INTRODUCTION
**PROTEASES IN GENERAL**

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories:

**Limited proteolysis**, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein e.g conversion of prohormones to hormones.

**Unlimited proteolysis**, in which proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists in the compartmentation of proteases e.g in lysosomes. Proteins transferred into this compartment undergo a rapid degradation.

The International Union of Biochemistry and Molecular Biology (1984) has recommended to use the term *peptidase* for the subset of peptide bond hydrolases (Subclass E.C 3.4.). The widely used term *protease* is synonymous with *peptidase*. *Peptidases* comprise two groups of enzymes: the endopeptidases and the exopeptidases, which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus respectively. The term *proteinase* is also used as a synonym for *endopeptidase* and four mechanistic classes of proteinases are recognized by the IUBMB as detailed below. The modern scheme of nomenclature is thus:
CLASSIFICATION OF PROTEINASES

Proteinases are classified according to their catalytic mechanisms. Four mechanistic classes have been recognized by the International Union of Biochemistry and Molecular Biology:

- The serine proteinases
- The aspartic proteinases
- The metallo proteinases
- The cysteine proteinases

This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationships of proteases (Rawlings and Barrett, 1993). In addition to these four mechanistic classes, there is a section of the Enzyme nomenclature, which is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified but the possibility remains that novel types of proteases do exist.

**Serine proteinases:** This class comprises of two distinct families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the substilisin family which include the bacterial enzymes
such as subtilisin. The general 3D structure is different in the two families but they have the same active site geometry and then catalysis proceeds via the same mechanism. The serine proteinases exhibit different substrate specificities which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Some enzymes have an extended interaction site with the substrate whereas others have a specificity restricted to the P1 substrate residue. Three residues which form the catalytic triad are essential in the catalytic process i.e. His 57, Asp 102 and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accept the OH group of the reactive Ser.

Aspartic proteinases: Most of the aspartic proteinases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). Second family comprises of viral proteinases such as the protease from the AIDS virus (HIV) also called retropepsin. Crystallographic studies shows that these enzymes are bilobed molecules with the active site located between two homologous lobes. Each lobe contributes one aspartate residue of the catalytically active diad of aspartates. These two aspartyl residues are in close geometric proximity in the active molecule and one aspartate is ionized whereas the second one is unionized at the optimum pH range of 2-3. Retropepsins, are monomeric, i.e. carry only one catalytic aspartate and then dimerization is required to form an active enzyme. In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two
simultaneous proton transfer: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism leads to the formation of a non covalent neutral tetrahedral intermediate

Metallo proteinases: The metallo proteinases may be one of the older classes of proteinases and are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without any loss of the activity. Bacterial thermolysin has been well characterized and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH, which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding of the Zn atom. The catalytic mechanism leads to the formation of a non covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group.

Cysteine proteinases: This family includes the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases (e.g Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family. Recent elucidation of the X-ray structure of the Interleukin-1-beta converting enzyme has revealed a novel type of fold for cysteine proteinases. Like the serine proteinases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys25 and His159 (papain numbering) plays the same role as Ser195 and His57 respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighbouring imidazolium group of His159. The attacking
nucleophile is the thiolate-imidazolium ion pair in both steps and then a water molecule is not required.

Cysteine proteases encompass a large and diverse group of enzymes and have been classified by Rawlings and Barrett (1994) into clans and families. A family is considered to be a group of enzymes which show an evolutionary relationship to at least one other enzyme, while a clan comprise a group of families for which there are indications of an evolutionary relationship despite the lack of statistically significant sequence similarities. The largest family of cysteine proteases identified to date by sequence homology is the papain family whose members include wide range of enzymes from both prokaryotes and eucaryotes, encompassing bacteria, plants, invertebrates and vertebrates (Berti and Storer ,1995). Mammalian papain-like cysteine proteases play a major role in the lysosomal protein degradation system (Kirschke and Barrett, 1987) and are also present in the extracellular matrix. This group includes cathepsins B,L,K,O,S and the recently discovered cathepsin W (Linnevers et al, 1997). Although the descriptor “cathepsin” includes protease of the serine (cathepsin G) and aspartic (cathepsin D and E) classes, by far the most abundant enzymes bearing this designation are cysteine proteases. It has become common to use “cathepsin” as a general term for the lysosomal cysteine proteases. Cathepsins are present in most cell types, although some appear to have selective cathepsin S, (Kirschke et al, 1989) or specific cathepsin K (Tezuka et al, 1994) cellular distribution. The best characterized of these mammalian enzymes are cathepsins B and L. The recent increased interest in cathepsin is due to accumulating evidence for their involvement in pathology where they play an extracellular role. Some of the processes in which they have been implicated include tumor invasion of metastasis (Yagel et al, 1989) bone resorption (Inui et al, 1997) and rheumatoid arthritis (Trabandt et al, 1991).

Essentially all papain like cysteine proteases are synthesized as inactive proenzymes which require processing to form the active enzyme. Their activation occurs through proteolytic cleavage of the N-terminal fragment of the enzyme, the so called pro-region, and its removal from the enzyme. The proregions are not only
inhibitors of the enzymatic activity but they likely fulfill other roles as well. In cathepsin L, the proregion is crucial for the correct folding of the newly synthesized enzyme and stabilizes the protein to the denaturing effects of neutral to alkaline pH, conditions which rapidly inactivate the mature enzyme (Tao et al, 1994). A detailed analysis of the sequence of papain like enzymes by Karrer et al (1993) led to the recognition of two subfamilies within this family. In terms of the mammalian members of the papain family, these subfamilies can be designated cathepsin L-like and cathepsin B-like enzymes, with the majority of enzymes belonging to the former group. While there is strong overall homology between the cathepsin L and cathepsin B subfamilies, and the main difference are restricted to a few short stretches where they have distinct fingerprint sequences.

