysozyme (Adams et al., 1969), trypsin (Lynn, 1971), and chymotrypsin (Lynn, 1972), tryptophan and tyrosine were found to be the residues first affected by radiolysis. In papain (Gaucher et al., 1971; Lynn and Louis, 1973) and ribonuclease (Schüssler and Jung, 1967), cysteine and cystine residues were modified. Pihl and Sanner (1963) demonstrated that inactivation of papain in solution was due almost exclusively to destruction of the active SH group. As destruction of SH group paralleled closely the loss of enzyme activity, specific protection of SH group provided a very strong protection against inactivation of enzymes. This has also been found in the case of other sulfhydryl enzymes, such as lactic dehydrogenase (Adelstein, 1965), phosphorylase b
(Damjanovich et al., 1967) and glyceraldehyde-3-phosphate dehydrogenase (Lange and Pihl, 1960). The loss of activity on irradiation could be almost completely accounted for by destruction of specific sulfhydryl groups. On the other hand, with RNase and chymotrypsin a general protein denaturation has been found to be of major importance for enzyme inactivation. Several studies have shown aggregation of ribonuclease on irradiation (Haskill and Hunt, 1967; Schuessler and Jung, 1967; Schuessler, 1973). It was demonstrated by Schuessler (1967) that EDTA had protective effects on ribonuclease, and prevented the formation of aggregates. From studies with chymotrypsin (Mounter, 1960) it was concluded that 50% of the loss of activity could be due to protein denaturation. With RNase, it has been found that the pattern of amino acid destruction was the same in active and inactive enzyme molecules (Jung, 1967).

Enzymes within cells or tissues resist irradiation much more strongly than those in homogenates or in pure solution. The ribonuclease and deoxyribonuclease activity of Tetrahymena geleii was not affected when a cell suspension was given 300,000 to 500,000 r X-rays, while an homogenate of the organism underwent 50% inactivation under the same conditions (Eichel and Roth, 1953). The mean lethal dose for catalase in crushed potatoes was 5,000,000 rep electrons as compared with only 25,000 rep for catalase in pure solution (Bellamy and Lawton, 1955). With 500,000 rep gamma irradiation, little or no reduction of proteolytic activity of beef
muscle tissue was noticed, with dosage of 1,600,000 rep about 50% of the activity was destroyed (Doty and Wachter, 1955).

The increased radiation resistance of enzymes in vivo may be attributed to the presence of various compounds in cellular environments which may act as radioprotectors or modifiers, etc. Enzymes complexed with other materials were found to be highly radioresistant (Lynn, 1972, 1974; Holladay et al., 1966). Hutchinson (1957) reported that enzymes embodied in lipoproteins were only slightly affected by radiation as compared with free enzymes. The 50% inactivation dose for cathepsin C, in crude extract, was significantly higher as compared with the enzyme in purified fractions (Giovannozzi-Sermani et al., 1969). Musch (1969) found that ionizing radiation of muscle of cod, red fish, coal fish, and haddock required doses of 10 - 20 Mrad for partial inactivation of cathepsin D in tissue, while after extraction, it became several times more sensitive to radiation. Several studies have demonstrated high radiation resistance of proteolytic enzymes in tissue (Klein and Altman, 1972b; Shults et al., 1975; Losty et al., 1973).

An increase in ATPase activity has been observed on low dose irradiation of actomyosin (Coelho, 1969), but higher doses resulted in progressive inhibition of enzyme. Myosin A showed a similar pattern of enhanced enzymatic activity up to a maximum followed by inhibition when submitted to increasing doses of X-rays (Szabolcs et al., 1964). There is ample evidence that under the influence of irradiation,
ATPase activity in tissues is considerably increased (Dale, 1952; Maxwell and Ashwell, 1953).

Giovannozzi-Sermani et al. (1969) found activation of invertase when irradiated in situ. This view was supported by some earlier work on plant tissues (Kuzin and Kopylov, 1960; Berizina, 1962; Vas, 1966). A seven-fold increase in catalase activity was observed in irradiated yeast several hours after irradiation (Aronson et al., 1956). Activation of enzymes has been reported mostly in tissues and not in homogenates or solutions, which indicates that there is some indirect mechanism involved. Desorption of enzymes from cell organelles plays an important role in the altered metabolism of the cell (Okada and Peachy, 1957), but cannot explain the general increase in activity observed when organelles are completely destroyed. Kuzin (1964) suggested the possibility of a decrease in concentration of inhibitors of the enzymes, either by disruption of their synthesis or by a change in the permeability of the cell, enhancing the removal of the inhibitors from tissue. This hypothesis was supported by Kurnick et al. (1959). According to Pauly and Rajewsky (1955), an alteration in permeability of cell membranes is closely related to an increase in enzyme activity after irradiation.

Roth et al. (1962) reported an increased specific activity of β-glucuronidase and acid phosphatase in rat spleen after 700 r of X-radiation. They interpreted this increase to be due to selective retention of enzymes during loss
of spleen nitrogen. Rahman (1962) also found an increase in specific activity of these enzymes in lysosome-rich fractions from rat thymus, after whole body irradiation. He suggested that this increase was due to selective nitrogen loss of the lymphoid tissue. He also noted an increase of dense bodies in the tissue. These dense bodies are considered to be lysosomes (Novikoff et al., 1956), and it was postulated that the increase in enzyme activity was due to a selective retention of enzyme or possibly de novo formation of the lysosomes.

1.2.4. Lysosomal Cathepsins

Cathepsins are a group of intracellular enzymes of animal tissue origin which hydrolyse proteins under acidic conditions. Currently five groups of cathepsins are recognized, although revision is likely as their functions are further characterized. Cathepsins have been reviewed by Barrett (1969, 1972), Barrett and Dingle (1971), and Mycek (1970). A summary of the cathepsin classes from the above reviews is presented here.

1.2.4.1. Cathepsin A (EC 3.4.2.-)

This enzyme splits N-carbobenzoxy-α-L-glutamyl-L-tyrosine, a synthetic substrate for pepsin. Its specificity is towards the carboxy terminal L-amino acid residue of a polypeptide. Cathepsin A probably has little action on proteins alone but acts synergistically with endopeptidases such as cathepsin D. Cathepsin A has optimum pH of 5.0 - 5.4; it is not activated by cysteine and iodoacetamide does not inhibit its activity.
1.2.4.2. **Cathepsin B (EC 3.4.4.-)**

Originally defined as the enzyme from bovine spleen splitting benzoyl-arginine amide (BAA), it now seems that at least two enzymes with this activity were present. The best known of these is cathepsin B1, or simply cathepsin B. It hydrolyses benzoyl-arginine p-nitroanilide (BAPA) and the corresponding 2-naphthylamide (BANA) as well as BAA. A second enzyme, cathepsin B2, hydrolyses BAA but not BAPA or BANA. It is not yet clear whether it is an endopeptidase. Endopeptidase activity as well as transpeptidation reactions are characteristic of cathepsin B. The pH optima for synthetic substrates range from 5.0 to 6.5, but with hemoglobin, its pH optimum is near 4.0. Since a cysteine residue is in the active site, activation can be achieved with cysteine and other sulfhydryls. Iodoacetamide and p-chloromercuribenzoate inhibit its activity.

1.2.4.3. **Cathepsin C (EC 3.4.4.9.).**

Cathepsin C first was recognized as the enzyme deamidating glycyl-L-phenylalaninamide, and is now known to act on glycyl-L-phenylalanine p-nitroanilide and 2-naphthylamide. Cathepsin C is an exopeptidase with broad specificity for splitting of dipeptide naphthylamides and removal of dipeptides sequentially from the amino terminus of a polypeptide chain (hence the alternative names dipeptidyl transferase and dipeptidyl-amino-peptidase I). Optimum pH is near 5.0; it requires a thiol reagent and Cl\(^-\) for maximum activity and is inhibited by thiol blocking reagents.
1.2.4.4. **Cathepsin D (EC 3.4.4.23).**

This is the major acid protease of animal tissue. It has few low molecular weight substrates and is inactive towards the synthetic substrates of cathepsins A, B and C. Cathepsin D is an endopeptidase, its assays are based on release of hydrolytic products from proteins. Maximum activity is toward hemoglobin in the range of pH 3.0 - 4.0. Cathepsin D activity is unaffected by standard thiol reagents, thiol blocking reagents, or metal activators or inhibitors.

1.2.4.5. **Cathepsin E (EC 3.4.4.-).**

An endopeptidase of much more limited tissue distribution than cathepsin D, but closely similar specificity. Cathepsin E is differentiated by higher activity at pH 2.5 on serum albumin as substrate.

1.2.4.6. **Neutral Proteinases (EC 3.4.4.-).**

There are many reports of neutral tissue proteinases, but little is known about their properties. A requirement for thiol reagent is common but not invariable, and some enzymes are activated by EDTA, while other require Ca$^{2+}$.

Other lysosomal proteolytic enzymes are: carboxypeptidase, dipeptidase, and dipeptidyl aminopeptidase. Enzymes acting on collagen represent a group of neutral proteinases in various tissues, but Schaub (1964) and Etherington (1972) described a collagenase from rat organs and muscle that was active on insoluble collagen at pH 3.3 - 3.5. The study of collagenase and neutral proteinases is made difficult due to instability of the enzymes and occurrence of
potent inhibitors in many tissues.

1.2.5. **Lysosomes and Lysosomal Cathepsins in Tissue**

Autolytic degradation of major tissue components has been attributed to the action of a group of enzymes of particulate hydrolases with acid pH optima (de Duve, 1963) as well as neutral protease (Okitani and Fujimaki, 1972). The cytoplasmic particles which envelop these enzymes are called lysosomes. The lysosomal complex includes several cathepsins as well as other hydrolytic enzymes.

Cathepsins have been isolated from a variety of tissues. Three enzyme fractions were separated from beef muscle, with optima at pH 5, 8-9, and 10, when using soluble protein from muscle of this species as substrate (Sliwinski et al., 1961). Parrish et al. (1969) presented evidence that cathepsin from bovine diaphragm muscle was lysosomal. The comprehensive work of Stagni et al. (1968) showed that lysosomes are present in the rat and bovine skeletal muscles. Landmann (1963) indicated that proteolytic activity in beef muscle was due to two enzyme systems: one strongly activated by ferrous ions with optimum activity at pH 5.0, and the other activated by EDTA with optimum activity at pH 9.0. They demonstrated the presence of cathepsins B and C. Randall and MacRae (1967) also reported presence of cathepsins B and C in water soluble proteins of bovine skeletal muscle.

Bodwell and Pearson (1964) were unable to attribute the proteolytic activity in an extract of bovine skeletal
muscle to either cathepsin B or C. They concluded that the enzyme fraction obtained displayed endopeptidase activity similar to that of cathepsin A. Lutalo-Bosa and MacRae (1969) demonstrated the presence of cathepsins B, C, and D in bovine skeletal muscle.

Caldwell and Grosjean (1971) reported the presence of cathepsins A, B, C, and D in chicken breast muscle, which was confirmed by the observations of Iodice et al. (1972). Martin and Whitaker (1968) isolated and purified cathepsin D from chicken leg muscle using ammonium sulfate fractionation and chromatographic techniques. Fukushima et al. (1971) were able to obtain highly purified cathepsin D from chicken muscle by column chromatography and gel filtration. Barret (1970) demonstrated the presence of three isoenzymes of cathepsin D in chicken liver.

Various chemical changes take place in muscle proteins during storage. Sarcoplasmic proteins are readily hydrolysed by cathepsins as compared with fibrous and extracellular proteins (Sharp, 1963; Bodwell and Pearson, 1964). Suzuki et al. (1969) reported that soluble protein was most rapidly hydrolysed, followed by myosin A, actin and myosin B, extracted from rabbit muscle.

It has been demonstrated that cathepsin D contributes to the first step of the degradation of intracellular proteins (Iodice et al., 1966). Caldwell (1970) suggested that hydrolysis of proteins by cathepsin D could be augmented by supplementary action of other cathepsins present in
tissue. This view was supported by the studies of Liao-Haung et al. (1971). Firfarova and Orekhovich (1971) suggested the existence of an inactive precursor of cathepsin D, which is activated on release from lysosomes.

Ever since the early report of Hoagland (Hoagland et al., 1917) that proteolysis was an important factor contributing to post-mortem changes in muscle, it has been attractive to suppose that proteolytic enzymes, possibly cathepsins, were causative agents of many of the important post-mortem changes observed in muscle. It has been observed that certain areas of the myofibrils such as junction of light and heavy meromyosin sections of myosin and the tropomyosin-troponin complex are very vulnerable to proteolytic cleavage (Ebashi and Kodama, 1966). The other site for post-mortem proteolysis of myofibrils is at or near the Z-line. Stromer et al. (1967a) have shown that trypsin very quickly removes the Z-lines from myofibrils, and they also showed evidence that post-mortem storage causes extensive degradation of the Z-line. Moreover, both Busch (1969) and Penny (1968) found that Z-line degradation occurred only during post-mortem storage of intact muscle, which would contain catheptic enzymes found in situ in either blood or muscle and not during storage of myofibrils prepared from at-death muscle; such myofibrils would not contain catheptic enzymes. Henderson et al. (1970) also observed degradation of Z-lines in post-mortem muscle. Reville et al. (1971) reported disruption of myofibrils accompanied by an increase in non-sedimentable
cathepsin D activity after 15 days post-mortem storage.

Even though some of the studies have discredited the role of proteolysis in post-mortem muscle changes (Goll et al., 1970; Sharp, 1963; Locker, 1960), the concept of limited proteolysis has been put forward by Goll et al. (1971). They have suggested that uncoupling of the Ca$^{2+}$ pump by proteolysis may result in loss of Ca$^{2+}$ accumulating ability of sarcoplasmic reticular membranes, causing an increase in free intracellular Ca$^{2+}$ concentration. An increased free intracellular Ca$^{2+}$ concentration causes shortening or isometric tension development in either living or post-mortem muscle. They also theorized that proteolysis involving Ca$^{2+}$ causes modification of actin-myosin interaction as well as loss of Z-disk integrity; these are considered to be possible causes of the resolution of rigor mortis (Goll et al., 1970).

Recently Busch et al. (1972) isolated an endogenous factor (CASF - Ca$^{2+}$-activated sarcoplasmic factor) from rabbit skeletal muscle which in the presence of Ca$^{2+}$ causes removal of Z-lines. They proposed that this factor might have been released from lysosomes and that following removal of Z-lines, the rest of the myofibrils may be susceptible to catheptic enzymes. Recently, Suzuki and Goll (1974) characterized CASF as a proteolytic enzyme which, besides removing Z-lines, solubilized proteins from myofibrils.
1.2.6. Lysosomal Concept

In the early 1950s, de Duve and co-workers realized that rat liver acid phosphatase was associated with a new class of cytoplasmic granule, the "lysosomes" (Appelmans et al., 1955; de Duve et al., 1955; de Duve, 1964, 1963a, 1963b). The lysosomes have since been shown to contain over 50 enzymes capable of catabolizing macromolecules of the cell. A partial list of the enzymes includes: deoxyribonuclease, ribonuclease, esterases, lipases, phosphatases, glucoside hydrolases and peptidyl amino acid hydrolases. Some of these enzymes may not be present in all lysosomes. In general these enzymes are hydrolytic and have acidic pH optima (Barrett, 1972).

Lysosomes are characterized by a general property, the structure linked latency of their enzymes. This latency was considered to be due to the presence of a limiting membrane-like barrier of lipid-protein which restricted the accessibility of the internal hydrolases to any external substrate (Tappel et al., 1963). The examination of pellets of "lysosome-rich" subcellular fractions of rat liver (Novikoff et al., 1956) and of rat kidney (Shibko and Tappel, 1965) showed they contained a large number of distinct particles, each limited by a single membrane. Such particles were either dense bodies or showed one or more internal cavities, sometimes lined with a broad layer of dense material or containing a clump of material. Lysosomes may vary in shape, but generally they have a diameter of 0.25 to 0.50 μm (Wilson
The major function of lysosomes appears to be the intracellular digestion of particles ingested into the cell by endocytosis (de Duve and Wattiaux, 1966; Strauss, 1964). Localized autolysis associated with lysosomes has also been demonstrated (de Duve and Wattiaux, 1966; Miller and Palade, 1964). Specific functions of lysosomes have been documented in detail by de Duve and Wattiaux (1966) and Dingle and Fell (1969a, 1969b).

1.2.7. Heterogeneity of Lysosomes

Lysosomes may differ quite widely from each other in a number of properties such as size, structure, enzyme content, density in various media, and sedimentation coefficient. Lysosomes from various species were shown to have lower specific activities of β-galactosidase, aryl sulfatase, and β-glucuronidase in muscle relative to organ tissue (Shibko et al., 1963; Shibko and Tappel, 1964). It is unlikely that all the lysosomal hydrolases are contained within each lysosome since, for example, rat liver lysosomes do not appear to behave as enzymically homogeneous particles (de Duve, 1963).

Shibko and Tappel (1964) reported that liver and kidney lysosomes appeared to have the same enzyme complement, but the liver lysosomes sedimented mainly in the light mitochondrial fraction, while kidney lysosomes sedimented between the nuclear and mitochondrial fractions. After gradient
centrifugation of a crude rat liver lysosomal fraction the ratios of three acid hydrolases were found to vary between different fractions of the gradient, indicating the heterogeneity of lysosomal enzyme content (Futai et al., 1972). Zonal gradient centrifugation of rat liver lysosomes by Rahman et al. (1967) indicated that acid phosphatase and cathepsin C belonged to one group of lysosomes, and acid ribonuclease and cathepsin D to another. Harikumar et al. (1974) found that chicken muscle lysosomes were relatively more stable than liver lysosomes under similar conditions of incubation and osmotic protection.

These observations indicate that lysosomes are heterogeneous organelles and that they differ among and within the tissues of the same animal.

1.2.8. Stability of Lysosomes

Stability of isolated lysosomes is affected considerably by environmental conditions like osmotic pressure, temperature, pH, ambient ions, and other physical and chemical treatments.

Appelmans and de Duve (1955) demonstrated the importance of osmotic protection by a rapid release of acid phosphatase from rat liver lysosomes that were suspended in distilled water. Increasing sucrose concentration at 0°-4°C had a stabilization effect on lysosomes; maximum stability could be achieved by 0.20 - 0.25M sucrose in the case of lysosomal suspensions of rat liver and spleen (Appelmans and de Duve,
1955; Gianetto and de Duve, 1955; Rahman, 1963), guinea pig liver (Turnbull and Neil, 1969), and muscle (Stagni and de Bernard, 1968).

Elevated incubation temperatures decrease the stability of lysosomes. Dingle (1961) found that incubation temperatures above 30°C increased the susceptibility of lysosomes to rupture, while this effect was not prominent when rat liver lysosomes were incubated in 0.25M sucrose at temperatures over the range of 1°C-30°C for 45 minutes; longer incubation times decreased the stability of lysosomes at temperatures from 5°C-30°C (Sawant et al., 1964c). Acid phosphatase was almost completely released from rat liver lysosomes after two hours at 37°C, but little release occurred at 0°C (Rahman, 1964). Higher temperature of 45°C caused comparatively more release of enzyme than at 37°C (Ignarro, 1971). To study the labilization, incubation at 37°C has been used (Weissmann and Thomas, 1963; Sawant et al., 1964a; Balasubramaniam and Deiss, 1965; Bird et al., 1968).

