NON-ENZYMATIC OXIDATIVE DEAMINATION OF LYSINES TO ALLYSINES BY ORTHOQUINONES

by

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A DISSERTATION

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ABBREVIATIONS

AHis	- Aldolhistidine
a.m.u.	- atomic mass units
BAPN	- Beta-amino propionitrile
BSAO	- Bovine serum amine oxidase
BSTFA	- N,O-bis-[Trimethylsilyl] trifluoroacetamide
deDHLNL	- Dehydrodihydroxylysinonorleucine
deHisHMD	- Dehydrohistidinohydroxymerodesmosine
deHLNL	- Dehydrohydroxylysinonorleucine
deHMD	- Dehydrohydroxymerodesmosine
deLNL	- Dehydrolysinonorleucine
DHI	- 5,6-Dihydroxyindole
DHLNL	- Dihydroxylysinonorleucine
DHNL	- Dihydroxynorleucine
DOPA	- 3,4-Dihydroxyphenylalanine
FAB	- Fast atomic bombardment
GC	- Gas Chromatography
HAHis	- Hydroxyaldol-histidine
HCl	- Hydrochloric acid
HLNL	- Hydroxylysinonorleucine
HLONL	- Hydroxylysino-5-oxonorleucine
HNL	- Hydroxynorleucine
HPLC	- High pressure liquid chromatography
HPTLC	- High pressure thin layer chromatography
Hyl	- Hydroxynorleucine
LNL	- Lysinonorleucine
LONL	- Lysino-5-oxonorleucine
Lys	- Lysine
MS	- Mass spectroscopy
$NaHCO_3$	- Sodium bicarbonate
NaOH	- Sodium hydroxide
PBS	- Phosphate buffered saline
PQQ	- Pyrroloquinoline quinone
pTSA	- para-Toluenesulphonic acid
tBC	- tert-Butyl catechol
TMCS	-Trimethylchlorosilane
TMS	-Trimethylsilyl
TOPA	-Trihydroxyphenylalanine; 6-HydroxyDOPA
TTQ	-Tryptophan tryptophylquinone

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I would like to thank my advisor Dr. Peter Bentley for giving me the opportunity to complete my thesis in Biochemistry, a field that has fascinated me through the years. Dr. Bentley is a great mentor and a wonderful person. I also thank him for teaching me the art of presentation and writing, two important skills for todays scientist. I greatly appreciate his foresight and willingness to fund my research to the very last date, even after the end of the project.

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ABSTRACT

Lysyl oxidase, the enzyme catalyzing oxidative deamination in collagen and elastin, has a covalently attached ortho-quinone cofactor. This thesis reports studies which show that in-vitro oxidative deamination may be carried out even in the absence of the enzyme, with the ortho-quinone of 3,4-dihydroxyphenylalanine (DOPA) which is capable of causing oxidative deamination of both peptidyl and free lysines. We demonstrate this by using collagen, pepsin-modified collagen and N-α-acetyl-L-lysine, respectively, as substrates. The lysyl aldehydes and condensation products thus generated were separated after reduction with tritiated sodium borohydride, extracted into ethyl acetate and characterized by mass spectroscopy. A cyclic product arising from N-acetyl hydroxynorleucine was produced. We propose that it originated when N-α-acetyllysine was oxidatively deaminated to the aldehyde which then condensed with another molecule of N-α-acetyllysine, giving an unstable Schiff base. The Schiff base spontaneously undergoes an elimination reaction to yield the cyclic product.

It is further shown that DOPA is capable of oxidatively deaminating the ϵ-amino group of 6-aminocaproic acid, which is an analog of lysine. The reduced aldehyde of 6-aminocaproic acid is identified by mass spectroscopy.