OVERALL STRUCTURE OF PAPAIN-LIKE ENZYMES

The three dimensional structures of several enzymes from the papain family are presently known. The structure of papain, the prototypical enzyme from this family, has been known for almost 30 years (Dreuth, 1968) and the structural determination of several other enzymes from this family followed (actinidin, Baker and Drenth, 1987; calotropin D, Heinemann et al, 1982; caricain, Pickersgill et al, 1991; cathepsin B, Musil et al, 1991; chymopapain, Maes et al, 1992; cathepsin L, Fujishima et al, 1997). They show a high degree of structural similarity corresponding to the level expected from sequence homologies. Two domain can be distinguished within the enzymes: the N-terminal domain is mostly α-helical, the C-terminal domain contains predominantly β-sheets (Fig 1). The active site consists of Cys25 and His159 (papain numbering) and is supplemented by Asn175 hydrogen bonded to His159, and by Gln19, the latter forming part of the oxyanion hole which stabilizes the negative charge on the tetrahedral intermediate. This site is located at the interface between the two domains. The substrate follows the extended groove between the domains in the direction from the bottom (N-terminal) to the top (C-terminal). The inhibitory action of prorregions in both subfamilies is similar and occurs by obstructing the access of the substrate to the active site. The N-terminal fragment of the proregion is attached to the enzyme through the interactions with the PBL on one side of the active site (top),
Fig 1 3-Dimensional structure of papain family: Asterereodrawing of the ribbon representation of cathepsin B in the standard orientation. The N-terminal, mostly β domain is on the left and the C-terminal, mostly α helical domain is on the right. The occluding loop insertion, typical for this subfamily and shown in shaded gray, is not present in the procathepsin L subfamily. The substrate binding groove is in the centre and extends from top bottom. The active site residues, Cys 29, His 199 and Asn 219 are shown in full.
opposite to the N-terminal residue of the mature form (bottom) to which the proregion is covalently attached. The extended segment of the proregion follows the groove between the two domains of the enzyme along the same path as taken by the substrate.

**Papain from papaya (Carica papaya)**

The latex of the papaya plant and its green fruits contains two proteolytic enzymes, papain and chymopapain. The latter is most abundant but papain is twice as potent as chymopapain. Papain, a powerful proteolytic enzyme, belongs to cysteine protease family and is a plant endo protease. Its amino acid sequence (Mitchel et al 1970) (Fig.2), detailed X-ray structure (Kamphuis,1984), three dimensional structure (Fig.3) and extensive kinetic data, have generated new concepts and data on structure function relationship of this enzyme. Although derived from a plant source, papain displays some structural and functional similarities to other cysteine protease of both plant and animal origin. Papain consists of a single polypeptide chain of mol. wt. 23 Kd with 212 amino acid residues (Mitchel et al 1970). The papain molecule is folded to form two interacting domains resulting in a cleft at the surface of the enzyme (Drenth et al, 1971; Glazer and Smith, 1971). The L-domain is alpha helical rich and
Fig. 2 Amino acid sequence of papain from papaya latex
Fig.3  3-Dimensional structure of papain
it contains residues 10-111 and 208-212. It has got three alpha-helics in the secondary structural features. The R-domain is mainly made up of anti parallel β-sheet structure, but it also contains 2 helics, which are located at the opposite ends of the central β-structure. The active site residues Cys-25 and His-159 are located at the interface of this cleft on opposite domains of the molecule. Here Cys-25 is part of the L1 alpha helix at the surface of the left domain, while His-159 is in a β-sheet of the right domain. Apart from Cys and His, Asn 175 also plays a very important role in the catalytic mechanism and also in thermal stability of papain (Vernet et al 1995)

**CATALYTIC MECHANISM OF CYSTEINE PROTEINASE, PAPAIN**

Catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys25 and His159 (papain numbering) play the same role as Ser195 and His57 respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighbouring imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps and then a water molecule is not required (Fig. 4).

**INDUSTRIAL AND THERAPEUTIC POTENTIAL OF PAPAIN AND CYSTEINE PROTEINASES OF PAPAIN FAMILY**

Papain is used in various industries for a variety of functions (see Table 1). The functions and applications in Table 1 are listed in random order. Some recent findings have shown the role of cysteine proteinases in cancer progression and their chemical relevance for prognosis (Lah and Kos, 1998). They have proposed that an imbalance between cathepsins and cystatins, associated with the metastatic tumor cell phenotype, may facilitate tumor cell invasion and metastasis and can be responsible for early relapse of the disease after removal of the primary tumor. Desser et al (2001) have shown that the therapy with oral proteolytic enzymes (OET) with combination drug products containing papain, bromelain, trypsin and chymotrypsin is beneficial in
Table I: Uses of papain

<table>
<thead>
<tr>
<th>Industry</th>
<th>Uses</th>
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<tbody>
<tr>
<td>Brewing</td>
<td>clarifying cold beer</td>
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<tr>
<td>Food</td>
<td>meat tenderization, spices, hydrolysates</td>
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<tr>
<td>Yeast hydrolysis</td>
<td>yeast hydrolysates</td>
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<tr>
<td>Pharmaceutical</td>
<td>contact lens cleaner</td>
</tr>
<tr>
<td>Pet Food</td>
<td>palatability, viscosity reduction</td>
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<tr>
<td>Health Food</td>
<td>digestive aid</td>
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<tr>
<td>Detergents</td>
<td>blood stain remover</td>
</tr>
<tr>
<td>Veterinary</td>
<td>antitussive agents in preparations</td>
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Fig. 4 Catalytic mechanism of cysteine proteinase, papain
clinical setting such as radiotherapy induced fibrosis, bleomycin pneumotoxicity and immunosuppression in cancer, all of which are nowadays known to be accompanied by excessive transforming growth factors-β (TGF-β) production. It has been demonstrated that proteolytic enzymes reduce TGF-β levels in serum by converting the protease inhibitor α2 macroglobulin (α2M) from the "slow" form to "fast" form, whereby the "fast" form binds and inactivate the TGF-β irreversibly. Plant extracts with a high content of proteolytic enzymes have been used for a long time in the traditional medicine of Central and South America (Vanhoof and Cooreman, 1997; De Feo, 1992). Cysteine proteinases are also effective in second-degree burns and inhibit inflammation (Latha et al, 1997, 1998). In a clinical study of 156 patients with rheumatoid arthritis, an effect of orally administered proteinases on the level of cytokines was found (Mazurov et al, 1997). Mazurov et al (1997) showed that the serum levels of interferons were reduced to almost normal values after 6 months of therapy in the enzyme treated group (a combination of pancreatin/papain/bromelain/trypsin/chymotrypsin in addition to methotrexate). Leipner and Sailer (2000) have shown the effect and mode of action of systemic enzyme therapy in oncology where endoproteinases stem bromelain and papain were found to be most effective among other proteinases. The effectiveness of enzyme therapy has been shown in inoperable broncho-pulmonary carcinoma, gastric carcinoma, carcinoma of head and neck, ovarian carcinoma, multiple myeloma and large bowel carcinoma (Wrbka and Kodras, 1978; Schedler et al, 1980; Lahousen, 1995; Sakalova et al, 1998). In these studies there was an improvement in general condition and quality of life, improvement in life expectancy, rise in ratio of T lymphocytes, total lymphocytes, more rapid fall in AST, ALT, AP and LDH in the enzyme treated groups, survival of patients with stage II multiple myeloma and no patients showed a toxic pulmonary reaction to bleomycin. Systemic enzyme therapy would be a variation of the successful use of proteinase inhibitors in oncology. This mode of action would explain the therapeutic efficacy of systemic enzyme therapy although low activities of administered enzymes were detected in the plasma of patients. It also explains the influence of systemic enzyme therapy on cytokines metabolism. Since proteinases are neither pro-oxidants nor antioxidants, their effect on the formation or on the scavenging of reactive oxygen species seems to be indirect.
or an artefact rather than a mode of action of orally administered enzymes in oncology (Leipner and Saller, 2000).