Sawant et al. (1964a) reported an increased availability of rat liver lysosomal enzymes in acid and alkaline pH ranges, with a maximum stability between pH 6.8 and 7.2. Rat liver lysosomes were found to be labile at or below pH 5.0 (Appelmans and de Duve, 1955; Dingle, 1961). Bovine thyroid lysosomes were most stable between pH 5.1 and 7.3 (Balasubramaniam and Deiss, 1965), rat kidney lysosomes between pH 6.0 and 7.0 (Shibko and Tappel, 1965), and chicken
muscle lysosomes between pH 5.0 and 6.0 (Caldwell and Grosjean, 1971).

Inorganic salts, depending on ionic strength, have been shown to enhance or retard the release of lysosomal enzymes. Gianetto and de Duve (1955) reported isotonic NaCl at 0°C was not sufficient to retain the integrity of rat liver lysosomes without the presence of 0.25M sucrose. Ignarro (1971) reported a decreased lysosomal stability in the presence of sodium ion. Rat liver lysosomal stability was unaffected by 1mM Ca\(^{2+}\), stabilized by Zn\(^{2+}\), and labilized by Cu\(^{2+}\) and Hg\(^{2+}\) (Chvapil et al., 1972). Sawant et al. (1964a) reported an increased availability of aryl sulfatase with addition of 5mM of Ca\(^{2+}\) or Mg\(^{2+}\), but this effect was reduced with EDTA. Hayashi et al. (1973) reported enhanced proteolytic activity caused by addition of Mg\(^{2+}\) to intact rat liver lysosomes. Increasing concentrations of Na\(^{+}\) and K\(^{+}\) had a labilizing effect on lysosomes, but Ca\(^{2+}\) and Mg\(^{2+}\) resulted in a biphasic solubilization of lysosomal hydrolases, indicating reduced solubility of enzymes at lower concentration (2 - 10mM) and increased solubilization at higher concentration (Verity et al., 1968). Allen and Lee (1972) reported a labilizing effect of K\(^{+}\) on isolated lysosomes.

Release of lysosomal hydrolases by severe mechanical blending is well known (Gianetto and de Duve, 1955; Baccino et al., 1971; Parrish and Bailey, 1967). Freezing and thawing also released lysosomal enzymes (Gianetto and de Duve,
1955; Sawant et al., 1964c; Parrish and Bailey, 1967; Baccino et al., 1971). Nonionic detergents such as Igepal-630 and Triton X-100 were found to be quite effective in labilizing the lysosomal membrane (Rahman, 1963; Stagni and de Bernard, 1968; Baccino et al., 1971).

Radiation treatment has been found to decrease the stability of lysosomes. Desai et al. (1964) reported release of lysosomal hydrolases after exposure to gamma radiation. Watkins (1970) reported solubilization of $\beta$-glucuronidase, acid phosphatase, and N-acetyl-$\beta$-glucosaminidase from rat liver, kidney, and spleen lysosomes after gamma radiation treatment. Electron and neutron irradiation also released lysosomal enzymes from rat spleen lysosomes (Watkins and Deacon, 1973). Release of lysosomal hydrolases in various tissues after whole body irradiation has been reported (Warrier et al., 1972; Rahman, 1963; Valet and Bauer, 1969; Kocmierska-Grodzka and Gerber, 1974; Krasnikov et al., 1973; Roth et al., 1962). However, Sottocasa et al. (1965) failed to find any release of $\beta$-glucuronidase or $\beta$-galactosidase from the mitochondrial-lysosomal fraction of heart after irradiation treatment.

The original lysosomal concept envisioned simultaneous release of the enzymes when the lysosomes ruptured. However, the release of various enzymes in response to the different labilizing treatments documented above was not always uniform. Weissmann and Thomas (1963) reported a release of $\beta$-glucuronid-
dase to the extent of 7% to 9%, while acid phosphatase increased from 15% to 23% during incubation. In rat liver, aryl sulfatase, acid phosphatase and ribonuclease showed differential availability after incubation at different times, pHs, osmotic pressures, and temperatures (Sawant et al., 1964a). Freeze-thawing treatment was effective in releasing all the aryl sulfatase and β-glucuronidase, but acid phosphatase and ribonuclease remained partly associated with the membrane (Sawant et al., 1964c). Five enzymes from beef heart lysosomes were liberated in different proportions with various concentrations of sucrose or Triton X-100 (Romeo et al., 1966). A graded release of β-galactosidase, β-glucuronidase, cathepsin, and ribonuclease from rat and beef skeletal muscle lysosomes resulted from increasing Triton X-100 concentrations (Stagni and de Bernard, 1968). Verity et al. (1968) found that acid phosphohydrolase, N-acetyl glucosaminidase and β-glucuronidase responded individually to mono and divalent ion concentrations.

Differences in solubilization of the same enzyme from different tissues was reported by Rahman (1964). Rat liver lysosomes almost completely released acid phosphatase in two hours at 37°C, but little release occurred at 0°C. Spleen and thymus lysosomes failed to release acid phosphatase into solution at either 0°C or 37°C. Watkins (1970) demonstrated that rate of post-irradiation release of β-glucuronidase differed among rat kidney, liver, and spleen.

Differential release of aryl sulfatase, β-glucuronidase,
and acid phosphatase from gamma irradiated lysosomes has been reported by Desai et al. (1964). Aryl sulfatase and β-glucuronidase were most readily released followed by acid phosphatase, while ribonuclease showed no release. Watkins (1970) found a two-fold increase in β-glucosaminidase and β-glucuronidase, but acid phosphatase had an increased soluble activity of only 50% after a dose of 20 Krads. Watkins and Deacon (1973) found a bi-phasic dose response curve for the release of β-glucosaminidase whereas the curve for β-glucuronidase was linear with the dose; moreover, response to exposure of electron or neutron irradiation was different.

The release of enzymes from lysosomes reflected both the membrane character of the organelle and the presence of different enzyme-membrane bonds conferring structure-linked latency upon individual lysosomal enzymes (Sawant et al., 1964a; Verity et al., 1968).

1.2.9. Ultrastructure of Lysosomes

The work of de Duve and his associates (1959, 1963) resulted in the concept of lysosomes as membrane-limited cytoplasmic particles containing hydrolytic enzymes. This concept has been further supported by morphological studies (Essner and Novikoff, 1961; Novikoff, 1963) which have shown that particles marked by a histochemical acid phosphatase reaction were surrounded by a single outer membrane. Novikoff et al. (1956) found the activity of acid phosphatase
associated with "dense bodies" 0.25 to 0.5μm in diameter in their isolated subcellular fraction. Baudhuin et al. (1965) observed similar "dense bodies" and found good correlation between specific activities of acid phosphatase and deoxyribonuclease and frequency of dense bodies in particulate fractions from rat liver. The lysosomal nature of these dense bodies is well established and supported by both biochemical data (Wattiaux et al., 1963) and cytochemical staining for acid phosphatase at the electron microscopy level (Essner and Novikoff, 1961; Trump and Ericsson, 1964).

Lysosomes form an extremely heterogeneous population, at least in the morphological sense, as a result of their functional activities being responsible for the digestion and for the storage of material ingested by the cell (Daems et al., 1972; de Duve, 1963; de Duve and Wattiaux, 1966).

However, lysosomes have a single limiting membrane which, because of its larger dimensions and its asymmetrical structure, often can be distinguished from the limiting membranes of other cell organelles (Daems et al., 1972). Primary lysosomes contain a number of hydrolases not directly observable in routine preparations, while secondary lysosomes contain, in addition to this, material previously ingested by the cell. The ingested material may vary widely in nature and also can be present in various stages of digestion (de Duve and Wattiaux, 1966; Daems and Wisse, 1966).
A general feature of lysosomes is electron density of their matrix, limited by a single membrane (Novikoff et al., 1956; Baggiolini et al., 1969). Most of the lysosomes could be characterized by the presence of an electron-lucid rim or halo beneath their limiting membrane (Daems, 1966). Electron microscopical methods for the demonstration of enzyme activity established the presence of acid phosphatase activity in structurally characterizable single-membrane-limited bodies in many types of cells (Straus, 1967). This group includes cell organelles known as multivesicular bodies (Sotelo and Porter, 1959). Acid phosphatase activity has been demonstrated in such multivesicular bodies in several types of cells (Novikoff et al., 1964; Smith and Farquhar, 1966; Björkman and Sibalin, 1967; Friend and Farquhar, 1967; Holtzman and Dominitz, 1968; Locke and Collins, 1968), and they are also reported to be involved in the uptake and digestion of proteins (Becker et al., 1967; Smith and Farquhar, 1966). It was first suggested by Bennet (1956) that as a result of lysosomal digestion of material ingested by the cell, indigestible residues remain behind in the secondary lysosomes. Since secondary lysosomes can perform successive acts of digestion (de Duve, 1963; Gordon et al., 1965; de Duve and Wattiaux, 1966), they show accumulation of residues. Secondary lysosomes, having reached the stage of being thus filled with the residues of digestion, develop into what de Duve and Wattiaux (1966) suggested to be called
telolysosomes. Residue-loaded bodies have been characterized by the abundant presence of myelin figures, electron dense pigments, and droplets having a lipid nature and do not show characteristically high enzymatic activities on the basis of their lysosomal origin (Beck et al., 1972; de Duve and Wattiaux, 1966; Smith and Farquhar, 1966; Beaulton, 1967; Parker et al., 1965; Lane and Novikoff, 1965; Miller and Palade, 1964; Frank and Christensen, 1968; Essner and Novikoff, 1961; Fischer et al., 1966). These telolysosomes still contain the lysosomal enzymes; these enzymes are not renewed and are destroyed, resulting in a functionally and enzymatically inactive structure consisting solely of digestive residue. For such bodies, the terms post-lysosome or residual bodies, have been preferred by de Duve and Wattiaux (1966).

Unfortunately, as yet knowledge of internal structure of lysosomes is rather limited and quite insufficient to explain many of the known metabolic features; however, Maunsbach (1969) has listed some structural features and functional equivalents of lysosomes in the proximal tubule cells of the rat kidney. Although spatial distribution within the organelle must await further information as to internal structure of lysosomes, their content of hydrolases capable of splitting various types of peptide bonds is well established (Barret and Dingle, 1971; Tappel, 1969; Barrett, 1972). Structure-linked latency of the lysosomal enzymes is a well-known phenomenon. Two mechanisms have been proposed
to account for the latency of these enzymes, the membrane theory and the matrix binding theory. The membrane theory has been suggested by de Duve and associates (de Duve et al., 1955; de Duve, 1959, 1963) and proposes that a lysosome is a simple osmotic sac which is delimited by a lipoprotein membrane. The impervious membrane serves as a physical barrier to restrict the physical freedom and substrate accessibility of the enclosed enzymes, which are thought to be present within the intact lysosomes in a diffusible fully active form (de Duve, 1963). Koenig (1962, 1964a) advanced the matrix binding theory as a membrane-limited polyanionic lipoprotein granule, containing hydrolytic enzymes in an inert state bound to lipoprotein matrix. Cytochemical observations revealed the acid phosphatase reaction product in electron micrographs associated with lysosomal matrix, but no enzyme product was found in the normally occurring electron-lucent vacuoles of neuronal lysosomes (Koenig, 1968a; Kreutzberg and Hager, 1966). \( \beta \)-glucuronidase reaction product seemed to be absent from electron-lucent vacuoles of liver lysosomes (Hayashi et al., 1968).

Electron microscopic studies have shown that the first ultrastructural change in lysosomes during dilution of the medium was a swelling and increased electron-lucency of the matrix; subsequently, lysosomes rupture in increasing numbers concomitantly with release of acid hydrolases (Koenig, 1967a, 1967b). Shibko et al. (1965) observed that initially rat kidney lysosomes were electron dense and had an intact
membrane. After three hours of incubation at 37°C, when most of the enzyme was solubilized, the membranes were intact, although the lysosomes lost their matrix completely. They concluded that release of acid phosphatase had occurred in two stages: firstly, the enzyme became available to the substrate but remained sedimentable; later, the quantities of soluble enzymes approximately equalled the total amount of available enzyme. Small membranous vesicles inside the lysosome "ghosts" could then be observed, and Shibko et al. (1965) concluded that these represented the remains of an internal membrane structure of unknown function. Electron micrographs of lysosomes that were repeatedly frozen and thawed revealed that complete disruption of particles occurred. Twenty per cent of the enzyme activity remained with the membrane fraction and it was presumed by these workers that lysosomal enzymes were non-specifically adsorbed or mechanically trapped with this fraction. Lucy (1969) suggested that association of enzyme activity with membrane fraction was due to membrane bound enzymes. Beck and Tappel (1968) have studied rat liver β-glucosidase and found that the enzyme remained firmly bound to the membrane after particles were ruptured. It is known that many acid hydrolases remain bound to some extent to the insoluble fraction; part of this binding may be non-specific adsorption, but it is difficult to rule out a genuine appertenance of some hydrolase activities to the lysosomal membrane or matrix (Thinès-Sempoux, 1973).
Experimental evidence based mostly on biochemical studies has accumulated to indicate that lysosomes can be labilized by a great number of substances and treatments (Weissman, 1969; Whiting, 1974; see also section on Stability of Lysosomes). In most of the studies of a biochemical nature, measurement of shifts between "free", "sedimentable", and "unsedimentable" activities of lysosomal enzymes was performed on the lysosome-rich fractions. In investigations of this type, the leakage of hydrolytic enzymes from lysosomes has been demonstrated, but changes occurring at the ultra-structural level have not been elucidated. Brunk and Ericsson (1972) observed leakage of acid phosphatase from structurally intact lysosomes while there was no leakage of thorium dioxide particles, concluding that the enzyme could diffuse through the lysosomal membrane when there were no large holes or ruptures in the membrane. Lodin et al. (1974) observed that at a terminal stage of degeneration, lysosomes lost their simple membranes and dense granular content was diffusely spread in cytoplasm of neurons and glial cells in vitro under malnutritional stress. They also noticed the disappearance of cytoplasmic particles and the appearance of empty spaces.

High doses of whole body or partial body radiation has been reported to produce changes in neuron lysosomes (Kagan et al., 1962; Pick, 1965; Roizin et al., 1966) and redistribution of lysosomal acid phosphatase (Greenspan et al., 1964). Changes in specific activity of lysosomal enzymes
and in the number or intracellular distribution of cytochemical demonstrable lysosomes have been observed in a number of irradiated tissues, both normal (Horvath and Touster, 1967; Hugon and Borgers, 1966) and malignant (Carney, 1965; Leener and Evans, 1969). Rahman (1962) observed an increase in specific activities of acid phosphatase and β-glucuronidase in the lysosome-rich fraction of rat thymus after whole body X-radiation; moreover, some of the lysosomes showed an internal cavity.

Disruption of the lysosome-rich granule fraction has been reported (Harris, 1966a, 1966b). Structural changes or altered permeability of lysosomal membrane have been suggested as the cause of enzyme leakage from lysosomes after radiation (Aikman and Wills, 1974a, 1974b; Wills and Wilkinson, 1966; Desai et al., 1964; Watkins, 1970; Harris, 1970), or due to other treatments (see section on Stability of Lysosomes). Most of these studies are based on biochemical and/or cytochemical data, but detailed ultrastructural studies have not been reported.

1.2.10. Ultrastructure of Muscle

Transmission electron microscopy has been used to study the ultrastructural details of striated skeletal muscle (Bendall, 1969; Slautterback, 1966). The presence of interdigitating filaments of actin and myosin are revealed in electron micrographs of longitudinal sections. The hexagonal arrangement of actin filaments around the
centrally located myosin can be seen in cross-sectioned fibres (Huxley, 1965).

During post-mortem storage of muscle, ultrastructural changes have been observed. Storage of muscle at low temperatures has been shown to cause cold shortening of the muscle during the onset of rigor (Bate-Smith and Bendall, 1949; Marsh, 1953). Stromer and Goll (1967) reported a decrease in the length of I bands, but some lengthening of sarcomeres in beef fibrils was observed between 24 and 312 hours post-mortem at 2°C. Some studies have indicated that during aging of beef and chicken muscle, the Z-line disappeared and the actin filaments were weakened at the Z-I junction (Davey and Gilbert, 1967; Fukazawa et al., 1969). It has been observed that treatment of myofibrils with trypsin results in a rapid loss of Z-lines (Goll et al., 1969; Stromer et al., 1967). In addition to Z-line removal, structural degradation of Z-lines is also observed during post-mortem storage of muscle (Davey and Gilbert, 1967, 1969; Fukazawa and Yausi, 1967; Henderson et al., 1970; Stromer et al., 1967). Post-mortem degradation of Z-lines has been most extensively studied by Henderson et al. (1970), who found that Z-lines in rabbit and porcine muscle were more susceptible to post-mortem degradation than Z-lines of bovine muscle. Busch et al. (1972) isolated a protein fraction from sarcoplasm of rabbit skeletal muscle which caused removal of Z-lines in the presence of Ca^{2+}, but had no ultrastructurally detectable effect on the myofibril.
Considerable attention has been given to transmission electron microscopy of sarcoplasmic reticulum, a complex network of vesicles on surfaces of fibrils (Peachy, 1970). The reticulum is considered to be capable of controlling the calcium ion concentration in the sarcoplasm. It has been shown that release of calcium ions from the reticulum causes muscle contraction (Ebashi, 1961; Weber et al., 1963; Ohnishi and Ebashi, 1964; Schmidt et al., 1970). It is known that sarcoplasmic reticular membranes lose their ability to accumulate Ca\(^{2+}\) during post-mortem storage of muscle (Greaser et al., 1967; Nauss and Davies, 1966), thus Busch et al. (1972) suggested that such a release of bound Ca\(^{2+}\) might activate the "sarcoplasmic factor", resulting in Z-line degradation observed during post-mortem storage of muscle (Henderson et al., 1970). Greaser et al. (1969) pointed out that structural features of mitochondria in porcine muscle were changed during a 24 hour aging period, but no structural alterations were observed in sarcoplasmic reticulum. West et al. (1974) observed a loss of Ca\(^{2+}\) accumulating ability in sarcoplasmic reticulum following degradation by trypsin, crude cathepsin fraction, and cathepsin Bl.