The effect of increasing concentration of metal ions, copper and iron, on oxidative deamination of N-α-acetyllysine by DOPA was studied. Quantitation of

the oxidative product, the cyclic compound, formed by mass spectroscopy was used as a structure specific and accurate measure of the oxidative deamination reaction. DOPA plus copper(II) appears to be better than DOPA plus iron(III), at catalyzing the oxidative deamination of N-α-acetyllysine. The effect of various ortho-quinones: DOPA, trihydroxyphenylalanine (TOPA), tert-butylcatechol (tBC) and pyrroloquinolinequinone (PQQ) was studied. It was found that the oxidative deamination of lysines is dependant on the nature of the o-quinone and the order of efficiency is: DOPA < TOPA < tBC < PQQ. The effect of time on oxidative deamination of lysines by DOPA and PQQ was studied, and it was found that the maximum of the plot between duration of incubation and the extent of oxidative deamination is at least 8 times greater for PQQ than DOPA.

Finally, incubation of N-α-acetyllysine with PQQ followed by reduction with sodium borohydride and extraction with ethyl acetate yielded a reduced aldehyde which was identified by GC-MS.

III. INTRODUCTION

Quinones and semiquinones are integral to biological systems and are present in many of the oxidases, dehydrogenases, oxygenases and decarboxylases. Their functions in these proteins are varied and many of them are topics of current research. Enzymes with covalently attached quinones are known as 'quinoproteins'. The most important property of quinones is an ability to function as both an oxidant and a reductant. The redox function of quinoproteins is often influenced by the presence of metal ions or other oxidants/reductants in the vicinity of the active site.

1.1 QUINOPROTEINS:

The interest in quinoproteins started in the late 1970s, when Duine and coworkers investigated the nature of the cofactor of methanol dehydrogenase. Using electron spin resonance, nuclear magnetic resonance, and mass spectroscopy they showed (1) that the cofactor was not a pteridine (as was initially thought) but was a quinone that contained two nitrogen atoms. At approximately the same time the structure of this cofactor was deduced by Salisbury (2) from X-ray diffraction studies to be pyrroloquinoline quinone, or

PQQ. (The I.U.P.A.C. name for PQQ is 4,5-dihydro-4,5-dioxo-1-H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid.) Subsequently, several similar PQQ-dependent bacterial enzymes were identified that catalyzed transformations of the following compounds: long-chain alcohols and polyethylene glycol (3,4), secondary and polyvinyl alcohols (5), polyhydroxy alcohols (6), hydroaromatics (7), lactate (8) and nitriles (9). Thus by the early 1980s it became clear that PQQ was a widely distributed and important cofactor in prokaryotic systems. Claims were later made that PQQ was covalently bound to certain mammalian enzymes designated as 'quinoproteins' (10).

The existence of covalently protein-bound PQQ has not been confirmed. There is, however, convincing evidence for the covalent attachment of other types of quinones in certain enzymes. These quinones are not related to PQQ. They arise either by the posttranslational modification of a tyrosine residue to a trihydroxyphenylalanine (TOPA) residue, as shown with bovine plasma monoamine oxidase (11), or by post-translational cross-linking of two sequence-separated tryptophan residues to form a quinonoid compound called tryptophan tryptophylquinone (TTQ) as shown in bacterial methylamine dehydrogenase (12). More recently, lysyl oxidase was shown to have a unique TOPA-like quinone at its active site (13), which also arises from a tyrosine residue. The term quinoprotein, initially applied to designate a protein with covalently bound PQQ, has been retained to now identify a protein that contains any type of covalently bound quinonoid compound.

Quinoproteins have been classified into four groups (10): dehydrogenases, oxidases, oxygenases and hydratases/decarboxylases. Oxidases or amine-oxidases as they are known, are involved in oxidative deamination. As mentioned earlier, a number of these amine oxidases have been proposed to contain covalently bound quinone derivatives. In this group are the copper containing amine oxidases (14): plasma amine oxidase, kidney and placental diamine oxidase , plant diamine oxidase, fungal amine oxidase and methylamine oxidase in prokaryotes, bovine serum amine oxidase and lysyl oxidase. Lysyl oxidase (EC 1.4.3.13) is a kind of copper amine oxidase that initiates the protein crosslinking steps in collagen and elastin.