Wald et al (2001a) have also shown that the proteinase administration reduces growth of human breast and pancreatic cancer in nude mice and diminishes expression of some tumorigenesis biomarkers. A specific mixture of serine and cysteine proteinases has been shown to significantly reduce tumor growth, metastatic potential and proliferation activity in animal tumors, as well as markedly prolong survival time in experimental animals. A very close correlation was found between the early start of treatment and growth of syngeneous melanoma B 16 in C57B16 mice (Wald et al, 2001b; Wald et al, 1998a, Wald et al 1998b; Wald et al, 1998c; Batkin et al, 1988). Zavadova (2001) have examined the in vitro effects of treating Dendritic Cells (DC) in peripheral blood samples and in ascites obtained from ovarian cancer patients using cytokines or Proteolytic Enzymes (PE) on leukocyte surface differentiation markers and on DC function. They have demonstrated that DC obtained from the peritoneal cavity and blood of ovarian cancer patients, cultured in the presence of PE undergoes differentiation to cells that show high expression of cell surface differentiation antigens and IL-12 production. These findings are important to ongoing multicancer clinical trials with PE in which it is being utilized as an oral agent that has an acceptable level of toxicity.

Recent technological advances, such as knockout mice, and the isolation of cysteine proteases with limited tissue distributions have contributed to the discovery of more sophisticated roles of these enzymes. Two enzymes have been shown to participate in MHC class I peptide presentation (Nakagawa et al, 1998; Riese et al, 1998), and the balanced activity of another is critical for bone health (Gelb et al, 1996). As detection and identification techniques become increasingly sophisticated and sensitive, more and more cysteine proteases that exhibit narrow tissue distribution are being characterized. Some will almost certainly function in crucial physiological processes. Thus, structural information is important for understanding the subtleties of how these proteins function and for the precise design of inhibitors to serve as therapeutics when activity leads to pathology.
Free radicals, also known as reactive oxygen species, are extremely reactive, unstable molecules or fragments of molecules, with unpaired electrons in their outer orbital. Free radicals strive to balance their unpaired electrons by combining with electrons in other substances that have opposite spins (Siodin et al, 1990). The property of readily accepting electrons, combined with the abundance of oxygen in the cells, is one reason that oxygen-centred free radicals are primary or secondary mediators of many free radical reactions (Alessio, 1993). Free radicals are generated by disturbances in the metabolic system as a result of oxidative stress. During low intensity activities most of the oxygen consumed during energy metabolism in the mitochondria combines with hydrogen to form water via the electron transport chain. If circumstances cause oxidative stress or if there is electron leakage in the electron transport chain, some of this oxygen is converted to free radicals (McArdle et al, 1996). Oxidative stress occurs during activities demanding high energy or oxygen utilisation such as intense exercise, tissue inflammation, cigarette smoking, environmental pollution, ionising radiation and certain medications (McArdle et al, 1996). This leads to an accumulation of hydrogen ions/electrons flowing through the electron transport chain in the mitochondria. Oxygen reacts highly with the hydrogen ions so with increased oxygen demand there is increased need for alternate electron acceptors.

**The Principles of Free Radical Chemistry**

In order to appreciate the role of free radicals in biology firstly it is necessary to understand their chemical nature. In general, molecular bond formation is dependent on the interaction of paired electrons. They have properties which enable them to spin about their own axes, and to confer bond stability they must be paired in opposite spins. Free radicals and molecular oxygen have properties which do not conform with their normal requirements of bonding. When a conventional molecular bond is broken, the two composite electrons usually split heterolytically.
However, under certain circumstances it is also possible for bonds to break homolytically:

\[ A:B \rightarrow A' + B \]

This is a free radical reaction. In non-biological systems, free radicals can be produced via the effects of ionising radiation, temperature and various photochemical events. Often, free radical reaction involve, either directly or indirectly, the formation of oxy radicals. Molecular oxygen is, in fact, a bi-radical possessing two unpaired electrons of parallel spin. These electrons exist in their lowest energy level when unpaired and when they have spins in the same direction. This configuration is called the ground or triplet state and it describes the paramagnetic and electronic behaviour of oxygen. Most molecules have electrons with opposite spins and are termed as diamagnetic, their ground states are described as singlets. The transfer of electrons to oxygen can also lead to the production of toxic free radical species. The best evidence of these is the superoxide radical. A two electron reduction of oxygen results in the formation of the peroxide ion. This is not a free radical, but is very reactive and at physiological pH protonates to form hydrogen peroxide (H\(_2\)O\(_2\)). The reaction of hydrogen peroxide with superoxide radical can lead to a series of reactions, which produce additional reactive species. These are described below.

**Haber - Weiss and Fenton reactions:** Haber proposed a reaction between superoxide radical and hydrogen peroxide

\[ H^+ + H_2O_2 + O_2^- \rightarrow O_2 + H_2O + OH^- \]

The product is the hydroxyl free radical (OH\(^-\)), reputed to be one of the most reactive molecules known in chemistry. This original reaction was later found to occur in two steps, now called "Fenton reaction". Firstly, the superoxide radical reduces the ferric
Secondly, this interacts with $\text{H}_2\text{O}_2$ to produce the $\text{OH}^-$ radical:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-$$

The total sequence of events can be summarized as:

$$\text{O}_2^- + \text{H}_2\text{O}_2 \xrightarrow{\text{Fe}^{2+/3+}} \text{O}_2 + \text{OH}^- + \text{OH}^-$$

There is still some controversy over the Fenton reaction particularly in vivo, and it is thought that $\text{OH}^-$ may not be the only oxidizing radical found in systems containing $\text{Fe}^{3+/2+}$, $\text{O}_2^-$ and $\text{H}_2\text{O}_2$. The high reactivity of these other species makes them very difficult to study and they have been aptly named the "crypto $\text{OH}^-$" radicals. When protonated in aqueous solutions, $\text{O}_2^-$ forms the reactive molecule, $\text{HO}_2^-$. It is thought that this species is also produced in vivo.