Scanning electron microscopy has proven to be a valuable tool for evaluating topographical details of a variety of biological systems. Schaller and Powrie (1971) studied the surface ultrastructure of cryofractured skeletal muscle
from rainbow trout, turkey, and beef at pre-rigor and various post-rigor times. In pre-rigor samples they found elevated transverse elements, presumably a part of sarcoplasmic reticulum which collapsed after storage, and breaks across the fibril occurred at the positions of transverse elements. They also observed perforation of the sarcolemma after post-mortem storage of muscle. Eino and Stanley (1973a) studied surface ultrastructure of post-mortem beef by scanning electron microscopy, and observed a depression of transverse striations followed by extensive fibre breakage during two week storage. Cathepsin treatment of muscle fibres has been reported to cause disappearance of transverse striations, multiple breakage, and some disruption of fibres (Eino and Stanley, 1973b). These authors also reported that collagenase treatment brought about shredding and dissolution of collagen fibres, followed by disintegration of sarcolemma, but had little effect on myofibrils.
2.1. INTRODUCTION

The existence of proteolytic enzymes of lysosomal origin in various skeletal muscle tissues is well established. The intracellular enzymes responsible for the activity in the tissues have been named cathepsins by Willstätter and Bamann (1929). There are several studies indicating that lysosomal cathepsins are capable of intracellular protein degradation (Bohley et al., 1971; de Duve, 1963a; Réville et al., 1971). Isolated lysosomal proteases have been shown to degrade large proteins to free amino acids or small peptides (Coffey and de Duve, 1968; Huisman et al., 1974).

The role of catheptic enzymes in post-mortem proteolysis and meat tenderization is not clear, but there are several reports which suggest release of cathepsins and other lysosomal hydrolases during aging (Dutson and Lawrie, 1974; Eino and Stanley, 1973a; Lutalo-Bosa, 1970; Ono, 1971). Proleolytic enzymes have been held responsible for aging-tenderization of meats (Zender et al., 1958; Davey and Gilbert, 1966) and for the proteolysis in post-mortem muscle (Sharp, 1963; Khan and van de Berg, 1964a, 1964b; Suzuki et al., 1967; Parrish et al., 1969; Okitani and Fujimaki, 1972; Okitani et al., 1973).

Protein breakdown by lysosomal extracts and proteolysis in tissue is considered to be due to the involvement
of various cathepsins as well as other lysosomal hydrolases. Caldwell (1970) suggested joint action of several muscle cathepsins on endogenous muscle proteins. Liao-Haung and Tappel (1971) observed that degradation of hemoglobin was initiated by cathepsin D, and products were further utilized by cathepsin C. Goettlich-Riemann et al. (1971) demonstrated a synergistic and concerted action of cathepsins A, B, and D. Similar results have been reported for cathepsins A and D (Iodice et al., 1966). Lazarus (1973) suggested that probably collagenase and the lysosomal protease such as cathepsin D (Dingle et al., 1971) work synergistically in connective tissue degradation.

The lysosome is a membrane-limited subcellular organelle containing cathepsins and other hydrolytic enzymes capable of intracellular degradation. While these enzymes remain within an intact lysosome they are inactive on external substrates (de Duve, 1963a). For the cathepsins to have a role in the post-mortem modification of muscle, they must be released from the lysosome.

Cathepsins have been thought to cause the proteolysis observed during extended storage of irradiation-sterilized meats (Cain et al., 1958; Pearson et al., 1960; Coleby et al., 1961; Bailey and Rhodes, 1964). Studies of Klein and Altman (1972b) showed an increase in free catheptic activity in chicken skeletal muscle after irradiation treatment. These observations indicate possible release of cathepsins by radiation-induced fragility of tissue lysosomes. However,
it is difficult to determine the extent of lysosomal damage and release of enzymes in intact tissue. Isolated lysosomes offer a good system for such studies, but it is difficult to obtain enough intact lysosomes from skeletal muscle due to few numbers of lysosomes present and the vigorous disruptive treatment needed to extract lysosomes from muscle. For these reasons, most of the studies have been conducted on isolated lysosomal fractions obtained from selected organ tissues.

The release of various enzymes from isolated lysosomes in response to the conditions like pH, osmotic strength, temperature, and other physiochemical treatments is not uniform (Sawant et al., 1964a; Verity et al., 1968; Stagni and de Bernard, 1968). Gamma radiation has been shown to result in differential release of acid phosphatase, β-glucuronidase and aryl sulphatase, but had no effect on ribonuclease from rat liver lysosomes (Desai et al., 1964). Similar differential release of β-glucuronidase, β-glucosaminidase, and acid phosphatase due to ionizing irradiation treatment has been reported by other workers (Watkins, 1970; Watkins and Deacon, 1973). Sottocassa et al. (1965), however, failed to find any effect of X-irradiation on release of lysosomal β-glucuronidase or β-galactosidase from the mitochondrial-lysosomal fraction of heart tissue. Large variations in radiation sensitivity of various lysosomal enzymes from different tissues as well as under different conditions have been encountered in the literature, which makes it
difficult to draw general conclusions regarding lysosomal enzymes, especially cathepsins. In the present study, release of cathepsins A, B, C, and D from isolated chicken liver lysosomes has been investigated.
2.2. EXPERIMENTAL

2.2.1. Materials

Commercial broiler-type chickens (8 - 12 weeks old) were obtained from the University Poultry Farm, and maintained under standard husbandry conditions for about a week prior to slaughter. The birds were sacrificed by exsanguination after fasting for 40 - 48 hours. The livers were rapidly removed and washed free of blood with cold 0.25M sucrose solution and chilled in crushed ice. Excessive fat and connective tissue were removed. All the subsequent operations were carried out at 4\textdegree C unless otherwise indicated.

2.2.2. General Sample Preparation

2.2.2.1. Chicken liver tissue

Chicken liver tissue, cooled in crushed ice, was minced into small slices with scissors prior to irradiation. One portion was kept as a non-irradiated control. Immediately after irradiation, the samples were homogenized in distilled water 1:20 (w/v), using a Sorvall Omnimixer. The homogenization was done at high speed for four 15 second bursts with 30 second cooling intervals between bursts. During homogenization, the mixer container was kept immersed in a crushed ice slurry. The tissue homogenate was centrifuged at 20,200 \times g at 4\textdegree C for 30 minutes in a Sorvall RC2-B refrigerated centrifuge. The supernatant was used for deter-
mination of free cathepsin D activity and expressed as per cent of non-irradiated control values.

2.2.2.2. Isolation of lysosomes.

Deionized water and analytical grade sucrose were used for preparation of sucrose solutions. The solutions containing 0.001M tetrasodium salt of EDTA (Ethylenediaminetetra acetic acid) were adjusted to pH 7.0. The livers were minced and homogenized in 0.25M sucrose (1:8, W/V). Homogenization was for 30 seconds in a Waring blender at top speed. The pH of the homogenate was adjusted to 7.2 with 5 N KOH. The homogenate was filtered through four layers of cheese cloth and then fractionated by sucrose density gradient and differential centrifugation technique according to the scheme outlined in Figure 1, using Sorvall RC2-B refrigerated centrifuge (Sawant et al., 1964c). The purified lysosomal fraction (F IV) was suspended in 0.7M sucrose solution and a 1:2 (w/v) dilution was made on the basis of liver tissue used for isolation of lysosomes. This final suspension was irradiated as described in the irradiation section.

2.2.3. Irradiation

The samples were subjected to varying doses of gamma radiation in a Gamma Cell-220 (Atomic Energy of Canada Ltd.). During irradiation, the samples were kept in crushed ice which was changed periodically when necessary. The chicken
Figure 1. Scheme for the preparation of lysosomes

Homogenate

1. Centrifuged at 750 x g for 10 min.
2. Centrifuged at 3,300 x g for 10 min.

Pellet (discard)  Supernatant
Centrifuged at 16,300 x g for 20 min.

Pellet  Supernatant (discard)
Light mitochondrial fraction (F I)

Resuspended carefully in 0.3M sucrose and centrifuged at 9,500 x g for 10 min.

Pellet  Supernatant (discard)
Light mitochondrial fraction (F II)

Resuspended in 0.45M sucrose and layered over a discontinuous gradient of 0.7M sucrose (bottom layer) and 0.6M sucrose (middle layer), centrifuged at 9,500 x g for 30 min.

Pellet (F III)  Supernatant (discard)

Resuspended in 0.7M sucrose, centrifuged at 5,900 x g for 30 min.

Pellet (discard)  Supernatant carefully decanted, centrifuged at 17,000 x g for 20 min. Pellet washed again and centrifuged to yield purified lysosomes (F IV)
liver tissue slices were subjected to radiation doses of 0, 0.05, 0.10, 0.25, 0.50, or 1.00 Mrad. The lysosomal suspensions received radiation doses of 0, 0.1, 0.25, 0.50, or 1.00 Mrad. The dose rate at the time of irradiation was 0.52 - 0.71 Mrad/hr., using $^{60}$Co as radiation source.

2.2.4. **Light Scattering Properties of Lysosomes**

After irradiation, the lysosomal suspensions were incubated at 37°C in a constant temperature water bath. The absorbance of the suspensions was read at 540 nm after 0, 15, 30, 60, 120, and 180 minutes of incubation using a Spectronic 20 spectrophotometer (Bausch and Lomb). The instrument was set at zero by using irradiated 0.7M sucrose solutions. The decrease in absorbance was used as an index of release of lysosomal enzymes (Sawant et al., 1964c).

2.2.5. **Release of Cathepsins from Lysosomes**

After irradiation, the release of catheptic enzymes from lysosomal suspensions was followed at two different temperatures. One portion of the suspension was incubated at 37°C in a constant temperature water bath and samples were drawn after 0, 15, 30, 60, 120, and 180 minutes. The other portion of lysosomal suspension was kept at 4°C and samples were drawn after 0, 24, 48, and 72 hours. To determine free catheptic activity as a measure of released enzyme, the samples were centrifuged at 17,000 x g for
20 minutes and catheptic activity of the supernatant measured. For determination of total catheptic activity of lysosomes, samples were given freeze-thaw treatment ten times and then centrifuged at 17,000 x g for 20 minutes and the supernatant used for determination of total catheptic activity. In some cases Triton X-100 was used to disrupt lysosomes (final concentration 0.2%).

2.2.6. Enzyme Assays

2.2.6.1. Cathepsin A

Cathepsin A activity was determined according to the procedure of Iodice et al. (1966), using N-carbobenzoxy-α-L-glutamyl-L-phenylalanine (CBZ-glu-phe; Sigma Chemical Co.) as substrate. The reaction mixture (1 ml), containing 0.0152M substrate, 0.04M sodium acetate buffer pH 5, and enzyme, was incubated at 37°C for 2 hours. The reaction was stopped by addition of 1 ml of 10% TCA. The TCA mixtures were heated for 10 minutes in a water bath at 50° - 55°C and centrifuged for 15 minutes at low speed; 0.1 - 0.2 ml aliquotes were used for color development by the ninhydrin procedure of Moore and Stein (1954). Controls without substrate were treated in the same manner.

2.2.6.2. Cathepsins B and C

Cathepsins B and C were measured with benzyol-L-arginine amide and glycyl-L-phenylalanine amide (Sigma Chemical Co.), respectively. The assays were performed at 37°C for 2 hours in 1 ml reaction mixtures containing 0.01M substrate,
0.1M sodium citrate buffer, pH 5.0, 0.04M cysteine (cysteine-HCl) and enzyme (Bowers et al., 1967). The reaction was stopped by addition of 1.0 ml of 5% TCA. The hydrolytic products were measured by microdiffusion technique (Seligson and Seligson, 1951). One ml of saturated potassium carbonate was added and liberated ammonia was absorbed in H$_2$SO$_4$ by rotating the vials on a rotator for 30 minutes and determined spectrophotometrically by the Nesslerization technique.

2.2.6.3. Cathepsin D.

Cathepsin D activity of tissue and of lysosomal preparations was determined according to the method of Anson (1938) with some modifications (Berman, 1967).

The reaction mixture contained 1 ml of supernatant and 2 ml of 2% hemoglobin (Bovine, Type II - Sigma Chemical Co.) in 0.2M acetate buffer pH 3.8. The reaction was carried out at 37°C for 2 hours in a constant temperature water bath with shaking and the reaction was terminated by addition of 2 ml of 10% TCA. The samples were kept overnight at 4°C and then filtered through Whatman No. 4 filter paper. The blanks were also prepared in a similar manner, but were kept at 4°C instead of incubating at 37°C. The absorbance of the filtrate was read against respective blanks at 280 nm on a Unicam SP 800B.U.V. spectrophotometer (Pye-Unicam Ltd.). The increase in absorbance was expressed as enzyme activity. All measurements were performed in duplicate.
2.3. RESULTS AND DISCUSSION

2.3.1. Proteolytic Activity of Tissue as Affected by Gamma Radiation

To evaluate the effect of irradiation on catheptic enzymes and their release from lysosomes, chicken liver was selected as the source of lysosomes because skeletal muscle contains a relatively low lysosomal content as well as low catheptic activity. In liver tissue, a low dose of irradiation (50 and 100 Krad) enhanced the catheptic activity significantly (P<0.05) as shown in Table 1, but higher doses (0.25, 0.5, and 1.00 Mrad) resulted in relatively smaller increases (Table 2).

Table 1. Effect of low doses of gamma radiation on free catheptic activity of chicken liver tissue.

<table>
<thead>
<tr>
<th>Dose (Krad)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0$^a$</td>
</tr>
<tr>
<td>50</td>
<td>118.7$^b$</td>
</tr>
<tr>
<td>100</td>
<td>122.2$^b$</td>
</tr>
</tbody>
</table>

Means with different letters are significantly different (P<0.05).
n = 4
Table 2. Effect of high doses of gamma radiation on free catheptic activity of chicken liver tissue.

<table>
<thead>
<tr>
<th>Dose (Mrad)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.25</td>
<td>113.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.50</td>
<td>112.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.00</td>
<td>111.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means with different letters are significantly different (P<0.05). n = 5

Higher doses of irradiation might have resulted in partial inactivation of enzymes or radiation denaturation of the tissue proteins, thus decreasing extraction of enzymes, resulting in a slight drop in free enzyme activity (Tables 1 and 2). These results are in agreement with the work of Coelho (1969), who noticed an increase in ATPase activity at lower doses and progressive inhibition at higher doses of gamma radiation. A similar pattern for ATPase was reported by Szabolcs et al. (1964) upon X-radiation of myosin A. Other studies have indicated extensive protein alterations in irradiated animal tissue (Cain et al., 1958; Bautista et al., 1961). Klein and Altman (1972b) observed an increased proteolytic activity in chicken leg and breast muscle at low irradiation doses up to 0.2 Mrad, but higher doses inhibited enzyme activity; they also observed changes in
soluble protein fractions after incubation using disc electrophoresis. Some studies have indicated that irradiation has fragmentary action on tissue proteins (El-Badawi et al., 1964); such changes would render the tissue proteins more susceptible to enzyme attack. Cathepsin D, an endopeptidase, may have a limited contribution to such changes, but other enzymes like cathepsins A, B, and C might be involved in degradation of fragmented proteins.

In studies with fish muscle, Musch (1969) reported no increase in catheptic activity, but noticed that cathepsins were highly radiation resistant and required very high doses of 10 - 20 Mrad for even partial inactivation. It is likely that small changes in enzyme activity were not detectable in the above studies, as muscle was used as an enzyme source, and it contains a very low level of catheptic activity. Secondly, small increases in enzyme activity probably would be cancelled out due to partial inactivation of enzymes after irradiation. This may explain, to some extent, why Doty and Wachter (1955) found little or no decrease in proteolytic activity of beef muscle after 500,000 rep gamma radiation.

Various explanations have been given for an irradiation-induced increase in enzyme activity. Okada and Peachy (1957) supported the view that desorption of enzymes from cell organelles plays an important role, but it can not explain the general increase in activity observed when the
organelles are completely destroyed. Kuzin (1964) suggested that irradiation treatment may decrease the enzyme inhibitors either by disruption of their synthesis or by a change in permeability of the cell enhancing removal of inhibitors from tissue. According to Pauly and Rajewsky (1956), an alteration in permeability of cell membranes is another possibility related to enzyme increase. Roth et al. (1962), as well as Rahman (1962), suggested that an increase in specific activity of acid phosphatase was due to selective retention of enzymes during loss of tissue nitrogen. In the present study, alteration in cell metabolism cannot be a cause of the increased enzyme activity as post-mortem liver tissue was used; also, cell organelles were disrupted, and there would be no synthesis of inhibitors. If changes in cell permeability enhance the removal of inhibitors from tissue, they would be extracted easily and would have depressed the catheptic activity of tissue supernatant. Loss of tissue nitrogen as a possible reason for the increase in the specific activity of tissue enzymes, as suggested by Rahman (1962) and Roth et al. (1962) would not affect the results of the present study as the results are not based on nitrogen content of tissue. Changes in cell permeability and desorption of the enzyme seem to be logical reasons for an increase in the extractability of enzymes from tissue and hence an increase of free enzyme activity as observed in this study.
It was obvious from these results that the complexity of the tissue system prevented definitive conclusions from being easily drawn. This complexity necessitated the use of relatively simpler and well-defined systems. For this purpose, isolated chicken liver lysosomes were used in subsequent studies.

2.3.2. Light Scattering Properties of Lysosomal Suspension

Light scattering has been used as an index of change in the shape of particles and the integrity of lysosomes under various pH and temperature conditions (Sawant et al., 1964c) or irradiation treatment (Harris, 1966a, 1966b). Badenock-Jones and Baum (1974) have recently shown that lysis of rat kidney lysosomes resulted in a decrease in the absorbance parallelled quantitatively and temporally the release of soluble acid phosphatase. The authors concluded that measurement of changes in absorbance is a valid method to measure the changes in lysosomal integrity.

In the present study, irradiation of lysosomal suspensions caused a significant (P<0.01) change in absorbance at 540 nm (Figure 2). There was a sharp decrease in absorbance during initial 15 - 30 minutes incubation at 37°C in all the irradiated and control samples, although the decrease due to irradiation treatment was significantly (P<0.05) greater than the non-irradiated controls up to 60 minutes of incubation. Upon longer incubation, there was a slow
decrease in absorbance, and doses up to 0.50 Mrad still had significantly (P<0.05) lower absorbance than the control, while 1.0 Mrad treatment did not produce any further decrease in absorbance after 60 minutes of incubation. An aggregation of the particles was observed in 1.0 Mrad treatment after 120 minutes of incubation, which might have been responsible for higher absorbance.

The correct interpretation of the decrease in absorbance of lysosomal suspensions after exposure to gamma radiation is not certain. It has been observed that a suspension of sarcosomes (Carney, 1965) and thymocytes (Myers and DeWolf-Slade, 1964) showed decreased turbidity as they swelled following irradiation. Mitochondria have a higher capacity for swelling and contraction accompanied by absorbance change (Packer et al., 1968). It is uncertain whether such an explanation would be applicable to irradiated lysosomes. Lysosomal suspensions have been reported to exhibit decreased absorbance after treatment with various agents such as acid (Cohn and Hirsch, 1960), Triton X-100 (Badenock-Jones and Baum, 1974), neutral steroids (Weissman, 1965; Badenock-Jones and Baum, 1974), ultraviolet radiation (Cohn and Hirsch, 1960), and gamma radiation (Harris, 1966a).