1.2 SOME GENERAL CHARACTERISTICS OF COPPER AMINE OXIDASE:

According to Knowles (15), the biological function of the copper amine oxidase depend upon its source:

In microbes:(eg. E.Coli)

- Utilization of amines from N and C sources

In mammals: (eg. Pig plasma)

- Detoxification of biogenic amines (16)
- crosslinking of collagen and elastin in connective tissue biogenisis (17)
- regulation of intracellular spermine and spermidine (18)

- Cell signalling events

In Plants: (eg. Pea seedling and Arabidopsis)

- Normal development

- Di and polyamine oxidation to generate H_2O_2 for peroxidases

- Cell wall crosslinking and lignification

The copper amine oxidases exist as dimers, with one copper and one quinone per subunit. The quinone is formed by oxidation of a tyrosyl residue. Spectroscopic studies have revealed Cu^{2+} coordinated by three equatorial His ligands, plus two waters, one in plane and one axial (Figure 1, reproduced from P.F.Knowles [15]). One of the water ligands is replaced by the TOPA during electron abstraction. Mechanistic studies have suggested hydrogen abstraction step during the reductive half reaction by a group with a pKa < 5. Therefore, this group is a carboxylate. Sequence comparison between the various copper amine oxidases has shown the presence of two Glu and five Asp residues: one of these residues probably contributes the important base.

The role of copper in amine oxidase was recently demonstrated. Dooley et al (19) presented evidence for the generation of Cu(I)-semiquinone state by substrate reduction of several amine oxidases under anaerobic conditions, and suggested that the Cu(I)-semiquinone may be the catalytic intermediate that reacts directly with oxygen. (Figure 2)

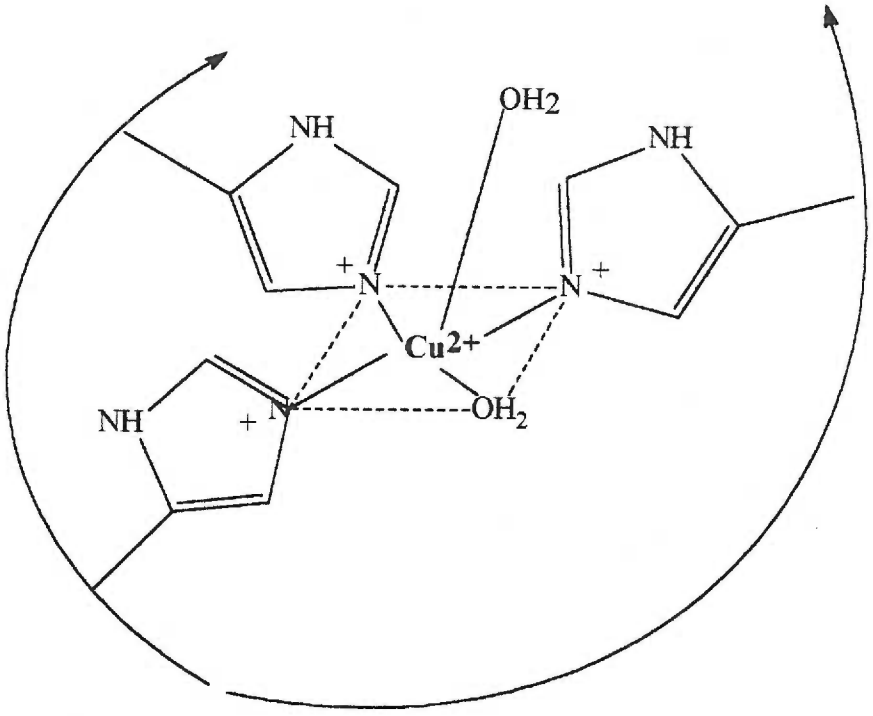


Figure 1 Spectroscopic studies have revealed that the copper in the amine oxidases is coordinated by three equatorial His ligands, and two waters, one in plane and one axial (courtesy of P.F.Knowles, 15)