**Termination and propagation reactions:** The fate of the free radicals is an important consideration when evaluating their toxicity. Because of the intrinsic need for their electrons to pair, they are highly reactive and have very short half life ($\text{OH}^-$ has half life of $10^{-9}$ seconds). Of course, some radicals such as semiquinone radical present in tobacco smoke has a half life of several days. The termination reactions of the free radical reactions have been categorized into several types:

1. **Dimerization**
   $$\text{A}^\cdot + \text{B}^\cdot \rightarrow \text{A-B}$$

2. **Disproportional**
   $$\text{A}^\cdot + \text{B-C-D} \rightarrow \text{A-B} + \text{C = D}$$

3. **Electron transfer**
   $$\text{A}^\cdot + \text{B} \rightarrow \text{A}^+ + \text{B}^- \text{ or } \text{A}^- + \text{B}^-$$
Free radicals often react with other molecules in a manner that initiates the formation of many more free radical species. It is this self-propagating ability that makes them so toxic to living organisms. Thus, free radicals are generated by several mechanisms:

1. Addition reaction to double bonds
   \[ A' + B = C \rightarrow A-B + C' \]

2. Cleavage
   \[ A'-B=C \rightarrow A' + B=C \]

3. Electron transfer
   \[ A' + B^- \rightarrow B' + A^- \]

A single initiation event which leads to the production of one free radical can soon produce many more; this is called "cascade", and it can be prevented via the termination steps as described above. In cells, termination also occurs by the interaction of protective mechanisms and antioxidants.

**Singlet oxygen:** However, as stated earlier, a second and most important reaction of oxygen is the spin reversal of one of the parallel electrons in the outer orbital. This new arrangement of the molecule is called the singlet state, and hence the term singlet oxygen \((^1\text{O}_2)\). Two singlet states exist. The first type of singlet oxygen is most important *in vivo* while second type is energetically unstable and usually decays to first state before it has time to react with other molecules. It is important, of course, to note that \((^1\text{O}_2)\) is not a free radical, but because of its electronic arrangement it displays properties which are similar to the free radical species of molecular oxygen, and there are some important non-photochemical reactions with this type of oxygen.
(1) Dismutation of $O_2^-$

$$HO_2 + O_2^- + H^+ \rightarrow ^1O_2 + H_2O_2$$

(2) Fenton type reactions

$$O_2^- + H_2O_2 \xrightarrow{\text{Fenton}} ^1O_2 + OH^- + OH^+$$

(3) Interaction of hypochlorite

$$OCl^- + H_2O_2 \rightarrow ^1O_2 + Cl^- + H_2O_2$$

(4) Reaction with hydroxyl radicals

$$O_2^- + OH^- \rightarrow ^1O_2 + OH^-$$

(5) Reaction with diacyl peroxides

$$2O_2^- + R\cdot C\cdot O\cdot O\cdot C\cdot R \rightarrow 2^1O_2 + RCO_2^-$$

**Biochemistry of Radical Mediated Protein Oxidation**

Radical-mediated protein oxidation has been studied throughout the century. In the first decade, Dakin (1906,1908) published detailed chemical studies of the oxidation of leucine and other amino acids by Fenton systems (transition-metal ions plus hydrogen peroxide), and protein aggregation and (apparently) fragmentation were detected by others. Soon after the discovery of glutathione, Hopkins appreciated that this reductant could be both an anti- and a pro-oxidant, the latter depending on the presence of transition-metal ions (Hopkins, 1925), and so could inactivate proteins. Several authors assessed the proteolytic susceptibility of oxidized proteins, and demonstrated biphasic effects, whereby limited oxidation leads to enhanced susceptibility, while more extensive oxidation may be associated with increasing resistance (Bevilotti, 1945; Drake et al, 1957). Recent findings have shown that glycated proteins accumulated *in vivo* provide stable active sites for catalyzing the formation of free radicals (Yim et al, 2001).

**Sources of radicals in experimental and biological systems:** The primary free radical in most oxygenated biological systems is the superoxide radical ($O_2^-$), which is in equilibrium with its protonated form, the hydroperoxyl radical (HO'). The major sources of these radicals are modest leakages from the electron transport chains of
mitochondria, chloroplasts and endoplasmic reticulum. Although $O_2^-$ is relatively unreactive in comparison with many other radicals, biological systems can convert it into other more reactive species, such as peroxyl ($ROO^-$), alkoxy ($RO^-$) and hydroxyl ($HO^-$) radicals. The last of these can originate from the Fenton reaction, in which the metal ion redox cycles, with reduction effected by $O_2^-$ and oxidation by its dismutation product, hydrogen peroxide ($H_2O_2$). Iron and copper are biologically important transition-metal ions, with their reduced forms capable of rapidly cleaving organic (including lipid) hydroperoxides, forming radicals that can initiate chain reactions, ultimately giving stable products such as lipid hydroxides (Kim et al, 2002; Courtois et al, 2002; Schoneich and Williams, T.D., 2002). Experimentally, many biologically important radicals can be generated in defined qualities and quantities by the c-radiolysis of water. Linear fluxes of $ROO^-$ can also be generated by decomposition of thermolabile azo compounds such as 2,2'- azobis-(2-amidinopropane) hydrochloride. More complex experimental systems can involve the metal-ion-catalysed autoxidation of a variety of molecules, such as sugars. In these cases, it is extremely difficult to quantify the radical fluxes.