Concerning the decrease in the absorbance of lysosomes, the present study agrees with the report of Harris (1966a, 1966b), indicating that radiation damages the lysosomes, perhaps causing alterations in lysosomal membrane, resulting
Figure 2. Effect of gamma radiation on light scattering properties of lysosomes. (n = 6)
in permeability changes, and subsequently causing disruption of lysosomes. Such radiation effects seem to be amplified by elevated temperatures (Harris, 1966a). A decrease in the absorbance of lysosomal suspensions has been accompanied by an increase in free activity of various lysosomal enzymes, which supports the concept of irradiation-induced damage to lysosomal structure. Release of lysosomal enzymes from irradiated lysosomes is discussed in the following sections.

2.3.3. Release of Lysosomal Cathepsins

It is well established that lysosomes are membrane-limited particles containing various hydrolytic enzymes. One important group of these is tissue proteases, commonly termed as cathepsins. These enzymes are considered to be responsible for protein breakdown in tissue. Cathepsin D is an endopeptidase which hydrolyses only proteins, while cathepsins A, B, and C are exopeptidases and degrade peptides, but concerted action of cathepsins A, B, and C along with cathepsin D on proteins has been demonstrated in several studies (Iodice et al., 1966; Goettlich-Riemann et al., 1971; Liao-Huang and Tappel, 1971). Isolated lysosomal proteases are capable of degrading large proteins to free amino acids or small peptides (Coffey and de Duve, 1968; Huisman et al., 1974). Intralysosomal protein degradation has been demonstrated by studies of Neely and Mortimore (1974), Brostrom and Jeffay (1970), Segal et al. (1974), and
Davies et al. (1971). All these studies indicate that lysosomal cathepsins are involved in protein degradation. Intralysosomal protein degradation is of limited importance in post-mortem muscle, but extralysosomal proteolysis seems to play a significant role; in the latter case, availability of lysosomal enzymes by way of leakage and release from lysosomes is a limiting factor. Some studies on the release of cathepsins A, B, C, and D as influenced by gamma radiation are presented.

2.3.3.1. **Cathepsin D.**

A decrease in absorbance of irradiated lysosomal suspension when incubated at 37°C (as shown in Figure 2) indicated that gamma radiation affects the integrity of lysosomes, and thereby might result in release of lysosomal enzymes. Release of cathepsin D from irradiated lysosomes was followed at two different temperatures. In lysosomal samples incubated at 37°C after gamma irradiation, there was a sharp increase in free cathepsin D in the first 15 - 30 minutes, almost reaching maxima at 60 minutes in all irradiated samples (Figure 3). In contrast, free enzyme activity of non-irradiated control increased more slowly, and reached a maximum after 180 minutes of incubation. The mean free enzyme contents of suspensions irradiated with 0.10, 0.25, or 0.50 Mrad were significantly higher (P<0.05) compared with the control throughout the 180 minutes of incubation at 37°C. Samples receiving a dose of 1.0 Mrad followed a similar
Figure 3. Free cathepsin D activity of lysosomal suspension as influenced by gamma radiation and incubation at 37°C. (n = 5)
pattern of increase in free enzyme content up to 60 minutes of incubation and then slowly decreased; a reverse pattern was also exhibited by the absorbance readings (Figure 2). An aggregation of particles in 1.0 Mrad samples was noticed after 120 minutes of incubation at 37°C, and may be responsible for higher absorbance values. The low free enzyme activity might be due to inactivation of the enzyme on prolonged incubation at 37°C and/or adsorption of the enzyme due to aggregation of particles.

Data summarizing the release of cathepsin D after irradiation at 4°C are presented in Figure 4. The increase in free enzyme activity was considerably slower at 4°C than at 37°C (Figure 3). There was a rapid increase in free enzyme activity in irradiated samples in the first 24 hours. The controls exhibited a negligible increase in free enzyme content during the initial 24 hour incubation at 4°C, then increased steadily during subsequent incubation of 48 and 72 hours. Control samples had significantly lower (P<0.01) free enzyme activity compared with irradiated samples throughout the incubation period of 72 hours. In Figure 5, results of the release of cathepsin D at 4°C are presented as percentages of the total activity of the respective treatments. In this case, the inactivation effect of irradiation is excluded and the data reflect activity in relation to the actual potential activity of the enzyme. The general pattern of enzyme release is similar to that shown in Figure 4.
Figure 4. Free cathepsin D activity of lysosomal suspension as influenced by gamma radiation and incubation at 4°C. (n = 5)
Release of cathepsin D in all the irradiated samples sharply increased during the first 24 hours of incubation, and then increased slowly during subsequent incubation. Control samples showed very slow release in the first 24 hours, but rate of release was higher between 48 - 72 hours of incubations. The enzyme release was 55.63, 87.22, 80.35, 84.17, and 81.56 percent in samples irradiated with 0.0, 0.10, 0.25, 0.50, and 1.00 Mrad respectively after 72 hours of incubation.

The observed increase in free cathepsin D activity from lysosomal particles is most probably due to the release of bound enzymes and leakage of enzymes due to radiation damage to lysosomes. Various radiation-induced mechanisms are possible for such changes in lysosomal structure. Desai et al. (1964) showed that aryl sulfatase, β-glucuronidase, and acid phosphatase were released after treatment of lysosomal suspension with gamma radiation. They suggested a free radical mechanism leading to enhanced lipid peroxidation, presumably of lysosomal membrane lipid constituents, resulting in lysosomal membrane damage. Similarly, release of lysosomal enzymes has been correlated with induced lipid peroxidation (Tappel, 1962). Wills and Wilkinson (1966) reported that electron radiation caused release of lysosomal enzymes, suggesting that lipid peroxide formation leads to rupture of lysosomal membrane, causing release of hydrolytic enzymes. Harris (1966a, 1966b) observed a decrease in absorbance of lysosomal suspensions after gamma irradiation, and concluded
Figure 5. Release of cathepsin D from lysosomes as influenced by gamma radiation. (n = 3)
that a release of enzyme had occurred on the basis of an increase in soluble protein after irradiation treatment. Watkins (1970) demonstrated that radiation (electron radiation 5 - 20 Krad) induced release of β-glucuronidase, N-acetyl-β-glucosaminidase, and acid phosphatase from rat spleen lysosomes. Watkins and Deacon (1973) observed that neutron irradiation of rat spleen lysosomes was substantially more effective than electrons under similar experimental conditions in stimulating release of enzymes.

In this study, the observed slow release of cathepsin D at 4°C, compared with 37°C is possibly due to radiation after-effects, such as free radical interactions, formation of secondary radicals, and the resultant changes in the system which would proceed at a slower rate at 4°C (Copeland et al., 1968). It has been suggested that lipid peroxide formation is involved in the release of lysosomal enzymes after irradiation (Desai et al., 1964; Wills and Wilkinson, 1966). Formation of lipid peroxide has been shown to be enhanced at 37°C (Wills and Wilkinson, 1966, 1967a, 1967b; Myer and Bide, 1966); this might account at least in part for the rapid release of cathepsin D at 37°C.

Sulfhydryl groups are important components of cell membranes and potential "radiosensitive" sites in intracellular membranes. The relatively high sensitivity of SH groups to irradiation has been pointed out in many studies. Wills and Wilkinson (1967a) found that 5,000 rads of electron irradiation decreases the DTNB-reactive SH content
of liver lysosomes by 23%. Sutherland and Pihl (1968) reported 20% oxidation of erythrocyte membrane SH at 100 Krad. DTNB and other substances which react with SH groups have been reported to increase the fragility of lysosomes (Van Caneghem, 1972). Their studies strongly suggest that radiation-induced oxidation of sulfhydryl groups could cause disorganization of lipoprotein structure resulting in increased membrane permeability and disruption of lysosomes.

Peroxidation of membrane lipids may also be responsible, to some extent, for change in membrane structure, resulting in increased permeability of lysosomal membrane, but it has been observed that lipid peroxidation requires higher doses of radiation than required for oxidation of membrane sulfhydryl groups (Sutherland and Pihl, 1968; Wills and Wilkinson, 1967b). Lipid peroxidation has been shown to be accompanied by an increase in reactive sulfhydryl groups, suggesting that SH oxidation and lipid peroxidation, treated as two distinct mechanisms, might actually be interrelated (Robinson, 1965, 1966).

Ionizing radiation would be expected to alter all intracellular components more or less indiscriminately, and many reactions could occur in the lysosomal membrane as well as in the lysosomal matrix. During irradiation of pure chemicals in water, one observes not only oxidation of free thiols to disulfide, but also disruption of disulfide bonds by reduction to thiol, by oxidation, by interchange of various
portions of disulfide molecules and even by reaction of moieties of a disulfide such as cystine with a purified protein or with an unsaturated fatty acid in solution (Markakis and Tappel, 1960; Kollman and Shapiro, 1966; Myers et al., 1969). In view of these studies, it seems likely that disruption of disulfide bonds could also play a role in radiation damage to lysosomal membrane by contributing to the unfolding membrane proteins. Radiation has been shown to render the proteins of intact erythrocyte membrane more susceptible to attack by trypsin and other proteolytic enzymes (Myers et al., 1967). Radiation also accelerated the tryptic hydrolysis of purified proteins such as ribonuclease (Solbodian et al., 1965) or albumin (Myers et al., 1967). In this case, the radiation effect was attributed to cleavage of disulfide bonds in the protein, thus exposing hidden arginine and lysine groups to tryptic attack (Meyers et al., 1967; Solbodian et al., 1965). Meyers (1970) observed that prior exposure of yeast cells to 100 - 400 Krad X-radiation rendered the cell wall susceptible to lysis by an enzyme preparation; moreover, this particular radiation effect could be simulated by pretreating the cell with dithiothreitol, which reduces disulfide bonds. It is tempting to assume that the same mechanism might be involved in radiation damage to lysosomal structure, resulting in enzyme release.

There are also reports suggesting that membrane-bound
cathepsins may be activated by irradiation, and it might partially digest the lysosomal membrane, thus causing the release of lysosomal enzymes (Wills and Wilkinson, 1967b). Activation of lipolytic enzymes may also be a contributing factor in such a mechanism (Beaufay and de Duve, 1959). In present studies, involvement of these enzymes in changing membrane permeability does not seem to be a likely cause as release is very rapid immediately after irradiation, especially at elevated temperatures (Figure 3). According to this hypothesis, there should be slow release initially, with a subsequent rise in free enzyme content on prolonged incubation. Moreover, it is hard to explain how cathepsins can cause their own release even though after prolonged incubation, it is possible that activation of other lysosomal hydrolases might play some role.

Firfarova and Orekhovich (1971) suggested that cathepsin D existed in an inactive precursor form in chicken liver; it is possible that this is true also for other lysosomal hydrolases. Lysosomal enzymes might be activated by radiation-induced dissociation of a precursor complex, thus triggering further release of enzymes by coordinated enzyme action. It has been demonstrated that the carbohydrate chains afford the lysosomal glycoproteins limited protection against proteolytic attack by cathepsins, and splitting of the carbohydrate moiety expedites the autolysis of the protein component of lysosomal glycoproteins (Goldstone and Koenig,
Radiation-induced damage to glycoproteins might make them susceptible to enzyme attack. In other cases of radiation activation of lysosomal hydrolases, liver neuraminidase and glucosidases might split the carbohydrate moiety, thus initiating the autolysis. Such autolysis would affect the structural integrity of lysosomes and might cause, to some extent, enhanced release of lysosomal enzymes.

Radiation-induced alterations in lysosomal proteins (Copeland et al., 1967, 1968), carbohydrates (Phillips, 1972), and lipids (Nawar, 1972; Scott et al., 1964) may cause permeability changes in the lysosomal membrane and/or dissociate bound lysosomal enzymes from intralysosomal structure.

2.3.3.2. Cathepsins A, B, and C.

Lysosomes are the source of proteolytic enzymes involved in intracellular protein breakdown (Coffey and de Duve, 1968). Caldwell (1970) suggested the joint action of several muscle cathepsins on endogenous muscle proteins. Liao-Haung and Tappel (1971) observed in the chromatographic analysis of hydrolytic products of hemoglobin that oligopeptides produced by prior action of cathepsin D were degraded by cathepsin C to smaller oligopeptides, dipeptides, and free amino acids. Goettlich-Riemann et al. (1971) reported a synergistic and concerted action of cathepsins A, B, and D in protein hydrolysis. These workers pointed out that cathepsin D initiated protein hydrolysis and cathepsins A and B utilized the hydrolysis products for further degradation. Iodice et al.
(1966) demonstrated that cathepsin A had little or no activity on hemoglobin unless cathepsin D was present, and concluded that cathepsin A was restricted in its activity to break down products of proteins resulting from the prior action of cathepsin D. It is evident from these studies that to the extent that cathepsins are involved in protein breakdown in post-mortem muscle, the availability of cathepsins A, B, and C likely would be important. In the previous section release of cathepsin D was discussed; this section deals with cathepsins A, B, and C as influenced by gamma radiation.

Free enzyme activity of cathepsin A is shown in Figure 6. Immediately following irradiation, a slight decrease in free cathepsin A activity was noticed, which might be due to partial inactivation of the enzyme already present in free state or released during irradiation. Free activity of cathepsin A in all the irradiated samples increased rapidly during incubation at 4°C, and the increases were directly dose-related. The increase in free enzyme activity of cathepsin A was significantly affected by radiation treatment as well as incubation time (P<0.01). After 72 hours incubation at 4°C, release of cathepsin A was 64.44, 75.00, and 80.19 percent for 0.25, 0.50, and 1.00 Mrad respectively, while in control samples, the release was very slow, reaching 36.78 percent after 72 hours of incubation.

Release of cathepsin B (Figure 7) was enhanced by
Figure 6. Release of cathepsin A from lysosomes as influenced by gamma radiation. (n = 5)
Figure 7. Release of cathepsin B from lysosomes as influenced by gamma radiation. (n = 5)
radiation dose and incubation time at 4°C (P<0.01). An increase in cathepsin B was observed immediately after irradiation (zero incubation time) at 0.5 and 1.0 Mrad, in contrast to cathepsin A (Figure 6) and cathepsin C (Figure 8), which showed slight declines immediately after irradiation. Free activity increased steadily over the entire incubation period, with a slight levelling off between 48 hours and 72 hours, especially in 0.25 Mrad treatment. At the end of 72 hours incubation, free enzyme content in 0.50 and 1.00 Mrad reached a similar level, but the 0.25 Mrad treated samples had a distinctly lower mean level of free cathepsin B. After 72 hours incubation at 4°C, the free activity of cathepsin B was 26.13, 54.70, 70.14, and 70.48 percent of total activity for radiation treatments of 0.0, 0.25, 0.50, and 1.00 Mrad respectively. Cathepsin B had a slightly slower release and a generally lower level of free activity compared with cathepsin A.

Release of cathepsin C following radiation treatment (Figure 8) exhibited a similar pattern to that for cathepsins A and B, but rate of release and level of free activity were both higher. Cathepsin C was released to the extent of 43.86, 72.68, 82.00, and 85.47 percent of total enzyme activity after 0.0, 0.25, 0.50, and 1.00 Mrad of irradiation respectively when incubated at 4°C. Free enzyme activity of irradiated sample increased rapidly during 48 hours of incubation, and then levelled off on further incubation.
Figure 8. Release of cathepsin C from lysosomes as influenced by gamma radiation. (n = 5)
In non-irradiated samples, release of the enzyme increased slowly during the first 48 hours of incubation, and more rapidly during subsequent incubation at 4°C. Radiation doses had significant effect (P<0.01) on the release of lysosomal cathepsin C.

Figure 9 compares the effect of a 1.00 Mrad dose on the release of cathepsins A, B, C, and D during incubation at 4°C. The highest rate of increase and level of free enzyme activity were observed for cathepsin D, followed by cathepsins C, A, and B. It is interesting to note that free activity of cathepsins C and D was 11% - 13% higher than cathepsins A and B immediately after irradiation. In the cases of all four enzymes, the rate of release was quite rapid up to 48 hours of incubation. The rate of release of cathepsins C and D declined after 48 hours of incubation, but cathepsins A and B increased much more steadily until the end of 72 hours incubation, indicating that cathepsins C and D are relatively easily released from lysosomes and reach a maximum level in less time than cathepsins A and B. A similar situation is observed when the effects of radiation dose on release of cathepsins are compared. Control samples consistently had a higher free activity of cathepsins C and D than A and B after 48 hours incubation and this difference increased with increasing radiation dose (Figure 10 and Table 3).

The present studies on cathepsins demonstrate that
Figure 9. Comparative effect of gamma radiation (1.0 Mrad) on the release of lysosomal cathepsins A, B, C, and D. (n = 5)
Figure 10. Comparative effect of various radiation doses on release of cathepsins from lysosomes after 48 hours incubation at 4°C. (n = 5)
their release is significantly (P<0.01) enhanced by gamma radiation, thus increasing the free enzyme content in the system. This is in agreement with other workers' observations (Desai et al., 1964; Wills and Wilkinson, 1966) that lysosomal enzymes like acid phosphatase and β-glucuronidase are released from gamma irradiated lysosomes. Watkins and Deacon (1973) demonstrated similar release of β-glucuronidase, β-glucosaminidase, and acid phosphatase after treating rat spleen lysosomes with 5 - 20 Krad of neutron or electron irradiation.

Release of lysosomal enzymes has been attributed to membrane damage caused by free radicals, formation of lipid peroxide (Desai et al., 1964; Wills and Wilkinson, 1966), as well as oxidation of SH groups of the intracellular membranes (Wills and Wilkinson, 1967a, 1967b). The possible mechanisms of lysosomal enzyme release has been discussed in detail in the previous section, regarding cathepsin D.

Present studies also demonstrated that lysosomal cathepsins are differentially released after irradiation treatment -- cathepsins C and D are more readily released than cathepsins A and B. This phenomenon of differential release has been reported by Desai et al. (1964). They demonstrated that aryl sulfatase and β-glucuronidase were most readily released, while acid phosphatase showed a slow release, and ribonuclease was not affected. Watkins and Deacon (1973) found that following a dose of 20 Krad,
electrons affected the release of 22.9, 13.0, and 9.9 percent of β-glucuronidase, β-glucosaminidase, and acid phosphatase activities respectively. Differential solubilization of lysosomal β-glucuronidase and acid phosphatase was also reported by Watkins (1970). Lysosomal enzymes have been reported to show differential release under various conditions, like incubation (Weissmann and Thomas, 1963), different pH, osmotic strength, or temperature (Sawant et al., 1964b), freeze-thaw treatment (Sawant et al., 1964a), Triton X-100 concentration (Romeo et al., 1956; Stagni and de Bernard, 1968), or change of mono and divalent ion concentrations (Verity et al., 1968). Most of the studies cited above have reported such a behaviour of lysosomal enzymes other than cathepsins. No previous reports on cathepsins regarding the influence of radiation treatment have appeared in the literature.