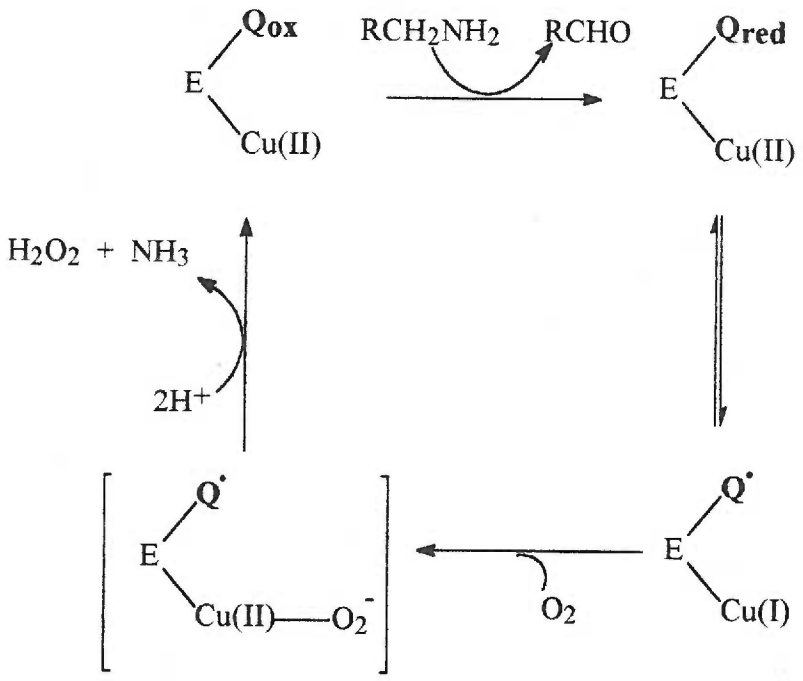


Figure 2 : Possible catalytic cycle of copper-containing amine oxidases (E). The species in the brackets is hypothetical. Q_{ox} = topaquinone, Q_{red} = topa aminoquinol, $\dot{Q}$ = topa semiquinone (courtesy of D.M.Dooley, 19)

1.3 LYSYL OXIDASE - the amine oxidase responsible for in-vivo oxidative deamination of lysine in collagen and elastin

Lysyl oxidase oxidizes peptidyl lysines to peptidyl α-aminoadipic-δ-semialdehyde (allysine) in collagen and elastin. Oxidative deamination of peptidyl lysines in these proteins is the first step for the formation of natural crosslinks in collagen. Lysyl oxidase can thus regulate the development and repair of the matrix in connective tissues.

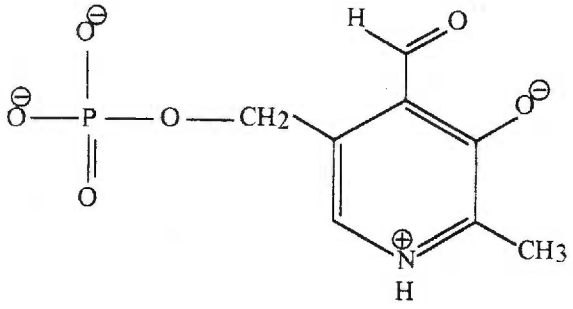
With respect to regulation, the activity of lysyl oxidase is influenced by hormones, factors that influence substrate conformation, and by environmental and nutritional factors. The enzyme has the highest affinity for collagens precipitated in the form of native fibrils. Lysyl oxidase has been isolated from human, pig, avian and bovine sources (22-25). Environmental factors and agents that elicit fibrotic responses, e.g. ozone or cadmium, are associated with increased lysyl oxidase activity (20). Lysyl oxidase is inhibited by vicinal diamines, heparin, semicarbazides, and hydrazines, presumably by interacting with the quinone at the active site of (21). The best characterized of these compounds are β-aminopropionitrile, semicarbazide and isoniazid (20-21).