Damage by radiation-induced radicals: Earlier and most recent radiation studies on lysozyme (Aldrich et al, 1969; Edward et al, 2002), ribonuclease (Adams et al, 1972) and other enzymes were carried out mainly in the absence of $O_2$. They showed that $HO^-$ was the most effective inactivator, and characterized other more selective (but less efficiently inactivating) species such as $(SCN)_2^-$, $Br_2^-$, $Cl_2^-$ and $I_2^-$. For example, $(SCN)_2^-$ was found to react with an important tryptophan residue in pepsin and so inactivate the enzyme, although damage could be reversed by the same radical (Adams et al, 1979). Inactivation by the hydrated electron has also been reported (Terato and Yamamoto, 1994), but its significance, and that of the above-mentioned selective radicals, for biological systems may be limited. In studies on D-amino acid oxidase, it was found that removal of the coenzyme FAD enhanced radical damage and inactivation, illustrating that conformation (Anderson et al, 1977) and ligands can affect the extent of inactivation.
**Damage by metal-ion catalysed systems:** Since 1981, Stadtman and colleagues have examined the inactivation of proteins in cell-free systems involving the metal-ion catalysed autoxidation of ascorbate and hydrogen peroxide; in some cases the metal ions were derived from metalloproteins. These systems are now termed as 'metal-catalysed oxidation systems', instead of the previous confusing term 'mixed function oxidation systems'.

These studies developed from earlier work on the oxidative inactivation of glutamine phosphoribosylpyrophosphate amidotransferase in extracts from *Bacillus subtilis* (Turnbough and Switzer, 1975a; 1975b). This system is O₂-dependent and is perturbed by metal-ion chelators, but its chemistry has not been fully defined. A similar inactivation occurs in a mutant strain lacking important proteinases; oxidation of a (4Fe-4S) centre appears to be important. Although metal-catalysed oxidation systems are mechanistically diverse, they are like Fenton systems and involve H₂O₂; thus they can be blocked by catalase. They inactivate a wide range of enzymes (Levine et al, 1981; Nakamura and Stadtman, 1984; Stadtman, 1991) and are suggested to be important for both proteolytic turnover (Levine et al, 1981) and the accumulation of proteins during aging (Fucci et al, 1983). The inactivation of one of the most studied enzymes, glutamine synthetase, is influenced by its adenylation state (Levine et al, 1981), which also regulates the enzyme and some multi-enzyme cascades (Stadtman and Chock, 1978; Stadtman, 1990). Some metal-catalysed oxidation systems can cause selective damage in cell-free systems, such as to the histidine residues in glutamate synthetase (Rivett et al, 1985), but this is accompanied by other alterations; in this case, both protein fragmentation (Kim et al, 1985; Breccia et al, 2002; Dubinina et al, 2002) and changes in hydrophobicity. Limited oxidation increases hydrophilicity, while further oxidation increases hydrophobicity (Cervera and Levin, 1988). Early studies suggested that modification of one (of 16) histidines, both inactivated and increased proteolytic susceptibility (Levine, 1983); later studies showed that enhanced proteolytic susceptibility requires the modification of at least two histidines per subunit (Rivett and Levine, 1990). But the stage at which this takes place, other modifications also occur, e.g. at Arg-344 (Climent and Levine, 1991), yet only 0.7 mol of carbonyl/mol of protein is present (Rivett and Levine, 1990), so most altered histidine residues do not contain carbonyls. Incorporation of tritium (from...
tritiated borohydride) into the enzyme at various stages of oxidation revealed eight (unidentified) radioactive peaks using amino acid HPLC after protein hydrolysis. Some of these may represent carbonyls. As the chromatographic pattern was relatively constant at different times, it was argued that the range of products is also constant. However, during the period when the first histidine was oxidized (60 min of oxidation), less than six of these species were formed. Bu at 120 min little further histidine oxidation had occurred, yet a further two or three amino acid derivatives were formed. Unfortunately, the earliest time reported was 20 min, making it difficult to conclude that the critical event for inactivation is the histidine oxidation, since other modifications had also occurred. Consumption of other individual amino acids was low when compared with that of histidine, and could not be detected; since the aggregate loss of all amino acids was not quantified, and may have been significant. In vivo studies of glutamine synthetase inactivation have been carried out in other species, such as the fungus Neurospora crassa, where the inactivated enzyme has been detected in vivo (Aguirre and Hansberg, 1986). Furthermore, when living Klebsiella pneumoniae (Chevalier et al, 1990) is switched from anaerobic to aerobic conditions (or exposed to H₂O₂), several enzyme activities, including that of glycerol dehydrogenase, are lost, apparently by oxidative inactivation. Purification of this enzyme when 90% inactivated (from cells exposed to H₂O₂) and comparison with the native form has shown no detectable differences in either subunit molecular size or amino acid composition (including -SH groups). The lack of such gross changes suggests that limited modifications are sufficient for inactivation, although the inactivated enzyme was more hydrophobic, and its intact oligomer migrated in gel filtration as if slightly larger than the native form. This emphasizes a major problem in studying oxidative inactivation in vivo: even analysis of the inactivated enzyme from these cells gave no evidence that inactivation was due to protein oxidation, although it was clearly initiated by an oxidative affront.

Inactivation of non-enzymic proteins by metal-catalysed oxidation has also been studied extensively. Thus a proteinase inhibitor is inactivated on oxidation of a susceptible methionine residue, and H₂O₂ inactivates a neutrophil cytosolic serine proteinase inhibitor (serpin), possibly via a similar process (Thomas et al, 1991). In some cases, these reactions of H₂O₂ and methionine may be nucleophilic (molecular)
rather than radical-mediated. The proteinase subtilisin is dependent on a methionine residue both for its activity and for its susceptibility to H₂O₂ in vitro; replacement of this residue by site-directed mutagenesis decreases inactivation of the enzyme by H₂O₂ (Estell et al., 1985). In contrast, Fenton systems produces a range of amino acid derivatives that inactivate α₁-proteinase inhibitor (Kwon et al., 1990); they also inactivate proteinase inhibitors and neutrophil elastase, which lack the susceptible methionine, with comparable efficiency (Dean et al., 1989). Thus methionine-containing inhibitors are not automatically more vulnerable, and susceptibility depends on the radical or oxidizing conditions, which systems are important in vivo are not known. Mattana et al. (2002) have shown that the metal catalyzed oxidation of extracellular matrix proteins promotes human mesengial cell apoptosis and is associated with the enhanced expression of bax and caspase activation. The protein fragmentation, enzyme inactivation and aggregation studies have also been performed by various metal catalyzed oxidation systems (Shiraishi and Nishikimi, 2002; Stadler et al., 2001; Sandgerg et al., 2002).