This differential release of cathepsins as well as other lysosomal enzymes is possibly due to differences in structure, binding sites, or localization of the enzymes in the lysosomes. The data suggest that enzymes are bound differentially within lysosomes, or that they might be localized in different particles (Dianzani, 1963) possessing varying sensitivity to external damage. Enzymes like cathepsin C might be localized in radiation-sensitive particles or in radiation-sensitive areas within the particle, or cathepsins A and B might be relatively tightly bound as compared with cathepsin C. Unfortunately, information on intralysosomal
Table 3. Release of cathepsins A, B, C, and D from lysosomes at 4°C after irradiation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Incubation time (hrs)</th>
<th>Radiation dose (Mrad)</th>
<th>Free enzyme activity (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0</td>
<td>0.25</td>
</tr>
<tr>
<td>Cathepsin A</td>
<td>0</td>
<td>20.9</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>22.2</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>27.8</td>
<td>51.8</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>36.8</td>
<td>64.7</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>0</td>
<td>11.9</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>14.0</td>
<td>25.1</td>
</tr>
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<td></td>
<td>48</td>
<td>21.6</td>
<td>46.9</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>26.2</td>
<td>54.7</td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>0</td>
<td>31.0</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>28.7</td>
<td>42.4</td>
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<td>48</td>
<td>33.5</td>
<td>61.9</td>
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<td></td>
<td>72</td>
<td>43.9</td>
<td>72.7</td>
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<tr>
<td>Cathepsin D</td>
<td>0</td>
<td>21.0</td>
<td>26.1</td>
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<td></td>
<td>24</td>
<td>29.0</td>
<td>64.4</td>
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<td>48</td>
<td>37.0</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>55.6</td>
<td>80.4</td>
</tr>
</tbody>
</table>

n = 5 for cathepsins A, B, and C  
n = 3 for cathepsin D
localization of enzymes is obscure. Lysosomes are generally considered as membrane-limited particles containing lysosomal hydrolases, which would be labilized by various treatments, causing an injury to particle membrane (Beaufay and de Duve, 1959).

Differential release of enzymes raises an interesting question concerning the enzymes of lysosomes -- whether they are free in the interior of the particles or bound to the enclosing membrane or lysosomal matrix. Sawant et al. (1964b) obtained differential availability of lysosomal enzymes at acid, alkaline, and neutral pH, which they considered indicated that lysosomal enzymes are bound to differing charged sites of the membrane. These workers also observed an increased availability of acid phosphatase on addition of 0.2M NaCl, indicating the possibility of salt linkages between the enzymes and the membranes. These observations are based on availability of enzymes rather than release of enzymes and increased availability could be due to either release of enzymes in soluble form, or to the greater accessibility of substrate to the bound enzyme. Beck and Tappel (1968) concluded from their studies that lysosomal enzymes might be bound to membrane. Verity and Reith (1967) reported that both inorganic Hg\(^{2+}\) ions and organic mercurials (p-chloromercuribenzoate, phenylmercuric acetate) induced an irreversible loss of the structure-linked latency of lysosomal enzymes in suspensions of lysosomes. A similar effect of
p-chloromercuribenzene sulfonate (pCMBS) has been reported on neutrophil granules (Hariss, 1968). This indicates the importance of the thiol groups of proteins in maintaining both the integrity of lysosomal membranes and the latent properties of lysosomal enzymes. It is also possible that proteins as well as lipids and lysosomal membranes may be involved in binding of some of the enzymes to the limiting membranes.

There is evidence that lysosomal enzymes might be bound to the lysosomal matrix. Shibko et al. (1965) observed loss of lysosomal matrix and release of acid phosphatase and aryl sulfatase during incubation of lysosomal suspension at 37°C. Koenig (1962) suggested that hydrolytic enzymes are contained within the lysosomes by electrostatic binding to the acidic groups of the lipoprotein matrix. Thiol groups in some hydrolases may play an ancillary role in the structural latency of some hydrolases, possibly through formation of disulfide bonds with thiol groups in lysosomal lipoprotein, or through some interaction with polyunsaturated fatty acids in the latter (Robinson, 1966).

Cytochemical observation of hydrolase activity at the fine ultrastructural level demonstrates association of reaction products with lysosomal membrane as well as the matrix (Daems et al., 1972). It is also well-established that a number of isoenzymes of lysosomal cathepsins exist, especially of cathepsins D and B (Barret, 1971; Keilova, 1973) which
might be differentially bound or localized within lysosomes.

In view of the above studies, it is obvious that various lysosomal enzymes are localized in a unique fashion with a differential affinity or binding for the intralysosomal structure. These differences in localization as well as binding might be responsible for differential radiation sensitivity and the resultant pattern of release of lysosomal cathepsins.

These studies demonstrate that radiation causes release of lysosomal cathepsins, thus making them available for hydrolysis of various components of animal tissue. It has been demonstrated that lysosomal cathepsins are capable of degrading proteins (Coffey and de Duve, 1968). Ethrington (1972) demonstrated that rat liver extracts degraded insoluble collagen and suggested the presence of collagenolytic cathepsin which was different than cathepsin B or cathepsin D, the two known endopeptidases. Cathepsin B1 has been considered a major contributor to the enzymic degradation of collagen by rat liver lysosomes (Anderson, 1969; Burleigh, 1973). Several studies on hemoglobin digestion by partially purified cathepsin preparations have suggested a synergistic action of cathepsins A, B, and C, and cathepsin D (Iodice et al., 1966; Liao-Huang and Tappel, 1971; Goettlich-Riemann et al., 1971). Tappel (1969) postulated that during the degradation of denatured proteins by lysosomal extracts, the action was initiated by cathepsin D, and large peptide fragments were broken down by
other proteases and dipeptidases. Coffey and de Duve (1968) have suggested an important role for cathepsin D in hemoglobin breakdown, and Dingle (1971) has shown its function in cartilage degradation. Recently Huisman et al. (1974) suggested that either cathepsin B1 or some thiol enzymes other than cathepsins B1, C, or D are involved in lysosomal protein degradation. Glycoproteins have been shown to be degraded by rat kidney lysosomes; moreover, it was observed that splitting of carbohydrate chains of glycoprotein by neuraminidase and glycosidases made the protein moiety more susceptible to proteinase attack (Aronson and de Duve, 1968; Goldstone and Koenig, 1974). The great majority of glucosidases are located in lysosomes (Barrett, 1969), and some of them have been shown to be released from lysosomes after irradiation treatment (Desai et al., 1964; Watkins, 1970; Watkins and Deacon, 1974).

Besides in vitro studies using purified substrates, there is evidence that lysosomal cathepsins are active on endogenous proteins. Eino and Stanley (1973a) reported catheptic activity on salt-soluble and insoluble proteins of beef muscle, moreover, incubating muscle fibres in a cathepsin solution at pH 5.5 and 0° - 5°C reduced the tensile strength of muscle fibres (Eino and Stanley, 1973b). Their crude enzyme preparation might have contained cathepsins A, B, and C besides cathepsin D, and there is a strong possibility that the effect was due to involvement of all the cathepsins, especially at pH 5.5, as cathepsins A, B, and C are able to degrade the
oligopeptides produced by cathepsin D attack (Goettlich-Reimann et al., 1971; Liao-Huang and Tappel, 1971). Autolysis of chicken skeletal muscle extract has been attributed to a combined action of cathepsins (Caldwell, 1970). There is ample evidence of proteolysis in post-mortem muscle (Sharp, 1963; Khan and van den Berg, 1964a, 1964b; Suzuki et al., 1967; Parrish et al., 1969; Okitani and Fujimaki, 1972; Okitani et al. 1973), but its extent and importance has been disputed.

Ultrastructural observations show a disorganization or absence of Z-line in aged muscle, and a tendency for a myofibril to be broken at the former location of the Z-line (Davey and Gilbert, 1967). Trypsin has been shown to quickly remove the Z-line from myofibres (Goll et al., 1970). Evidence that cathepsins may cause the degradation of Z-lines was presented by Penny (1968) and Fukazawa et al. (1969). They found that fibrils prepared post-mortem without the presence of cathepsins and other soluble cellular material did not show a loss of Z-line. Busch et al. (1972) demonstrated that Z-line degradation was caused by CASF (Ca$^{2+}$-activated sarcoplasmic factor) isolated from muscle; it has been suggested that CASF might be localized in lysosomes, and the leakage might have occurred during their isolation procedure. Recently, CASF has been characterized as proteolytic enzyme, having optimum pH and optimum Ca$^{2+}$ concentration of 7.0 and 1.0 mM respectively, and it removes soluble material from myofibrils (Suzuki and Goll, 1974).
West et al. (1974) observed that incubation of isolated sarcoplasmic reticulum with cathepsin fraction at pH 7.0 resulted in loss of calcium accumulating ability of sarcoplasmic reticulum. Such an effect was lower with cathepsin B1 treatment as compared with treatment with a fraction containing cathepsins A, B1, B2, and C. However, no studies have been reported with cathepsin D or other lysosomal proteases, but brief tryptic digestion produced similar results (West et al., 1974). Loss of calcium accumulating ability of sarcoplasmic reticular membranes causes the onset of rigor mortis (Greaser et al., 1969; Schmidt et al., 1970), which in turn is associated with the post-mortem changes in muscle (Herring et al., 1965; Marsh and Leet, 1966). Degradation of sarcoplasmic reticulum in muscle would result in release of calcium, thus activating CASF, which is capable of removing Z-lines (Busch et al., 1972) and solubilizing myofibrils (Suzuki and Goll, 1971). However, it remains to be demonstrated whether muscle cathepsins can cause such changes in sarcoplasmic reticulum in post-mortem muscle.

Evidence of proteolysis affecting the texture was noted in studies with irradiation-sterilized meat (Cain et al., 1958; Pearson et al., 1958, 1960; Coleby et al., 1961; Bailey and Rhodes, 1964). Radiation did not inactivate the proteolytic enzymes, and with storage, extensive degradation of muscle occurred. Klein and Altman (1972b) found an increased proteolytic activity in chicken breast and leg muscle after
0.2 Mrad of ionizing radiation. Extensive degradation and increased proteolysis in irradiated meat is possibly due to lysosomal fragilization and release of lysosomal cathepsins.

Results of this investigation suggest that irradiation-induced damage to lysosomal structure markedly enhanced the release of lysosomal cathepsins. Release of other lysosomal enzymes such as collagenase and neutral protease is a strong possibility. Similar damage to lysosomes and increases in free enzyme contents might occur in irradiated meats, resulting in an extensive breakdown of tissue proteins. Such an effect would considerably influence the ultimate quality of irradiation-preserved foods of animal origin.
2.4. SUMMARY AND CONCLUSIONS

The effect of gamma radiation on chicken liver tissue and on isolated lysosomes was studied. The release of chicken liver cathepsin D by gamma radiation treatment was observed. The enzyme release was greater at low doses (50 and 100 Krad) compared with high doses (0.25, 0.50, 1.0 Mrad); possibly at high doses partial inactivation occurred, or radiation denaturation of tissue proteins might have decreased the extraction of the enzyme.

Lysosomes were isolated from chicken liver by sucrose gradient and differential centrifugation techniques. Light scattering studies of isolated lysosomes showed a rapid drop in absorbance at 540 nm after irradiation treatment and post-irradiation incubation at 37°C, indicating a strong possibility of leakage of lysosomal enzymes.

The results of studies on release of lysosomal enzymes indicate that free activity of cathepsins A, B, C, and D was substantially increased by irradiation treatments of isolated lysosomes. Release of cathepsin D was very rapid at 37°C compared with 4°C incubation. Release of cathepsin D from irradiated lysosomes almost reached maximum level in one hour at 37°C, while the non-irradiated control showed a slow release over 3 hours of incubation.

Release of cathepsins A, B, C, and D from irradiated lysosomes was maximal during the first 24 - 48 hours of incubation at 4°C, but non-irradiated controls showed slow
initial release (during first 24 - 48 hours) and a higher rate of release after 48 hours; the level of free enzyme content remained considerably lower than the irradiated lysosomes. Lysosomal cathepsins A, B, C, and D exhibited a differential release pattern under the influence of irradiation. Cathepsins C and D were more readily released than cathepsins A and B.

Radiation-enhanced release of lysosomal enzymes is indicative of damage to lysosomal structure from such treatment. It is likely that radiation damage to membrane structure causes permeability changes, and possibly the creation of weak points results in disruption of some lysosomes during incubation. Another possible reason may be disassociation of lysosomal cathepsins from intralysosomal structure by radiation-induced damage to binding sites. Radiation-induced oxidation of SH groups, disruption of disulfide bonds, or lipid peroxidation also may be responsible for damaging lysosomal structure. Denaturation or fragmentation of lysosomal proteins could also contribute to radiation-induced alterations in lysosomes.

Radiation-induced release of cathepsins A, B, C, and D makes them freely available for degradation of tissue proteins. Synergistic action of these enzymes may cause extensive protein degradation. The observation that release of cathepsin D was very rapid at the initial stage of incubation makes it available for initiation of protein breakdown as suggested
by various workers. It is also important to point out that other lysosomal enzymes like neutral proteases, a proteolytic enzyme called Ca$^{2+}$-activated sarcoplasmic factor (CASF), or some other cathepsins yet uncharacterized, might also be released and have similar degradative action on tissue proteins.
CHAPTER 3. RADIATION SENSITIVITY OF LYSOSOMAL CATHEPSINS
AND HEMOGLOBIN SUBSTRATE

3.1. INTRODUCTION

The application of ionizing radiation for the preservation of foods is of great interest to radiation research workers, as micro-organisms responsible for food spoilage can be effectively eliminated by sterilizing doses of radiation. Results of various investigations show that a number of enzymes are radiation resistant (Coelho, 1969; Klein and Altman, 1972b; Losty et al., 1973). The residual proteolytic activity has been held responsible for autolytic degradation of irradiated foods of animal origin (Drake et al., 1961; Coleby et al., 1960). Several studies have established the presence of lysosomal cathepsins in skeletal muscle tissue (Caldwell and Grosjean, 1971; Parrish et al., 1969; Randall et al., 1967); moreover, joint action of different cathepsins in degradation of proteins have been suggested (Caldwell, 1970; Iodice et al., 1966). It is quite likely that residual activity in irradiated meats is not only due to cathepsin D, but also cathepsins A, B, and C. Inactivation of tissue proteolytic enzymes has been interpreted as inactivation of cathepsin D, but information on radiation sensitivity of cathepsins A, B, and C is needed. Due to low enzyme activity of skeletal muscle, isolated lysosomal fraction from chicken liver was used as a source of enzymes.

Tissue proteins undergo various changes due to irradiation-
tion treatment (Klein and Altman, 1972a; Uzunov et al., 1972), which might affect the course of proteolysis during storage of irradiated meats. It has been shown that irradiated albumin and casein become susceptible to tryptic digestion (McArdle and Desrosier, 1955), but studies on collagen indicate that protein becomes resistant to collagenase attack, possibly due to intermolecular cross-linking. Studies on degradation of irradiated proteins by cathepsins seems highly appropriate to better understand proteolysis occurring in irradiated meats. In tissue, it is difficult to differentiate between the effects of irradiation on various protein components, and to distinguish the separate effects of individual enzymes towards autolysis. In the present study, an attempt has been made to evaluate the effect of irradiation on lysosomal cathepsins and model substrate, and its digestion by lysosomal cathepsins.
3.2. EXPERIMENTAL

2.2.1. Treatment of Samples for Enzyme Study

Chicken livers were removed from 3 or 4 birds immediately after killing, washed free of blood with 0.25M sucrose solution, and chilled in crushed ice. Excessive fat and connective tissue were removed; tissue was minced with scissors and homogenized in 0.25M sucrose (1:8, w/v) using a Waring blender at top speed for 30 seconds at 4°C. The lysosomes were isolated by sucrose density gradient and differential centrifugation techniques (Sawant et al., 1964c). The lysosomal pellet was suspended in 0.7M sucrose solution and a 1:2 (w/v) dilution was made on the basis of liver tissue. Samples of this final suspension received varying doses of gamma radiation in a Gamma Cell-220. Details of these procedures are given in Chapter 2.

3.2.2. Enzyme Assay

Conditions for determination of cathepsins A, B, C, and D are outlined in Table 4. Detailed procedures are given in Chapter 2 under "Enzyme Assays" section.

3.2.3. Enzyme Activity in Lysosomal Suspensions

3.2.3.1. Residual activity.

After irradiation, lysosomal suspensions received freeze-thaw treatment ten times, or were treated with Triton X-100, 0.2% final concentration, and kept at room temperature for
at least 30 minutes to disrupt the lysosomal particles. This suspension was centrifuged at 17,000 x g for 20 minutes, and the supernatant used for determination of enzyme activity. Catheptic activity remaining after irradiation treatment was expressed as percentage of control value and designated "residual activity".

3.2.3.2. **Enzyme availability after irradiation of lysosomes in the intact and ruptured state.**

The lysosomal pellet was suspended in 0.7M sucrose solution at pH 7.2. One portion was given freeze-thaw treatment ten times to disrupt lysosomal particles prior to irradiation, while the other portion was subjected to freeze-thaw treatment after irradiation. Doses of 0.25, 0.50, and 1.00 Mrad were given to the above samples, and non-irradiated samples were kept as controls. Available enzyme activity of cathepsins A, B, and C was determined using the disrupted particle suspensions without centrifugation.

3.2.3.3. **pH during irradiation.**

The lysosomal pellet was suspended in 0.7M sucrose solution. Lysosomes were disrupted by Triton X-100 treatment and centrifuged at 17,000 x g for 20 minutes. pH of the supernatant was adjusted to 4.0, 5.5, 7.0, or 8.5 prior to irradiation. Irradiation doses of 0.10, 0.25, 0.50, or 1.00 Mrad were given to these samples. Non-irradiated samples were used as controls. Cathepsins A, B, C, and D
Table 4. Substrates and incubation conditions for cathepsin assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate*</th>
<th>Buffer</th>
<th>pH</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin A</td>
<td>0.0152M N-carbobenzoxy-α-L-glutamyl-L-tyrosine</td>
<td>0.04M acetate</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>0.01M benzoyl-L-arginine amide</td>
<td>0.1M citrate</td>
<td>5.0</td>
<td>0.04M cysteine HCl</td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>0.01M glycyl-L-tyrosine amide</td>
<td>0.1M citrate</td>
<td>5.0</td>
<td>0.04M cysteine HCl</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>2.0% Hemoglobin</td>
<td>0.2M acetate</td>
<td>3.8</td>
<td>-</td>
</tr>
</tbody>
</table>

Reaction time: 2 hours at 37°C.

* All substrates were purchased from Sigma Chemical Company.
activities were determined. Enzyme activity of irradiated samples was expressed as a percentage of the respective controls.

3.2.3.4. Sucrose concentration during irradiation.

The lysosomal pellet was suspended in 0.10, 0.25, 0.50, or 7.0M sucrose solution pH 7.2, and given a dose of 0.50 Mrad, while non-irradiated samples served as controls. After irradiation, lysosomes were disrupted with Triton X-100 treatment and cathepsin D activity was determined.

3.2.3.5. Temperature during irradiation.

Lysosomes were suspended in 0.7M sucrose solution pH 7.0 and administered a dose of 0.50 Mrad of gamma radiation either at room temperature or crushed ice temperature and then stored at 4°C. Samples were drawn at 0 hours and 48 hours after irradiation, and centrifuged at 17,000 x g for 20 minutes; cathepsin D activity of the supernatant was designated as "free activity". Total cathepsin D activity was determined after Triton X-100 treatment.

3.2.3.6. Irradiation - heat combination treatment.

Lysosomal suspension was treated with Triton X-100 and centrifuged at 17,000 x g for 20 minutes. The supernatant samples were subjected to 0.0, 0.25, 0.5, or 1.00 Mrad gamma radiation. Samples were drawn from each dose and heated at 50, 60, 70, 80, or 90°C for 10 minutes in a constant temperature water bath, and then cooled to room temperature immediately. Cathepsin D activity was determined according to the procedure described in Chapter 2.
3.2.4. **Hydrolysis of Irradiated Hemoglobin**

3.2.4.1. **In solution.**

A 10% aqueous solution of hemoglobin was irradiated for 0.0, 0.25, 0.5, or 1.00 Mrad at ambient chamber temperature. Hydrolysis of 2.5% hemoglobin by cathepsin D was followed at pH 3.8 or pH 5.0 in 0.2M acetate buffer or pH 7.0 in 0.2M phosphate buffer.

3.2.4.2. **In dry state.**

Dry powdered hemoglobin was irradiated at room temperature for 0.0, 0.25, 0.5, 1.00, or 5.0 Mrad. The irradiated hemoglobin was used as substrate for cathepsin D hydrolysis at pH 3.8. The procedure for determination of cathepsin D has been outlined in the "Enzyme assay" section of Chapter 2.

3.2.5. **Absorption Spectrum of Irradiated Hemoglobin**

Absorption spectrum of hemoglobin substrate irradiated for 0.0, 0.25, 0.5, and 1.00 Mrad in solution were taken after appropriate dilution. Samples were scanned using Pye-Unicam SP-800B spectrophotometer (Pye-Unicam Ltd.).

3.2.6. **Agarose Gel Electrophoresis of Hemoglobin after Hydrolysis by Cathepsin D**

Irradiated as well as non-irradiated hemoglobin was hydrolysed by cathepsin D at pH 3.8 in 0.2M acetate buffer using 5% substrate and 5 hours reaction time at 37°C. Control samples were kept at 4°C. After hydrolysis, 10μl
of sample was applied to agarose gel.

3.2.6.1. Electrophoresis procedure.

Fifty grams of urea were dissolved in 200 ml of Barbitral buffer pH 8.6, 0.05M with 0.035% EDTA. Agarose gel film (Agarose Universal Electrophoresis Film – ACI) was soaked in the above buffer for 15 minutes; 0.6 ml of 2-mercaptoethanol was added and soaking continued for another 15 minutes. The gel was dried and 10μl of hydrolysed hemoglobin was applied into the dried sample slots. The cassette cell compartments were filled with Barbital buffer pH 8.6 and the agarose gel film slipped into a cassette cell cover. The samples were run at 4°C for one hour. After electrophoresis, staining was done in 0.2% amido black in 5% acetic acid for 15 minutes, followed by 30 seconds washing in 5% acetic acid. The film was dried for 20 minutes in an air oven, cleared by soaking in 5% acetic acid for one minute, and then dried at 70°C - 85°C for 20 minutes.
3.3. RESULTS AND DISCUSSION

3.3.1. Radiation Inactivation of Cathepsins A, B, C, and D

3.3.1.1. Lysosomal suspension.

Radiation doses of 0.25, 0.5, and 1.00 Mrad significantly (P<0.01) inactivated cathepsins A, B, and C (Figure 11). A dose of 0.25 Mrad caused a marked decrease in residual enzyme activity and the curves levelled off as the dose was increased to 0.5 and 1.00 Mrads. Cathepsin B was most sensitive to radiation, followed by cathepsins C, D, and A (Figure 11). After 1.00 Mrad radiation, the residual enzyme activity dropped to 78.7, 67.0, and 70.2 percent of control in the case of cathepsins A, B, and C respectively. Higher radiation sensitivity of cathepsins B and C might be due to the presence of sulfhydryl groups in their active site. This is in agreement with the report that in sulfhydryl enzymes like papain, destruction of SH groups leads to rapid inactivation of enzymes (Pihl and Sanner, 1963; Lynn and Louis, 1973).

Radiation doses of 0.25, 0.50, and 1.00 Mrad caused appreciable inactivation of cathepsin D (Figure 11). Enzyme activity of irradiated samples was significantly (P<0.01) lower than that of control, and the 1.00 Mrad treatment had significantly lower activity compared with other irradiation treatments. Doses of 0.25, 0.50, and 1.00 Mrad caused a decrease in the enzyme activity of 15.42, 18.46, and 27.42 percent respectively. The results
Figure 11. Radiation inactivation of cathepsins A, B, C, and D in intact lysosomes. (n = 5)
of this report are in general agreement with other workers' observations of radiation inactivation of enzymes. It has been observed that tissue proteases are relatively radiation-resistant (Doty and Wachter, 1955; Drake et al., 1957b; Landman, 1963). Klein and Altman (1972b) reported 50% inactivation of proteolytic enzyme in chicken breast and leg muscle after 1.0 Mrad gamma irradiation, and 85% - 100% inhibition by 5.0 Mrad. Losty et al. (1973) observed up to 75% reduction of proteolytic activity in ground beef after 2 - 6 Mrad irradiation.

Giovannozzi-Sermanni (1969) reported that cathepsin C was highly radiation-resistant in ox spleen tissue, compared with purified fractions. In tissues, the low catheptic activity makes it difficult to conduct such studies; moreover, vigorous extraction procedures are required to extract lysosomes. Purified cathepsins might exhibit different radiation sensitivity due to change of environment, and partially due to purification procedure. Isolated lysosomes from soft organ tissue such as liver provide the optimum system to study radiation sensitivity of cathepsins close to their natural environments. Information on radiation inactivation of cathepsins, especially in lysosomes, is lacking in literature. However, Desai et al. (1964) reported that lysosomal β-glucuronidase was readily inactivated compared with aryl sulfatase and acid phosphatase, while ribonuclease did not show inactivation after 10 - 50
Krads of gamma radiation administered to isolated rat liver lysosomes. In the present study, lysosomal cathepsins A, B, C, and D showed slight differences in their radiation inactivation. The difference observed by Desai et al. (1964) in the case of non-catheptic lysosomal hydrolases was quite large as compared with the present study.

Inactivation of enzymes could be due to general denaturation, destruction, or modification of active centres (Sanner and Pihl, 1969) and formation of inhibitory substances by radiolysis. Variations in enzyme localization, active centre, and general structure would affect the extent of inactivation of each enzyme. In view of these studies, it seems that net inactivation would be complex, and the mode of inactivation might be different for each of the cathepsins.

The results indicate that lysosomal cathepsins are fairly radiation-resistant, and even higher resistance has been reported when irradiated in tissue (Rhodes and Meegungwan, 1962; Siebert and Musch, 1969), thus residual proteolytic activity may cause extensive protein breakdown during post-mortem storage.

3.3.1.2. Soluble enzyme fraction at various pHs.

The disrupted lysosomes were centrifuged and the supernatant fraction irradiated at pH of 4.0, 5.5, 7.0, or 8.5. The results of the experiment are presented in Table 5. Comparative effects of pH 5.5 and 7.0 on radiation inactiva-
tion of cathepsins A, B, C, and D are shown in Figures 12 and 13 respectively. pH during irradiation significantly (P<0.01) affected the radio-sensitivity of cathepsins A, B, C, and D. Although the extent of inactivation

Table 5. Effect of pH on radiation sensitivity of soluble lysosomal cathepsins A, B, C, and D.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dose (Mrad)</th>
<th>pH 4.0</th>
<th>pH 5.5</th>
<th>pH 7.0</th>
<th>pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin A</td>
<td>0.10</td>
<td>59.28</td>
<td>55.77</td>
<td>63.57</td>
<td>68.00</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>41.07</td>
<td>37.62</td>
<td>50.00</td>
<td>51.11</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>17.14</td>
<td>25.08</td>
<td>31.78</td>
<td>40.89</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>7.78</td>
<td>22.77</td>
<td>15.35</td>
<td>23.11</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>0.10</td>
<td>91.80</td>
<td>81.82</td>
<td>98.44</td>
<td>83.75</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>72.13</td>
<td>71.20</td>
<td>90.62</td>
<td>78.75</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>68.85</td>
<td>53.63</td>
<td>85.93</td>
<td>70.00</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>65.57</td>
<td>48.20</td>
<td>68.75</td>
<td>47.50</td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>0.10</td>
<td>61.60</td>
<td>94.42</td>
<td>73.78</td>
<td>91.58</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>51.10</td>
<td>47.37</td>
<td>68.92</td>
<td>87.50</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>32.54</td>
<td>36.53</td>
<td>50.95</td>
<td>65.49</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>23.48</td>
<td>28.48</td>
<td>35.52</td>
<td>47.28</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>0.10</td>
<td>94.30</td>
<td>77.29</td>
<td>69.45</td>
<td>75.79</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>76.25</td>
<td>54.46</td>
<td>53.17</td>
<td>63.57</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>46.56</td>
<td>38.07</td>
<td>50.28</td>
<td>46.04</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>28.50</td>
<td>15.59</td>
<td>26.86</td>
<td>30.20</td>
</tr>
</tbody>
</table>

n = 5
Figure 12. Radiation inactivation of soluble lysosomal cathepsins at pH 5.5. (n = 5)
Figure 13. Radiation inactivation of soluble lysosomal cathepsins at pH 7.0. (n = 5)
varied with the change of pH, cathepsin B showed highest radiation-resistance over the entire range of pH, followed by cathepsin C. Cathepsin A was found to be most sensitive to irradiation under these conditions.

Effect of pH on irradiation inactivation of lysosomal cathepsins was different for cathepsins A, B, C, and D (Table 5). Cathepsins A and C were most sensitive at pH 4.0 and least sensitive at pH 8.5. Cathepsins B and D had the highest inactivation at pH 5.5 and the lowest at pH 7.0 and 8.5 respectively. All the enzymes studied showed relatively high radiation sensitivity at acidic pH, the optimum pH range for their reactions. Results of the present study for cathepsin C are contradictory to studies of Giovannozzi-Sermanni et al. (1969) on purified cathepsin C from ox spleen; they found an increased radiation sensitivity with increasing pH. These differences might be due to origin of cathepsin C as well as state of purity, as presence of other components will greatly affect the radiosensitivity of enzymes (Sanner and Pihl, 1969). Results of this study agree with the report of Robins and Butler (1962), who found an increased radiation resistance of trypsin with increasing pH of solution, and the study of Delincee and Radola (1974), who observed higher irradiation inactivation of horseradish peroxidase at pH 4.0 as compared with pH 7.2 and 10.0. The effect of pH is largely due to its influence on dissociation
of proteins, which in turn may influence the radiation sensitivity by altering protein conformation (Sanner and Pihl, 1969), and distribution of radical species produced by radiolysis in aqueous system (Robins and Butler, 1962).

The difference in inactivation pattern between cathepsins irradiated in lysosomal suspension and in soluble form indicates that different mechanisms of inactivation are probably involved in different environments. In lysosomal suspension, the direct effect of radiation might be relatively greater than in free enzyme systems where the enzyme is in soluble form, and more likely subject to indirect effects.

The inactivation pattern of soluble cathepsins (Figure 13) is the reverse of that obtained with irradiation of intact lysosomal suspensions (Figure 11). Such a change may be due to dissociation of enzymes from lysosomal structure, where enzymes would be protected to a varying degree by binding or association with other lysosomal components; but after disruption of lysosomes and fractionation of soluble enzyme as used in this experiment, no such protective effect is involved, hence change in environment might be responsible for this marked change in response to irradiation of cathepsins. Nature and extent of radiation damage to the cathepsins might be different under changed environments as Lynn (1972) observed that trypsin, when complexed with silica before irradiation, showed a change
in its relative activity to synthetic substrates. Radiation damage to amino acid residues of the complexed enzymes has been shown to be different as compared with dissolved enzyme (Holladay et al., 1966; Copeland et al., 1967).

Comparison of Figures 11 and 13 shows that radiation inactivation was substantially increased when soluble enzymes were irradiated. This is in general agreement with reports that enzymes present in tissue (Giovannozzi-Sermanni, 1969) or complexed with other materials (Lynn, 1972, 1974) show higher radiation resistance, due to protective effects of the other compounds present in the system, thus decreasing the damage due to indirect effects of irradiation, especially by free radicals.

Variation in radiation sensitivity of cathepsins A, B, C, and D may be partially due to the difference in localization as well as binding mechanisms within lysosomes (Koenig, 1969).

3.3.2. Availability of Cathepsins A, B, and C after Irradiation of Intact and Disrupted Lysosomes

Lysosomes were irradiated with various doses of gamma radiation, before or after disruption, to study the effect of radiation on available enzymes. Determinations of activity of cathepsins A, B, and C were made using disrupted lysosomes without centrifugation, so that activity of partially bound or adsorbed enzyme could be included.
The results for cathepsin A are presented in Figure 14. Cathepsin A activity was found to decrease significantly (P<0.01) after irradiation of disrupted or intact lysosomes. Available enzyme content in disrupted lysosomes was found to be significantly (P<0.05) higher than intact lysosomes after 0.25 and 0.50 Mrad doses, but at a higher dose of 1.0 Mrad no significant difference was noted.

Cathepsin B activity of intact or disrupted lysosomes decreased significantly (P<0.01) after irradiation treatment, but the difference in available enzyme content of intact and disrupted lysosomes was not significant at any dose (Figure 15).

Cathepsin C also followed the familiar pattern of radiation inactivation (Figure 16), showing significant (P<0.01) decrease in activity in both intact and disrupted lysosomes. Enzyme activity in disrupted lysosomes was higher than in intact lysosomes for the 0.5 Mrad dose.

These observations suggest that the lysosomal membrane does not have much protective effect. However, enzymes in the disrupted system might have been adsorbed or bound to lysosomal fragments and have thus been protected from irradiation inactivation. It is also possible that the pre-irradiation disruptive treatment increased the availability of enzymes, and thereby compensated for the higher subsequent inactivation. Presence of lysosomal fragments in the suspension medium would provide some
Figure 14. Availability of cathepsin A after irradiation of disrupted and intact lysosomes. (n = 4)
Figure 15. Availability of cathepsin B after irradiation of disrupted and intact lysosomes. (n = 4)
Figure 16. Availability of cathepsin C after irradiation of disrupted and intact lysosomes. (n = 4)
protection to enzymes against indirect effects of irradiation by trapping water radicals and preventing formation of secondary radicals.

3.3.3. Radiation Inactivation of Cathepsin D under Various Conditions

3.3.3.1. Sucrose concentration.

Presence of other compounds in the suspension medium considerably affects the radiation sensitivity of enzymes when irradiated in solution (Sanner and Pihl, 1969). In the present study, sucrose concentration was varied from 0.1M to 0.7M at pH 7.0 prior to irradiation at 0.50 Mrad. The results are shown in Figure 17. At 0.1M sucrose concentration, residual activity of cathepsin D fell to 52.75% of the control value, but an increase of sucrose concentration in suspension medium to 0.25M significantly (P<0.01) protected the enzyme from radiation inactivation. Increasing sucrose concentration to 0.5M did not provide any further increase in radiation protection. The maximum protection was observed at 0.7M sucrose level, when residual activity was found to be 82.48% of unirradiated control value. These results are consistent with other studies (Jung, 1967; Schuessler, 1973; Dale, 1942; Sanner and Pihl, 1967), showing protective effects of various compounds present in the suspension medium. Such a protective effect is due mainly to interaction of these compounds with free
Figure 17. Effect of sucrose concentration on radiation sensitivity of cathepsin D after 0.5 Mrad dose. (n = 3)
radicals which would otherwise interact with enzyme molecules and cause inactivation.

3.3.3.2. Temperature.

Irradiation of lysosomal suspensions was carried out at ambient chamber temperature or 0°C to determine the influence of temperature on inactivation of lysosomal cathepsin D. After an irradiation dose of 0.50 Mrad at ambient temperature and 0°C, the residual activity of cathepsin D was 69.59 and 83.38 percent of the control value respectively. This increased radiation-resistance of cathepsin D at 0°C is in agreement with the findings of Schults et al. (1975), who observed a marked decrease in radiation sensitivity of proteolytic enzymes at low temperature in raw beef, pork, and chicken muscle. A similar effect was found on proteolytic activity of ground beef after irradiation at low temperature (Losty et al., 1973). It has been suggested that low irradiation temperatures should be used to avoid irradiation-induced flavour changes in radiation processed meats. However, the increased radiation-resistance of proteolytic enzymes at low temperatures suggests a hazard from undesirable residual proteolytic action.

3.3.4. Radiation - Heat Combination Treatment for Inactivation of Lysosomal Cathepsin D

Proteolytic enzymes are more radiation-resistant in
tissue than in lysosomal suspension and their total inactivation would require very high doses of radiation. However, the enzymes are heat labile and a combination of gamma radiation and heat might be expected to produce a synergistic effect based on similar finding by other workers (Glew, 1962; Farkas and Goldblith, 1962). The results of a radiation - heat combination treatment are shown in Table 6 and demonstrated that although both irradiation and heat alone could reduce the residual activity of soluble cathepsin D significantly (P<0.01); both failed to inactivate the enzyme completely at the levels used.

Table 6. Effect of irradiation - heat combination treatment on inactivation of cathepsin D.

<table>
<thead>
<tr>
<th>Irradiation dose (Mrad)</th>
<th>Room temp.</th>
<th>Residual enzyme activity (% of control)</th>
<th>Heat treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50°C</td>
</tr>
<tr>
<td>0.00</td>
<td>100.00</td>
<td>89.60</td>
<td>57.70</td>
</tr>
<tr>
<td>0.50</td>
<td>31.90</td>
<td>22.40</td>
<td>6.90</td>
</tr>
<tr>
<td>1.00</td>
<td>15.23</td>
<td>11.47</td>
<td>9.32</td>
</tr>
<tr>
<td>2.00</td>
<td>5.73</td>
<td>2.11</td>
<td>0.00</td>
</tr>
</tbody>
</table>

n = 5
* 10 minutes heating at given temperature in constant temperature water bath.
A combination treatment of 2.0 Mrad gamma radiation followed by 10 minutes heating at 60°C resulted in complete inactivation of cathepsin D. Irradiation of 1.0 Mrad alone reduced the enzyme activity to 15.23%, while the radiation of 0.5 Mrad followed by 10 minutes heating was much more effective in reducing residual enzyme activity to 6.9%; thus by radiation - heat combination treatment, the irradiation requirement can be reduced markedly. Glew (1962) has shown that heating at 50°C after radiation treatment had a synergistic effect on milk phosphatase inactivation and Farkas and Goldblith (1962) have found similar effects on lipoxidase. To reduce the residual activity of proteolytic enzymes in tissues, some studies have been conducted using various combination treatments. Cain and Anglemier (1969) reported that heating of beef to 140°F before irradiation significantly reduced the soluble nitrogenous constituents during storage. Losty et al. (1973) found that 2 - 6 Mrad gamma irradiation alone destroyed proteolytic activity of ground beef up to 75%, but a combination of 4.5 - 5.2 Mrad plus blanching at 65°C or 75°C reduced residual activity to 5%. Results of present experiment are in agreement with the above-mentioned reports in that residual activity can be considerably reduced by combination treatment. Due to higher radiation-resistance of proteolytic enzymes in tissue, higher radiation dose as well as elevated temperature would be required.
3.3.5. Radiation-induced Changes in Substrate

Proteolysis has been shown to be a cause of degradation of irradiated tissue (Cain et al., 1958; Drake et al., 1961), and much attention has been given to the enzymes responsible for it. Irradiation of tissue would also affect the endogenous proteins which are later attacked by tissue proteolytic enzymes. In tissue autolysis it is difficult to differentiate between the relative effects of irradiation on enzymes and endogenous proteins. In the previous section, studies on irradiation of lysosomal cathepsins have been presented. This section deals with the effects of irradiation on hemoglobin used as substrate for cathepsin D.