**FLAVONOIDS/ POLYPHENOLIC COMPOUNDS**

The flavonoids are a large and complex group of polyphenolic plant metabolites found in human foods (Hollman and Arts, 2000; Lu and Foo, 2002; Cotelle, 2001). The term flavonoids was first used for the family of yellow-coloured compounds with a flavone moiety. It was later extended to various plant polyphenols to include less intensively coloured flavanones, colorless flavon-3-ols and more colored red and blue anthocyanidins. So far, over 8,000 varieties of flavonoids have been identified. Individual differences within each group result from the variation in number and arrangement of hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups. The flavonoids are formed in plants, but not in animals, from the amino acids phenylalanine and tyrosine by combination with acetate units. Flavonoids are benzo-γ-pyrene derivatives and consists of benzene ring (commonly known as A-ring) attached to a six-membered heterocycle (named C-ring) which at C2 carries a phenyl group (named B-ring) as a substituent. The C-ring is either a γ-pyrene (flavones and flavonols) or its 2,3-dihydroderivative (flavanones and
flavanols). The most commonly occurring flavones and flavonols are those with dihydroxylation in position 5 and 7 (A-ring) and in position 3' and 4' (B-ring).

![Chemical Structure of Flavonoid]

Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification.

Examples of the 6 major subgroups are:

1. Chalcones

![Chalcone Structure]

2. Flavone (generally in herbaceous families, e.g. Labiatae, Umbelliferae, Compositae).
Apigenin (Apium graveolens, Petroselinum crispum).
Luteolin (Equisetum arvense)
3. Flavonol (generally in woody angiosperms)
Quercitol (Ruta graveolens, Fagopyrum esculentum, Sambucus nigra)
Kaempferol (Sambucus nigra, Cassia senna, Equisetum arvense, Lamium album,
Polygonum bistorta).
Myricetin.

4. Flavanone

5. Anthocyanins
6. Isoflavonoids

**Intestinal Absorption of Polyphenols:** The major sources of flavonoids are shown in Table 2. Amongst the most common and thoroughly studied of these flavonoids are the flavonols, quercetin, myricetin and kaempferol. It has been estimated that even in industrialised societies, intakes of flavonoids could be as high as 1 gram per day (Kuhnau, 1976), but this is probably an overestimation based on inadequate analytical data (Hertog and Hollman, 1996). The more conservative estimates of 23 mg per day in the Netherlands, 64 mg per day in Japan and 6 mg per day in Finland are probably much more realistic. (Hollman and Arts, 2000; Hertog et al, 1995). In Western Europe, flavonols and flavones are consumed predominantly in the form of water soluble glycosides in apples and onions, or as free solutes in black tea and wine (Hertog et al, 1993). Until very recently, it was believed that flavonol glycosides must be hydrolyzed prior to absorption and the low solubility of the liberated aglycones was regarded as a major rate-limiting factor for uptake into the circulation (Manach et al, 1996). Hollman et al (1996) have obtained direct evidence to show that they are readily absorbed in the human small bowel. Subjects were fed a meal of cooked onions containing quercetin aglycone. The level of quercetin in plasma, determined after deconjugation, rise rapidly within 1 hour of ingestion, peaked at approximately
Table 2: Flavonoids and their Sources

<table>
<thead>
<tr>
<th>FOOD</th>
<th>FLAVONOID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apples</td>
<td>catechins, quercetin</td>
</tr>
<tr>
<td>Broccoli</td>
<td>kaempferol</td>
</tr>
<tr>
<td>Celery</td>
<td>apigenin</td>
</tr>
<tr>
<td>Eggplant skin</td>
<td>delphinidin</td>
</tr>
<tr>
<td>Endive</td>
<td>kaempferol</td>
</tr>
<tr>
<td>Grapes</td>
<td>cyanidin</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>kaempferol, naringin</td>
</tr>
<tr>
<td>Leek</td>
<td>kaempferol</td>
</tr>
<tr>
<td>Onions</td>
<td>quercetin</td>
</tr>
<tr>
<td>Orange juice</td>
<td>hesperidin</td>
</tr>
<tr>
<td>Parsley</td>
<td>apigenin</td>
</tr>
<tr>
<td>Raspberries</td>
<td>cyanidin</td>
</tr>
<tr>
<td>Red wine</td>
<td>oenin</td>
</tr>
<tr>
<td>Soybeans</td>
<td>isoflavones</td>
</tr>
<tr>
<td>Strawberries</td>
<td>cyanidin</td>
</tr>
<tr>
<td>Tangerine peel</td>
<td>tangeretin</td>
</tr>
<tr>
<td>Tea</td>
<td>catechins</td>
</tr>
</tbody>
</table>
200 ng/ml after 2.9 hours, and fell slowly to around 10 ng/ml after 48 hours. Several other studies have also shown that average concentration of quercetin in plasma after the meal is quite high and elimination of quercetin from the plasma is relatively slow, so that accumulation can occur if repeated oral doses of quercetin are taken over a prolonged period (Manach et al, 1996; Hollman and Arts, 2000; Hollman and Katan, 1997; Hollman et al, 1997).

FLAVONOIDS AS ANTIOXIDANTS

Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory and vasodilating actions. These pharmacological effects are generally linked to the antioxidant properties of these molecules. Flavonoids can express these properties by (i) suppressing ROS formation by inhibiting some enzymes or chelating trace elements involved in free radical production. (ii) Scavenging radical species and more specially the ROS. (iii) Up-regulating or protecting antioxidant defence. Flavonoids have been identified as fulfilling most of the se criteria described above. Thus their protective effects are multiple. In a study performed by Roig et al (2002), it has been shown that flavonoids such as catechin, epicatechin, quercetin and procyanidins protect the Fao cells against hydrogen peroxide induced oxidative stress. Cardoon (Cynara cardunculus) is a species containing considerable amounts of polyphenolic compounds, namely flavonoids and phenolic acids, which shows its antioxidant activity against superoxide radical, hydroxyl radical, and hypochlorous acid (Valentao et al, 2002). There are numerous evidences to show the beneficial, antioxidant, anti-inflammatory and anticancer effects of various naturally occurring flavonoids (Perez et al, 2002; Ficarra et al, 2002; Shahat et al, 2002; Salucci et al, 2002; Rezk et al, 2002; Aviram and Fuhrman, 2002). The data obtained by these different findings confirm that the radical scavenging activity depends on the structure and the substituents of the heterocyclic and B-ring, as suggested by Bors et al (1990). The major determinants for radical scavenging capability are (i) the ortho-dihydroxy structure on the B-ring which has the best electron-donating properties, confers higher stability to the radical form and participates in electron delocalization. (ii) The 2,3-
double bond in conjugation with a 4-oxofunction in the C-ring is responsible for electron delocalization from the B-ring. The antioxidant potency is related to the structure in terms of electron delocalization of the aromatic nucleus. (iii) The 3- and 5- hydroxyl group with a 4-oxofunction in the A and C-ring for maximum radical scavenging potential. The maximum effectiveness for radical scavenging apparently requires a 3-OH group attached to the 2,3 double bond and adjacent to the carbonyl in the C-ring. The 3-hydroxyl Group interacts with the B-ring through a hydrogen bond between the 3-OH and the 2' or 6' proton which, conformationally maintain the B-ring in the same plane as the A- and C-rings favoring the conjugation between the B- and the C-ring. This might well explains the excellent antioxidant activity of flavonols. In flavones the B-ring is slightly twisted in relation to the plane of the A- and C-rings (Cody and Luft, 1994). The glycosylation of this position (as in rutin) reduces greatly the radical scavenging capacity.