3.3.5.1. Hydrolysis of irradiated hemoglobin at different pHs.

Hemoglobin was irradiated at ambient temperature in 10% aqueous solutions. Hydrolysis of 2.0% irradiated hemoglobin substrate by lysosomal cathepsins was followed at pH 3.8, 5.0, and 7.0. Results of the study are presented in Table 7. Irradiation doses of 0.25, 0.5, and 1.0 Mrad each significantly (P<0.01) reduced the hydrolysis of hemoglobin at the three pH conditions used.
Table 7. Hydrolysis of hemoglobin by lysosomal cathepsin D at various pHs after gamma radiation in soluble state.

<table>
<thead>
<tr>
<th>Irradiation dose (Mrad)</th>
<th>pH 3.8</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>0.25</td>
<td>84.39</td>
<td>95.17</td>
<td>97.50</td>
</tr>
<tr>
<td>0.50</td>
<td>67.87</td>
<td>93.79</td>
<td>96.26</td>
</tr>
<tr>
<td>1.00</td>
<td>44.57</td>
<td>84.14</td>
<td>84.11</td>
</tr>
</tbody>
</table>

n = 3

The maximum inhibitory effect was observed at pH 3.8, where cathepsin D has maximum activity; extent of hydrolysis of hemoglobin at pH 5.0 and 7.0 was similar, but significantly (P<0.05) lower than that observed at pH 3.8.

Hemoglobin hydrolysis was maximum at pH 3.8 due to cathepsin D attack; irradiation-induced denaturation, and changes in conformation of protein molecules might have rendered it unavailable for cathepsin D attack. Considerable activity was observed at pH 5.0 and 7.0, where cathepsin D would split hemoglobin to a lesser extent, but other enzymes including cathepsins A, B, and C might attack protein or oligopeptides produced by cathepsin D, thus the inhibitory effect of irradiation is reduced at higher pH. Concerted action of various cathepsins has been reported by various workers (Liao-Huang et al., 1971; Goett-
lich-Riemann et al., 1971; Iodice et al., 1966). Moreover, protein breakdown by cathepsin B at pH 5.0 (Otto, 1971; Huisman et al., 1974) and by neutral protease at pH 7.0 (Okitani and Fujimaki, 1972; Okitani et al., 1973) has also been reported. A radiation-induced decrease in high molecular weight component and an increase in low molecular weight components of chicken muscle soluble protein fractions have been observed (Klein and Altman, 1972a). This finding is consistent with the present results of enzyme hydrolysis of irradiated hemoglobin. Low molecular weight components are not attacked by cathepsin D at pH 3.8, thus resulting in decreased hydrolysis. Besides the effect of reduction in molecular weight denaturation might also have contributed to reduced susceptibility to protein hydrolysis by various cathepsins present in lysosomal extract used.

3.3.5.2. Hydrolysis of hemoglobin after irradiation in soluble or dry state.

Irradiation of hemoglobin was carried out in soluble (10%) form or in the dry state at room temperature. After irradiation, digestion of 2.0% hemoglobin substrate by lysosomal cathepsins was determined by incubation at 37°C for 2 hours. Results of this study demonstrated that the irradiation-induced inhibitory effect was significantly (P<0.01) higher on hemoglobin irradiated in soluble form than in the dry state (Table 8). Doses ranging from 0.25
to 5.00 Mrads progressively rendered the protein more resistant to attack by cathepsin D. A high dose of 5.0 Mrads given to dry hemoglobin resulted in 66.38% hydrolysis, while an equivalent effect (67.87%) was observed with only 0.5 Mrad dose administered to protein in the soluble state.

Table 8. Effect of irradiation of hemoglobin in the dry and soluble state on its hydrolysis by lysosomal cathepsin D at pH 3.8.

<table>
<thead>
<tr>
<th>Irradiation dose (Mrad)</th>
<th>Percent hydrolysis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry state</td>
<td>Soluble state</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>100.00</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>86.58</td>
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</tr>
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<td>0.50</td>
<td>82.68</td>
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</tr>
<tr>
<td>1.00</td>
<td>77.92</td>
<td>44.57</td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>66.38</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>

n = 3

Radiation-induced changes in substrate might decrease its affinity for enzyme binding. In dry hemoglobin, radiation damage would be caused by "direct effects" but in soluble state the protein would undergo additional changes due to "indirect effects" of irradiation. Moreover, irradiation damage to hemoglobin in dry and soluble state might be of a different nature. This variation in extent and nature of irradiation damage to hemoglobin is reflected in its
3.3.5.3. **Electrophoretic pattern of irradiated hemoglobin after hydrolysis by cathepsin D.**

Irradiated hemoglobin solution was diluted to 5% and used as substrate for hydrolysis by cathepsin D at pH 3.8. After incubation of 5 hours at 37°C, samples were drawn and 10μl was employed for electrophoresis. The results are presented in Figure 18. The electrophoretogram of control samples showed changes in various bands (Figure 18, slots 1, 3, and 5) after irradiation. Intensity of band A decreased with an increase in band C and D in 0.5 and 1.0 Mrad irradiated hemoglobin (slots 3 and 5 respectively) compared with unirradiated sample (slot 1). Hydrolysis of non-irradiated and irradiated hemoglobin showed that bands A and C primarily were decreased after digestion by cathepsins. A slight decrease in band D was also observed (slots 2, 4, and 6). After digestion of irradiated hemoglobin (slots 4 and 6), band A showed a slightly higher intensity and some smearing between bands A and B occurred, as compared with digestion of non-irradiated sample (slot 2). It is interesting to note that mainly bands A and C are affected by irradiation treatment (slot 1 compared with slots 3 and 5), and the same bands are reduced due to catheptic digestion (slots 2, 4, and 6). It is likely that changes in hemoglobin molecules as observed in the change of electrophoretic pattern after irradiation treatment are
Figure 18. Electrophoretic pattern of irradiated hemoglobin before (1, 3, and 5) and after (2, 4, and 6) catheptic digestion at pH 3.8.
responsible for its reduced digestion by lysosomal cathepsin D at pH 3.8 (Table 8). Delincee and Radola (1974) observed extensive modification of charge properties in horseradish peroxidase protein after gamma irradiation as studied by thin-layer isoelectric focusing. They also noted aggregation of protein. Alteration in electrophoretic mobility of irradiated horse heart myoglobin (Paul and Kumta, 1973), casein and egg albumin (McArdle and Desrosier, 1955), myoglobin (Satterlee et al., 1972), and soluble muscle proteins (Klein and Altmann, 1972a; Uzunov et al., 1972) also have been observed. It has been suggested that protein structure is altered by radiation-induced breakage of sulfur linkages or hydrogen bonds (McArdle and Desrosier, 1955), covalent bonds of the polypeptide chains or hydrogen and ionic bonds between the side chains (Uzunov et al., 1972).

3.3.5.4. Spectral changes in irradiated hemoglobin.

After irradiation of a 10% solution of hemoglobin, measurements of the visible spectrum were made on diluted samples for evaluation of radiation-induced changes in the protein used as a substrate for cathepsin D. The effect of various doses is shown in Figure 19. Increase in absorbance in the 520 - 540 and 560 - 580 nm regions, and decreases in the 480 - 500 and 600 - 630 nm regions were observed. Moreover, the Soret band peak around 400 nm decreased as a result of irradiation. These observations
Figure 19. Radiation-induced spectral changes in hemoglobin substrate.
are in agreement with the studies carried out on myoglobins (Clarke and Richards, 1971; Satterlee et al., 1971).

Spectral changes have been attributed to alterations in the protein moiety (Lycometros and Brown, 1973; Brown and Akoyunoglou, 1964) as well as to rupture of the hemin nucleus (Clarke and Richards, 1971). Radiation-induced polymerization of myoglobin has been reported by Lycometros and Brown (1973). Satterlee et al. (1972) observed that aggregation of metmyoglobin increased with radiation dosage. They also reported that irradiated metmyoglobin had a lower α-helical content, indicating that some of the irradiated molecules might exist in a partially unfolded state. It seems quite likely that such radiation-induced alterations in heme-proteins may be partially responsible for decreased hydrolysis of hemoglobin by lysosomal cathepsins, observed in the present study.
3.4. SUMMARY AND CONCLUSIONS

Radiation inactivation of lysosomal cathepsins A, B, C, and D has been studied in this chapter. It was found that all of the cathepsins showed a high resistance to radiation inactivation when irradiated in lysosomal suspension. It was noticed that cathepsin B was slightly more sensitive to radiation compared with cathepsins A, C, and D, and the highest resistance was shown by cathepsin A. Residual activity after 1.0 Mrad dose was found to be 78.7, 67.0, 70.2, 72.58% for cathepsins A, B, C, and D respectively.

A soluble enzyme fraction isolated from disrupted lysosomes was found to be considerably more sensitive than the enzyme irradiated in the lysosomal environment. Irradiation of disrupted and intact lysosomes showed similar effects on availability of the enzymes, suggesting that association of enzymes with lysosomal fragments imparted a radioprotective effect. However, disruption of lysosomes prior to irradiation may increase the availability of the enzyme, and thus compensate for the inactivation effect.

Soluble enzymes exhibited radiation-sensitivity at acidic pH (4.0 and 5.5), which decreased with increasing pH (7.0 and 8.5), thus demonstrating that these enzymes will be more radiation-resistant at physiological pH and drop in pH of post-mortem muscle might have some sensitization effect.
Studies on cathepsin D revealed that sucrose concentration in the suspension medium and temperature of irradiation influenced radio sensitivity; 0.5M sucrose concentration significantly (P<0.01) increased radio-protection and 0°C provided a greater degree of radio-protection than 23°C. Heating of irradiated cathepsin D solution decreased the residual activity, demonstrating that radiation - heat combination process could be used to reduce the irradiation dose level, which would be beneficial in avoiding the undesirable effects of high doses of irradiation required to reduce residual proteolytic activity in meats. The mechanism involved in radiation-inactivation of cathepsins is not clear. Its elucidation is made more difficult by insufficient characterization of lysosomal cathepsins and information regarding their localization within lysosomes.

Most of the other studies have concentrated their efforts in residual proteolytic activity, and less attention has been paid to tissue proteins and their hydrolysis by proteases. In the present investigation the observations made on irradiated hemoglobin used as substrate for cathepsin D indicated that hemoglobin irradiated in soluble form became resistant to enzyme degradation. Such resistance was higher at pH 3.8 as compared with pH 5.0 or 7.0, thus indicating that irradiated hemoglobin would be markedly resistant to cathepsin D attack but not to other proteolytic enzymes such as cathepsins A, B, or C, which
are active at pH 5.0, or neutral proteases active at pH 7.0. Hemoglobin irradiated in the dry state exhibited similar resistance to degradation, but the level of resistance was lower, indicating that such changes are caused by direct as well as indirect effects of irradiation treatment. Electrophoretic pattern and spectral characteristics support the conclusion that irradiation caused considerable changes in hemoglobin, which might result in decreased hydrolysis by proteolytic enzymes. Susceptibility of tissue proteins to catheptic attack after irradiation treatment remains to be established.

This study demonstrated that cathepsins are relatively radiation-resistant but the extent of resistance varies among lysosomal cathepsins. Presence of other compounds and pH of the medium influence the radiation inactivation of these enzymes. Radiation-induced changes in protein substrate would also influence the course of proteolysis. These results indicate that irradiation inactivation of tissue enzymes is a complex phenomenon and residual proteolytic activity of tissue would be influenced by various factors. Irradiation - heat combination treatment seems to be a promising choice to reduce the residual enzyme activity.
CHAPTER 4. EFFECT OF GAMMA RADIATION ON ULTRASTRUCTURE OF LYSOSOMES AND CHICKEN SKELETAL MUSCLE

4.1. INTRODUCTION

4.1.1. Lysosomes

The role of lysosomal cathepsins in post-mortem meat tenderization is not well elucidated, but several studies have demonstrated a release of lysosomal cathepsins and other hydrolases during the aging period (Lutalo-Bosa, 1970; Ono, 1971; Eino and Stanley, 1973a; Dutson and Lawrie, 1974). Release of lysosomal enzymes from isolated particles under various conditions have been reported (Sawant et al., 1964a; Hayashi et al., 1973; Verity et al., 1968). Ionizing radiation has also been shown to decrease the stability of lysosomes and enhance the release of enzymes (Desai et al., 1964; Watkins, 1970; Watkins and Deacon, 1973). Release of these enzymes is probably a result of alterations in lysosomal structure, especially lysosomal membranes. Shibko et al. (1965) reported that lysosomes lost electron-dense material as a result of incubation at 37°C for 3 hours, but retained intact outer membranes as observed in electron micrographs. Brunk and Ericsson (1972) demonstrated cytochemically that acid phosphatase could leak through ultrastructurally intact lysosomal membranes. In the present study, an attempt has been made to observe the influence of ionizing irradiation on ultrastructure of lysosomes,
and its possible effects on leakage of lysosomal contents.

4.1.2. Muscle

During post-mortem storage of muscle, ultrastructural changes have been observed. Studies have indicated that during aging of beef and chicken Z-I junction in myofibrils is weakened and breaks across the fibrils occur near Z-line (Davey and Gilbert, 1967; Fukazawa et al., 1969). Similar breakage of myofibrils occurs in aged bovine and chicken muscle when subjected to mechanical stress (Davey and Dickson, 1970; Sayre, 1970). Schaller and Powrie (1971) demonstrated that besides breaks occurring at weak points like Z-I junction in aged muscles, disruption of sarcolemma and sarcoplasmic reticulum is also possible. Ionizing radiation deposits energy at random, and it is difficult to predict its effects on ultrastructure of muscle, but the above-mentioned weak points might be vulnerable to radiation damage. In the present study, effort has been made to assess the alterations in ultrastructure of irradiated chicken skeletal muscle with the help of transmission electron microscopy (TEM) and scanning electron microscopy (SEM).
4.2. EXPERIMENTAL

4.2.1. General Sample Preparation

4.2.1.1. Lysosomes.

Irradiated and non-irradiated samples of lysosomal fraction were incubated at 4°C or 37°C. Samples were drawn at 0, 48, and 72 hours after incubation at 4°C and after 1 hour at 37°C. All the lysosomal suspensions were centrifuged at 17,000 x g for 20 minutes at 4°C, and the pellet was used for fixation and further processing for EM studies.

4.2.1.2. Tissue.

Breast muscle (Pectoralis major) samples were rapidly removed and subjected to gamma radiation in the Gamma Cell-220 unit. The cryofracture technique of Schaller and Powrie (1971) was used to prepare samples for fixation. Cubes of tissue, about 1 cm along each edge, were immersed in liquid nitrogen, a frozen sample was placed between two sheets of plexiglass and struck with a hammer head to fracture the friable material. Frozen fragments approximately 3 mm in diameter were taken for fixation and further preparation for scanning electron microscopy.

Tissue samples from chicken breast muscle, about 2 mm in diameter, were removed immediately after irradiation and further processed for transmission electron microscopy.
4.2.2. Sample Processing for Electron Microscopy

Lysosomal pellets and tissue samples were fixed by immersion in 2% glutaraldehyde in 0.1M cacodylate-HCl buffer pH 7.2 containing 0.1M sucrose at 4°C for 1 hour (Brunk and Ericsson, 1972). The specimens were washed 3 times with the buffer and placed in 1% OsO₄-0.1M sucrose in 0.1M cacodylate-HCl buffer pH 7.2 at 4°C. The specimens were dehydrated serially in water-ethanol solutions of 30%, 50%, 70%, 80%, 90%, and 100% ethanol.

4.2.2.1. Scanning electron microscopy.

After dehydration, the specimens were placed in amyl acetate-ethanol solutions of 25%, 50%, 75%, and 100% amyl acetate to replace ethanol and then dried in a critical point drier (Parr Instrument Co.) by flushing with CO₂ for 20 minutes under pressure, followed by evaporation for 20 minutes. The dried fragments of tissue and lysosomal pellet were mounted to an aluminum holder with silver cement and were coated with 60% gold, 40% palladium in a vacuum evaporator (Hummer I-Technics Inc.) using D. C. Sputtering system for coating. Samples were examined with an ETEC Autoscan - scanning electron microscope operated at 20 KV. Images were recorded on Polaroid type 55P/N film.

4.2.2.2. Transmission electron microscopy.

After dehydration, the specimens were infiltrated with araldite-epon mixture. The embedded samples were
cured for 24 hours at 70°C. Sections were cut from the hardened resin blocks on a Carl Reichert Om U3 Ultramicrotome.

Thin sections (approximately 600Å) were mounted on uncoated 300 mesh copper grids and stained with uranyl acetate followed by lead citrate. The sections were examined with an AEI Corinth 275 transmission electron microscope operated at 60 KV. The sample chamber was kept cool with liquid nitrogen during observations. A photographic record of the images was made using Agfa Gevatex T51P 70 mm film.
4.3. RESULTS AND DISCUSSION

4.3.1. Radiation-induced Changes in Ultrastructure of Lysosomes

4.3.1.1. Internal ultrastructure of lysosomes.

The present communication will consider the effect of radiation on ultrastructural changes in lysosomes under different incubation conditions, and their comparison with non-irradiated particles. Transmission electron microscopy was used to observe the internal structure, while scanning electron microscopy was employed to observe the surface ultrastructure of the lysosomal particles.

Figures 20 and 21 are TEM micrographs of non-irradiated control lysosomal particles. Different shape and size of particles is apparent: most of them have a spherical appearance. Some particles have a dense matrix (Figures 20 -21, L1), while others have a much lighter matrix (Figure 20 -21, L2). In most of the particles, a single layer membrane is distinguishable. This feature is characteristic of lysosomes (de Duve, 1963a). Some amorphous material, possibly cell debris or remains of damaged particles, can be found among the intact particles. Almost all of the lysosomal particles observed were turgid with a dense granular matrix contained within a limiting membrane.