Pro-oxidant activity of flavonoids: The scavenging properties of antioxidant compounds are often associated with their ability to form stable radicals after being effective. In fact, those flavonoids containing hydroxyl groups, especially those having ortho, di- or trihydroxy-functions on the A-ring (Gao et al, 1999) or B-ring (Cotelle et al, 1996), which can scavenge radicals effectively usually give rise to semiquinonic radicals in alkaline solution stable enough to be detected by EPR spectroscopy. Nevertheless, this molecular oxidative process (or autooxidation which can also take place in the presence of transition metal catalysis) can be accompanied by the production of pro-oxidant molecules such as reactive oxygen species (Hodnick et al, 1988) or secondary flavonoids radicals (Roginsky et al, 1996), which are more reactive than the primary aroxyl radicals. Consequently these species are able to propagate chain reactions. The proposed mechanism generally accepted for antioxidant activity or for autooxidation involves the formation of an intermediate aroxyl radical of flavonoids. This aroxyl radical can interact with oxygen or glutathione, generating an oxidized flavonoid and superoxide anion (Metodiewa et al, 1999). Galati et al (2002) have observed that dietary polyphenolics with phenol rings were metabolized by peroxidase to form prooxidant phenoxy radicals which, in some cases were sufficiently reactive to cooxidize GSH or NADH accompanied by...
extensive oxygen uptake and reactive oxygen species formation. The order of catalytic effectiveness found for oxygen activation when polyphenolics were metabolized by peroxidase in the presence of GSH was phloretin>phloridzin>4,2'-dihydroxychalcone>p-coumaric acid >narigenin>apigenin>curcumin>resveratrol >isoliquiritigenin>capsaicin>kaempferol. Ueda et al (2002) have reported that aiclin induces apoptosis via mitochondrial pathway as prooxidant. Induction of apoptosis by baicalin was accompanied with the marginal generation of intracellular reactive oxygen species (ROS), the increase of the cytosolic fractions of cytochrome c, and the disruption of mitochondrial transmembrane potential prior to the activation of caspase-3. Ferguson (2001) has demonstrated that not all polyphenols and not all actions of individual polyphenols are necessarily beneficial. Some have mutagenic and/or pro-oxidant effects, as well as interfering with essential biochemical pathways including topoisomerase enzyme activities, prostanoid biosynthesis and signal transduction. A small number of adequately controlled human intervention studies suggested that some, but not all polyphenol extracts or high polyphenol diets may lead to transitory changes in the antioxidative capacity of plasma in humans.

**ENZYME IMMOBILIZATION**

Enzymes are normally tightly packed in cellular organelles or in enzyme cascade, such as fatty-acid synthetase complex, thus enabling catalytic processes to take place precisely when and where they are needed. Artificial applications of such compartmentation or packing go back to 1950s, when immobilized enzymes (enzymes with restricted mobility) were first prepared intentionally by inclusion in polymeric matrices or binding onto carrier materials. Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes or alternative technologies, and the steadily increasing number of applications for immobilized enzymes (Kamori et al, 2002; Gorokhova et al, 2002).

There are several reasons to use immobilized enzymes. In addition to the conventional handling of enzyme preparations, the two main targeted benefits are (1) easy separation of enzyme from the product and (2) reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and permits reliable
and efficient reaction technology. Enzyme reuse provides a number of cost advantages, which are often an essential prerequisite for establishing an economically viable enzyme-catalyzed process. Biotechnological applications of immobilized biocatalysts include several fields of general interests and in particular, clinical and analytical chemistry, medicine, food and pharmaceutical technology, organic synthesis and industrial production of chemical compounds (Rai and Taneja 1998; Fields 2001; Zhu and Snyder 2001; MacBeath 2001; Nazarov 2001). The immobilized enzymes can also be used in understanding the action and effect of various toxic molecules on enzymes, such as respiratory enzymes of mitochondria, the enzymes participating in photosynthesis and in protein synthesis, and the enzymes responsible for active transport, act in vivo while embedded in membranes or attached to subcellular particles. With the aid of simple model systems in which enzymes are bound to various synthetic carriers or embedded in simple membranes, it may be possible to study some of the parameters individually and thus obtain a better insight into the factors governing the activity of enzymes in biological membranes. The success of enzyme immobilization technology is dependent on the choice of carrier and method of immobilization. Several organic, inorganic and synthetic polymeric materials have been used as support for the immobilization of enzymes (Svobodova et al, 2002; Yakovleva et al, 2002; Yu and Ju, 2002; Xie et al, 2002).

METHODS OF ENZYME IMMOBILIZATION

Methods used for the immobilization of enzymes fall into four main categories:

- Physical adsorption onto an inert carriers
- Entrapment in the lattices of a polymerized gels
- Cross-linking of the protein with a bifunctional reagents
- Covalent binding to a reactive insoluble supports

Physical adsorption of an enzyme onto a solid supports is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix,
brought about by mixing a concentrated solution of enzyme with the solid supports. Confining enzymes within the lattices of polymerized gels is another method for immobilization. This allows the free diffusion of low molecular weight substrates and reaction products. The usual method is to polymerize the hydrophilic matrix in an aqueous solution of the enzyme and break up the polymeric mass to the desired particle size. Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support, resulting in relatively low enzymic activity. The most intensely studied of the insolubilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be insolubilized, the choice is limited by the fact that the binding reaction must be performed under conditions that do not cause loss of enzymic activity, and the active site of the enzyme must be unaffected by the reagents used.