After incubation of lysosomal particles at 4°C, the lysosomal matrix appears less dense (Figure 22). The lysosomal membrane of some of the particles is diffused and
Figure 20. Transmission electron micrographs of non-irradiated lysosomes without incubation.
L1 = lysosomes with dense matrix;
L2 = lysosomes with light matrix;
MV = multi vesicular body 75,000X.
Figure 21. Transmission electron micrographs of non-irradiated lysosomes without incubation.  
L1 = lysosomes with dense matrix;  
L2 = lysosomes with light matrix;  
A, 75000X;  B, 80000X;  C, 80000X;  D, 120000X.
Figure 22. Transmission electron micrographs of non-irradiated lysosomes after 48 hour incubation at 4°C; arrow = leakage of lysosomal content; dm = diluted matrix; mv = multivesicular body. A, 50,000X; B and C, 75,000X.
irregular, suggesting that loss of intralysosomal material could have occurred as a result of weakening of the membrane or a change in its permeability. Apparent leakage of internal material can be seen in Figure 22-C (arrow), through weakened but intact lysosomal membrane. Dilution of matrix appears to occur in some particles (Figure 22-B, dm). Upon prolonged incubation, most of the particles were disrupted (Figure 23); particles with a low density matrix appeared to disintegrate readily. Lysosomes with a dense matrix were also subject to disruption (Figure 23-A and C; arrows), but the dense matrix appeared less readily dispersible and tended to clump together. Similar resistance to dispersion was exhibited by Triton X-100 treated lysosomes (Figure 24). All of the particles are disrupted, but few clumps of dense material are still present.

Leakage of lysosomal matrix material appeared to be enhanced by gamma radiation treatment of lysosomal particles. After 48 hours of incubation at 4°C, most of the particles appeared as hollow rings of lysosomal membrane with very little dense material contained within (Figure 25). After 72 hours, very few particles showed intact lysosomal membrane; even the dense type lysosomal particles were disrupted (Figure 26). A considerable amount of scattered lysosomal debris was observed in the TEM micrographs, with some dense matrix clumps (Figure 26,
Figure 23. Transmission electron micrographs of non-irradiated lysosomes after 72 hours incubation at 4°C; arrow = disrupted dense lysosomes; dmv = disrupted multi vesicular body; rm = residual material. A, 30,000X; B, 50,000X; C, 80,000X.
Figure 24. Transmission electron micrographs of lysosomes disrupted by Triton X-100 treatment; arrows = clumps of lysosomal dense matrix. A, 50,000X; B, 80,000X; C, 30,000X
Figure 25. Transmission electron micrographs of irradiated (1.0 Mrad) lysosomes after 48 hours incubation at 4°C. A, 75,000X; B, 50,000X; C, 150,000X.
Figure 26. Transmission electron micrographs of irradiated lysosomes (1.0 Mrad) after 72 hours incubation at 4°C; Lr = lysosome after release of its contents; rm = released material from lysosome; arrows = clumps of dense lysosomal matrix. A, 45,000X; B, 120,000X; C and D, 50,000X.
arrows), showing extensive disruption of the particles.

Incubation of lysosomal particles at 37°C seemed to enhance the leakage of lysosomal matrix material and disruption of lysosomes (Figures 27, 28). In non-irradiated samples, a few of the particles showed almost complete leakage of intralysosomal material after 1 hour of incubation (Figure 27-A), but most of the particles contained part of their dense material in their matrix (Figure 27, b and c). However, some disruption of particles was also observed. Irradiation of particles considerably enhanced the leakage of material (Figures 29, 30, 31). The majority of particles appear as hollow rings containing very little matrix material. Integrity of the membrane seemed to be affected by irradiation treatment, as the membrane looked disorganised. In Figures 29-B and 30-C (arrows), two irradiated particles show leakage of material through weak points in the lysosomal membrane. The dense particles, which were found to be relatively resistant to disruption and leakage of the internal material on incubation (Figures 27, 28), seem to have been affected greatly by irradiation treatment, resulting in either disruption (Figure 31, dL), or extensive leakage of lysosomal content (Figure 30, Lc) through a weakened and disorganised lysosomal membrane. In Figure 31-C (dm), a particle with a disrupted membrane and leaking dense material is shown. Another segment of the membrane appears to be still organised, with only a few
Figure 27. Transmission electron micrographs of non-irradiated lysosomes after 1 hour incubation at 37°C; a = complete leakage of lysosomal content; b and c = partial leakage; cv = cavity. A, B, and D, 50,000X; C, 30,000X.
Figure 28. Transmission electron micrographs of non-irradiated lysosomes after 1 hour incubation at 37°C showing various stages of leakage of lysosomal contents (a, b, and c). A, 30,000X; B and C, 80,000X; D, 200,000X.
Figure 29. Transmission electron micrographs of irradiated lysosomes (1.0 Mrad) after 1 hour incubation at 37°C; arrow = weak point in lysosomal membrane showing leakage. A, B, and C, 80,000X; D, 120,000X.
Figure 30. Transmission electron micrographs of irradiated lysosomes (1.0 Mrad) after 1 hour incubation at 37°C; Lc = leaked lysosomal contents; arrow = weak point in lysosomal membrane showing leakage. A, 80,000X; B and C, 50,000X; D, 60,000X.
Figure 31. Transmission electron micrographs of irradiated lysosomes (1.0 Mrad) after 1 hour incubation at 37°C; dL = disrupted lysosomes; mv = multivesicular body; dm = damaged membrane; arrows = weak points in the membrane. A, 30,000X; B, 80,000X; C, 120,000X; D, 200,000X.
weak points (arrows). Another particle in Figure 30-D shows a general weakening and disorganisation of the lysosomal membrane, indicating that membrane structure could be extensively damaged by irradiation.

The present observations agree with the results of Shibko et al. (1965), who observed the loss of electron-opaque contents but retention of intact outer membranes in rat kidney lysosomes after 3 hours incubation at 37°C. They also observed a membranous structure and numerous small vesicles present within the limiting membrane after incubation. In the present study, no such vesicles or other material indicating intralysosomal membranes, was observed. The present study also indicated that lysosomal membranes might be disrupted from weak points and lysosomal contents might leak from such points; this cannot be observed in TEM micrographs unless the section is cut through such a plane (Figures 29-B and 30-C). Moreover, irradiation might cause severe damage at such weak points, thus making the mechanism of leakage more apparent when examined under EM. Multi-vesicular bodies or residual bodies which contain undigested material might show presence of small vesicles after leakage of soluble contents (Figure 31-B). Ericsson and Brunk (1972) demonstrated that, following severe damage to in vitro cultivated cells induced by photosensitization injury, leakage of Thorotrast particles from lysosomes occurred through apparent ruptures or holes in the lysosomal
membranes; but under osmotic shock, release of acid phosphatase from lysosomes occurred by diffusion (Brunk and Ericsson, 1972), as detected by cytochemical study. As discussed in Chapter 2, release of cathepsins is greatly enhanced after irradiation treatment; it is possible that irradiation treatment causes damage resulting in rupture or hole formation at the site of already existing "pores" in lysosomal membranes. Radiation treatment might cause dissociation or solubilization of lysosomal matrix, thus speeding up the release of lysosomal contents.

4.3.1.2. Surface ultrastructure of lysosomes.

In SEM micrographs (Figures 32, 33) surface ultrastructure of non-irradiated lysosomal fractions is presented. After incubation at 4°C for 48 hours, some particles show apparent leakage of lysosomal content, but the majority of the particles seem to be intact and no extensive damage is observed. At a few places, amorphous material presumably arising from the particles, can be seen. Many of the particles are spherical but some show elongation. Irradiated particles appear extensively damaged and leakage of lysosomal contents is evident (Figure 34, 35). A considerable amount of amorphous material can be seen in the vicinity of disrupted particles. Some of the particles show very clearly the leakage of the material from holes occurring in the lysosomal membrane (Figure 35-A).

Comparison of the surface ultrastructure of irradiated
Figure 32. Scanning electron micrographs of non-irradiated lysosomal particles after 48 hours incubation at 4°C; Lc = leakage of contents; arrows = damaged particles; rs = rough-surfaced particle. A and B, 24,000X; C, 60,000X; D, 50,000X.
Figure 33. Scanning electron micrographs of non-irradiated lysosomal particles after 48 hours incubation at 4°C; am = amorphous material; Lc = leakage of contents; dp = damaged particles; arrows = site of leakage. A and B, 24,000X; C, 90,000X; D, 360,000X.
Scanning electron micrographs of irradiated (1.0 Mrad) lysosomal particles after 48 hour incubation at 4°C; am = amorphous material; dp = damaged particles; Lc = leakage of contents. A, 60,000X; B, 90,000X; C and D, 50,000X.
Figure 35. Scanning electron micrographs of irradiated (1.0 Mrad) lysosomal particles after 48 hour incubation at 4°C; am = amorphous material; dp = damaged particles; Lc = leakage of contents; arrow = point of leakage; de = depression. A, 90,000X; B, 240,000X of particle encircled in A.
and non-irradiated particles indicates that radiation damages the lysosomal structure most probably by weakening the lysosomal membranes, and resulting in the formation of "holes" at the weak points. These observations confirm and support the results obtained with TEM as discussed in the preceding section. In general, these ultrastructural observations provide further evidence that radiation damage to lysosomal structure is responsible for the marked increase in the free activity of lysosomal cathepsins as discussed earlier in Chapter 2.

4.3.2. Effect of Irradiation on Ultrastructure of Skeletal Muscle

4.3.2.1. Transmission electron microscopy.

Transmission electron micrographs of untreated control muscle 3 hours post-mortem are presented in Figures 36 and 37. Various bands can be easily distinguished with normal inter-fibril spaces. In contrast to control muscle, the irradiated myofibrils show severe disorganisation (Figures 38, 39, 40). Transverse breaks in irradiated myofibrils can be observed in Figures 38-A, B, and C. The most vulnerable site for breaks seems to be I band, but general disorganisation of the myofibril has occurred. Twisting of unattached ends of myofibrils occurred (Figure 38-D). In Figure 39, a transverse break at the I band through a number of myofibrils is shown. The I band, and in some
Figure 36. Transmission electron micrographs of non-irradiated chicken pectoralis muscle, 3 hours post-mortem; A = A band; I = I band; Z = Z line. A, 30,000X; B, 20,000X; C, 80,000X; D, 20,000X.
Figure 37. Transmission electron micrographs of non-irradiated chicken pectoralis muscle, 3 hours post-mortem; A = A band; I = I band; Z = Z line. A, 18,000X; B, 75,000X.
Figure 38. Transmission electron micrographs of irradiated (1.0 Mrad) chicken pectoralis muscle, 3 hours post-mortem; br = breaks; tf = twisted free ends of fibrils. A and B, 20,000X; C, 45,000X; D, 30,000X.
Figure 39. Transmission electron micrographs of irradiated (1.0Mrad) chicken pectoralis muscle, 3 hours post-mortem; Z = Z line; dz = disintegrated Z line. A, 30,000X; B, 120,000X; (area from A).
Figure 40. Transmission electron micrographs of irradiated (1.0 Mrad) chicken pectoralis muscle, 3 hours post-mortem; sf = separated fibrils. 25,000X.

Figure 41. Transmission electron micrographs of cytoplasmic particles in irradiated (1.0 Mrad) chicken pectoralis muscle 3 hours post-mortem; dp = damaged particles. 75,000X.
cases, Z-line material, is found to be disintegrated, and precipitation of this material seems to have occurred in the irradiated samples. Some workers have indicated that during aging of beef and chicken muscle, the Z-line dis­appeared (Davey and Gilbert, 1967; Fukazawa et al., 1969), and breaks across fibrils occurred near the Z-line (Davey and Gilbert, 1969; Sayre, 1970). In the present study, irradiation resulted in breaks occurring in the fibrils near the Z-line, but intact Z-lines could be observed in most cases (Figures 38-C, 39). In some cases, the Z-line seems to have disintegrated, but removal or dissolution of Z-line as found in aged muscle was not observed in irradiated muscle. In some areas, interfibril distance has increased, indicating that myofibrils have been pulled apart under the influence of irradiation (Figures 38-A, C, and 40). Transmission electron micrographs of lysosome-like cytoplasmic particles from irradiated and control muscle are shown in Figures 41 and 42 respectively. The particles found in control muscle exhibit clearly an intact limiting membrane, but particles found in irradiated muscle appear partially disrupted and their ultrastructure dis­organised. This effect is similar to the observations already discussed regarding isolated lysosomes, i.e., dissolution of lysosomal membranes and disruption of the particles occurs after irradiation treatment.
Figure 42. Transmission electron micrographs of cytoplasmic particles in non-irradiated chicken pectoralis muscle, 3 hours post-mortem. A, 30,000X; B, 120,000X; C, 150,000X; D, 120,000X.
4.3.2.2. Surface ultrastructure of muscle.

Surface ultrastructure of cryofractured control and irradiated muscle was examined by SEM. Non-irradiated muscle samples had a smooth fibre surface and the transverse break across the fibres was clean (Figure 43). A closer view of the transverse break in Figure 44-A reveals a smooth surface with some amorphous material and some bead-like structure, possibly formed by the disintegrated material arising from the breaks. Figure 44-B shows part of sarcolemma of non-irradiated muscle. The surface of the sarcolemma appears rough, but no breaks in the structure can be observed. Irradiation of muscle with 1.0 Mrad caused marked ultrastructural changes as shown in Figures 45 - 47. The muscle fibres appear considerably contracted, and alternately elevated and depressed areas appeared along the length of the fibres (Figure 45). Fissures between fibres were also observed (Figures 45-B, 46-A). This is possibly due to irradiation-induced weakening of connective tissue, which has subsequently broken during the cryofracture treatment. Higher magnification of the fibre surface (Figure 46-B) indicated that the sarcolemma of irradiated samples was extensively disrupted. Moreover, the fibrils thus exposed showed extensive longitudinal separation between them (Figure 46-B).

The transverse breaks occurring in irradiated fibres were different than those in non-irradiated fibres.
Figure 43. Scanning electron micrographs of non-irradiated and cryofractured chicken pectoralis muscle. A, 480X; B, 1,600X.
Figure 44. Scanning electron micrographs of non-irradiated and cryofractured chicken pectoralis muscle, 3 hours post-mortem; A, transverse break, 2,000X; B, sarcolemma, 20,000X.
Figure 45. Scanning electron micrographs of irradiated and cryofractured chicken pectoralis muscle, 3 hours post-mortem; ct = connective tissue; sf = space between fibres. A, 160X; B, 320X; C, 800X.
Figure 46. Scanning electron micrographs of irradiated (1.0 Mrad) and cryofractured chicken pectoralis muscle, 3 hours post-mortem; A, arrow = separation of fibres, 120X; B, perforated sarcolemma, sf = separated fibrils, 16,000X.
verse breaks in control fibres were straight with a smooth surface (Figure 44-A), whereas those in irradiated fibres showed an uneven surface at the breaks, and also a "spikey" structure coming out of the plane (Figure 47-A). Such an effect might be due to irradiation-induced separation of the fibrils, or breakdown of connective tissue, which could result in breakage of fibrils at different points. An enlarged area of the transverse break of irradiated fibres is shown in Figure 47-B; fissures between individual fibrils and remains of sarcoplasmic reticulum can be seen.

After aging of bovine longissimus dorsi, breaks across fibrils and collapse of sarcoplasmic reticulum have been reported by Schaller and Powrie (1971). Davey and Gilbert (1969), Davey and Dickson (1970), and Sayre (1970) showed that in aged bovine and chicken muscle, breakage of fibrils occurred near the Z-disc during mechanical stress. Irradiation-induced ultrastructural changes as presented in this report seem to be similar to those resulting from aging, but breaks across fibrils as observed in TEM micrographs are more severe. SEM revealed weakening and disruption of the sarcolemma as well as sarcoplasmic reticulum. It is difficult to determine whether the mechanism involved in irradiation-induced changes and that responsible for changes observed in aged muscle are similar, but the rapid and extensive damage to muscle ultrastructure caused by irradiation is indicative of a difference in mechanisms.
Figure 47-A. Scanning electron micrograph of irradiated (1.0 Mrad) cryofractured chicken pectoralis muscle, 3 hours post-mortem; f = fissures. 2,000X.
Figure 47-B. Area of rectangle in figure 47-A at higher magnification (20,000X); f = fissures; arrows = part of sarcoplasmic reticulum.
Damage to sarcoplasmic reticulum by irradiation could result in release of calcium ions and cause contraction of muscle (Ebashi, 1961; Schmidt et al., 1970).
4.4. SUMMARY AND CONCLUSIONS

It was found that ultrastructure of lysosomes can be significantly altered by irradiation treatment. Observation of lysosomal ultrastructure indicated that lysosomal contents leaked readily after irradiation treatment. Dilution of dense lysosomal matrix occurred in non-irradiated particles during incubation, but release of lysosomal contents was relatively slow. On prolonged storage (72 hours at 4°C), non-irradiated lysosomes showed some disruption, but dense particles retained their shape although their membranes were not clearly distinguishable. Irradiated lysosomes showed extensive release of dense material from the particles after 48 hours of incubation; further incubation caused disruption of most of the particles. Clumps of dense lysosomal matrix were found in the medium, indicating disruption of dense lysosomes, which in control samples showed resistance to disruption. Triton X-100 treatment disrupted the lysosomes extensively. Incubation of lysosomal particles at elevated temperatures enhanced the leakage of lysosomal contents, especially from irradiated lysosomes, and extensive damage to irradiated particles was observed. Leakage of lysosomal contents is probably due to increased permeability of lysosomal membranes, as most of the irradiated particles had diffused or disorganized membranes. Holes in the membranes of a few irradiated particles were observed. Irradiation might create weak points in the membranes and
such effect would be exaggerated due to incubation and might result in formation of holes in the membranes, and also cause disruption of particles. SEM studies showed additional evidence that lysosomal contents leaked from weak areas; deposits of amorphous material still attached to these areas could be observed.

Transmission electron microscopy of irradiated muscle revealed considerable damage occurring to myofibrils. Most of the damage was observed at the I band, where breaks across the fibrils were noticed. Disintegration of Z-lines was observed at a few breaks, but in other cases Z-lines remained intact. In certain areas, an increase in interfibril spaces was observed. Some cytoplasmic particles in tissue resembling lysosomes were found to be damaged by irradiation treatment.

Surface ultrastructure of cryofractured muscle was studied under scanning electron microscope. Radiation was found to cause extensive contraction of muscle fibres, while non-irradiated muscle did not show such an effect. Transverse breaks in non-irradiated muscle had a smooth surface, but in irradiated muscle, fibres showed an uneven surface at these breaks with a spike-like appearance. Fissures between fibrils were caused by irradiation treatment, which supports the earlier finding of TEM study that interfibril spaces tend to increase due to irradiation. There were indications that the sarcoplasmic reticulum was
also damaged in irradiated samples. It was observed that the sarcolemma was extensively perforated due to irradiation treatment.

Radiation-induced structural alterations in lysosomes and subsequent release of lysosomal contents, which contain powerful hydrolytic enzymes like cathepsins, might cause extensive proteolysis in tissue during post-mortem storage. Moreover, radiation-induced damage to myofibril structure might increase the availability and susceptibility of myofibrillar protein to cathepsin attack. This could be used beneficially for meat tenderization, but extensive damage caused directly by irradiation or brought about by enhanced proteolysis as a result of radiation-induced release of cathepsins could deteriorate meat quality during long-term storage of irradiation-preserved meats.
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