A great number of techniques for the immobilization of enzymes exist (Ruan et al., 2002; Podgornik and Tennikova, 2002; Xie et al., 2002). By selecting the proper carrier material and coupling procedure, it is usually possible to obtain immobilized derivatives with good activity and stability. However, most of them contain irreversibly bonded enzyme, which means that the carrier cannot be regenerated and reused. This problem can be circumvented by immobilization based on ion exchange (Chibata et al., 1972), hydrophobic interaction (Caldwell et al., 1975) or thiol disulfide interchange (Carlsson et al., 1975). Chaga (1994) has described a method for enzyme immobilization on immobilized metal-ion carriers. The method is based on the covalent modification with histidine and immobilization on bivalent metal ion-iminodiacetic acid-agarose. Immobilized-metal-chelate regenerable carriers have also been used for the immobilization of pencillin G amidohydrolase (Anspach and Altmann-Haase, 1994).

Calcium alginate gels are now one of the most widely used supports for the immobilization of enzymes and whole microbial cells (Klein et al., 1983; Smidsrod and Skjak-Braek, 1990). Entrapment of enzymes and cells in alginate is one of the simplest methods of immobilization (Kierstan and Bucke, 1977). Alginates are
available commercially as water soluble sodium alginate and have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying, film forming and gelling agents (Bickerstaff, 1997). Entrapment in insoluble calcium alginate gels is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilization. Saccharomyces cerevisiae cells, Kluyveromyces marxianus cells, inulase, glucose oxidase, chloroplasts, and mitochondria were immobilized in calcium alginate gels by Kiersten and Bucke (2000). Ethanol production from glucose solutions by an immobilized preparation of S. cerevisiae was demonstrated over a total of twenty-three days, and the half-life of such a preparation was shown to be about ten days. Immobilized K. marxianus, inulase, and glucose oxidase preparations were used to demonstrate the porosity and retraining properties of calcium alginate gels. Calcium alginate-immobilized chloroplasts were shown to perform the Hill reaction. Das et al (1998) have entrapped urease from pigeonpea in polyacrylamide gel with 50% immobilization at 10% total monomer (containing 5% cross-linker) with high mechanical stability of the gel. The immobilized enzyme had a t1/2 of approx. 200 days when stored in 0.1 M Tris/acetate buffer, pH 6.5, at 4 degrees C. The gel strips were used 4-5 times for urea assay over a period of 6 h with less than 2% loss of activity. Approximately 50% immobilization of urease in calcium alginate was observed at 3% alginate with 0.12 mg protein/ml alginate. The resultant enzyme beads showed a t1/2 of approx. 75 days when stored in 0.1 M Tris/acetate buffer, pH 6.5, at 4 degrees C. The beads were used 4-5 times for urea assay over a period of 6 h with about 40% loss of activity.

**IMMOBILIZATION OF PAPAIN**

Papain, a thiol protease, is well characterized kinetically and structurally (Liu and Hanzlik, 1993; Mellor et al. 1993; Vernet et al. 1995) being a suitable model to compare the efficiency of various immobilization procedures. The need for the immobilization of papain has been due to its great industrial and medicinal potential. For example, papain is used as a chill proofing agent during beer finishing operations in the brewing process (Wiseman, 1993). This enzyme also facilitates the tenderization of meat in the meat industry (Swanson et al. 1992). The potential uses of
papain include its frequent use as a biocatalyst for amino acid ester and peptide synthesis (Lozano et al. 1993), as well as the treatment of acute destructive lactation mastitis (Storozhuk et al. 1985). The biopharmaceutical potential of immobilized papain can be well illustrated by the interaction of papain digested HLA class-I molecules with alloreactive cluster of differentiation and cytotoxic-T-lymphocytes in transplantation immunology (Hausmann et al. 1993) and in the treatment of red blood cells with immobilized papain prior to its use in antibody-dependent cell mediated cytotoxicity (ADCC) assays with lymphocytes (Kumpel and Bakacs, 1992). A carrier containing primary amine groups was synthesized by the reaction between nitrilon fibre and diethylenetriamine, and papain was immobilized on this kind of carrier by using glutaraldehyde as a coupling agent. The factors involved with the activity recovery of the immobilized papain and the enzymic properties of the resulting immobilized papain were studied in comparison with free papain, for which casein was chosen as a substrate. The results show that the activity recovery of immobilized papain can reach 47.3%, and that the immobilized papain exhibits better environmental adaptability and reusability than free papain (Li et al, 2001).

Khan and Iqbal (2000) have insolubilized papain as enzyme-antibody adducts with the gamma-globulin fraction derived from the antiserum for its usefulness in pharmaceutical and other industries. In a study performed by Losel et al (1999), covalent attachment to papain was achieved via a thiol-specific thiosulfonate residue and, for the second anchor point, via a nonspecific photoreactive azido function. The thiosulfonate formed a reversible disulfide linkage, which could be cleaved again reductively by dithiothreitol. The spin label, a pyrroline-1-oxyl radical, was highly immobilized after attachment to papain by both functional groups and showed little if any relative motion with respect to the protein. The enzyme papain was utilized for experimental investigation both in the homogeneous state and on a modified polysulfone (MPS) membrane by Ganapathi-Desai et al (1998). Huckel et al (1996) have selected four different proteases (trypsin, chymotrypsin, papain and pepsin) and were covalently attached to the surface of a new type of porous zirconia, as well as a conventional porous silica, activated with 3-isothiocyanatopropyltriethoxy silane (NCS-silane). The immobilization efficiency onto the porous zirconia material was evaluated in terms of the amount of enzyme attached to the particles and from the
biological activity remaining after the immobilization step. Cysteine-proteinases (CP) of the papain family has also been adsorbed by egg white cystatin C coupled to Sepharose 4B, thus allowing their selective isolation from either tissue or cultured cell extracts as well as biological fluids and culture media. The method may be useful in the field of biochemistry, cell biology, and, possibly, clinical chemistry to perform rapid analysis of papain-like enzymes and to monitor changes in both cellular and extracellular CP profiles along with different physio pathological conditions (Tombaccini et al, 2001). Afaq and Iqbal (2001) have selected a method for immobilization of papain based on the interaction between its histidine, cysteine and tryptophan residues with the immobilized metal ion (IMI) carrier for maximum binding on a small volume of the carrier. The immobilized papain retained high activity has improved thermal stability and the carrier could be recovered from the spent bound enzyme, to be reused.