The resulting inhibition of collagen crosslinking in vivo (known as Lathyrism) can be significantly reversed by pyridoxal treatment while pyridoxine-deficient diets result in crosslinkage anomalies in chicks and in cell culture systems (27). Such results, as well as spectral analyses of chick aorta lysyl

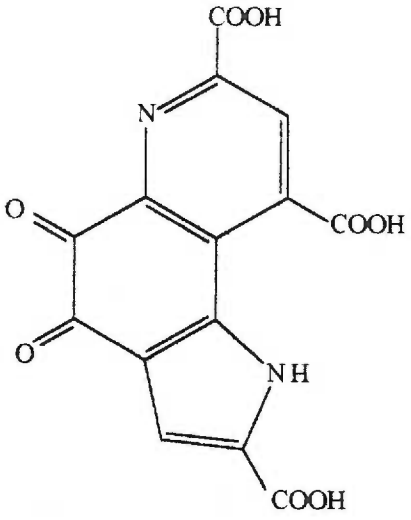
oxidase (28), led to the hypothesis that pyridoxal phosphate (PLP) (Figure 3 A) was the carbonyl cofactor of lysyl oxidase. Following research with other amine oxidases which appeared to contain PQQ (Figure 3 B) as the cofactor, considerable interest has developed in lysyl oxidase research. It was found that PQQ-deficient rats develop growth anomalies and lathyritic symptoms, which were associated with reduced levels of lysyl oxidase protein and activity (29). Feeding of PQQ to these deficient animals tended to reverse these effects (29) supporting a vitamin role and a cofactor function for this o-quinone in lysyl oxidase.

About five years ago, a new quinone cofactor was isolated in another amine oxidase, bovine serum amine oxidase. Janes et al. (11) showed by mass spectroscopic and NMR analyses of an active site peptide of bovine plasma amine oxidase that a quinone form of a TOPA residue (Figure 3 B), which is present in the X-position in the sequence: -Leu-Asn-X-Asp-Tyr-, was the site of attack by carbonyl reagents and therefore was the carbonyl cofactor in this enzyme. Investigators had earlier believed this cofactor to be PQQ (30). This was not surprising because the TOPA quinones with free α-amino and α-carboxylate functions released in digests of TOPA-containing proteins can spontaneously convert to a compound structurally very similar to PQQ (11).

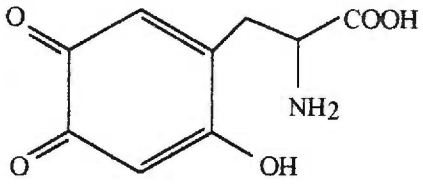
Lysyl oxidase is not nearly as abundant as plasma amine oxidase and it is more difficult to obtain sufficient quantities of active site peptides. Nevertheless, some preliminary studies showed strong similarities between the resonance Raman spectra of the phenylhydrazones of bovine plasma amine oxidase and an



PYRIDOXAL PHOSPHATE



PYRROLOQUINOLINE QUINONE



TOPAQUINONE

FIGURE 3 : Structures of proposed cofactors in Lysyl oxidase

active site peptide isolated from bovine aortic lysyl oxidase (11). This led to the proposal that lysyl oxidase might also contain a TOPA cofactor.

It was shown recently (13), that the resonance Raman spectra of lysyl oxidase peptide fragment compares well with the spectra of reduced aminoquinone form of the model compound shown in Figure 4 and does not have enough similarities to the spectra of Bovine serum amine oxidase (BSAO). Further, both lysyl oxidase and the model compound C had the UV-Vis λmax at 504 (13).

The discovery of the cofactor in lysyl oxidase is an example of yet another unique quinone cofactor. A decade ago, it was believed that most of these quinones were PQQ. But over the last 5 years three of the well known amine oxidases have been shown to have different quinone cofactor: TOPAquinone (TPQ) in BSAO, Tryptophan tryptophylquinone (TTQ) in methylamine dehydrogenase and now this TOPA-like quinone in Lysyl oxidase. The third still hasn't been given a name!

OXIDATIVE DEAMINATION BY LYSYL OXIDASE

Mechanism:

Steady state kinetic analyses (31) indicated that catalysis by lysyl oxidase follows a ping-pong kinetic course. Its action on collagen may be divided into two

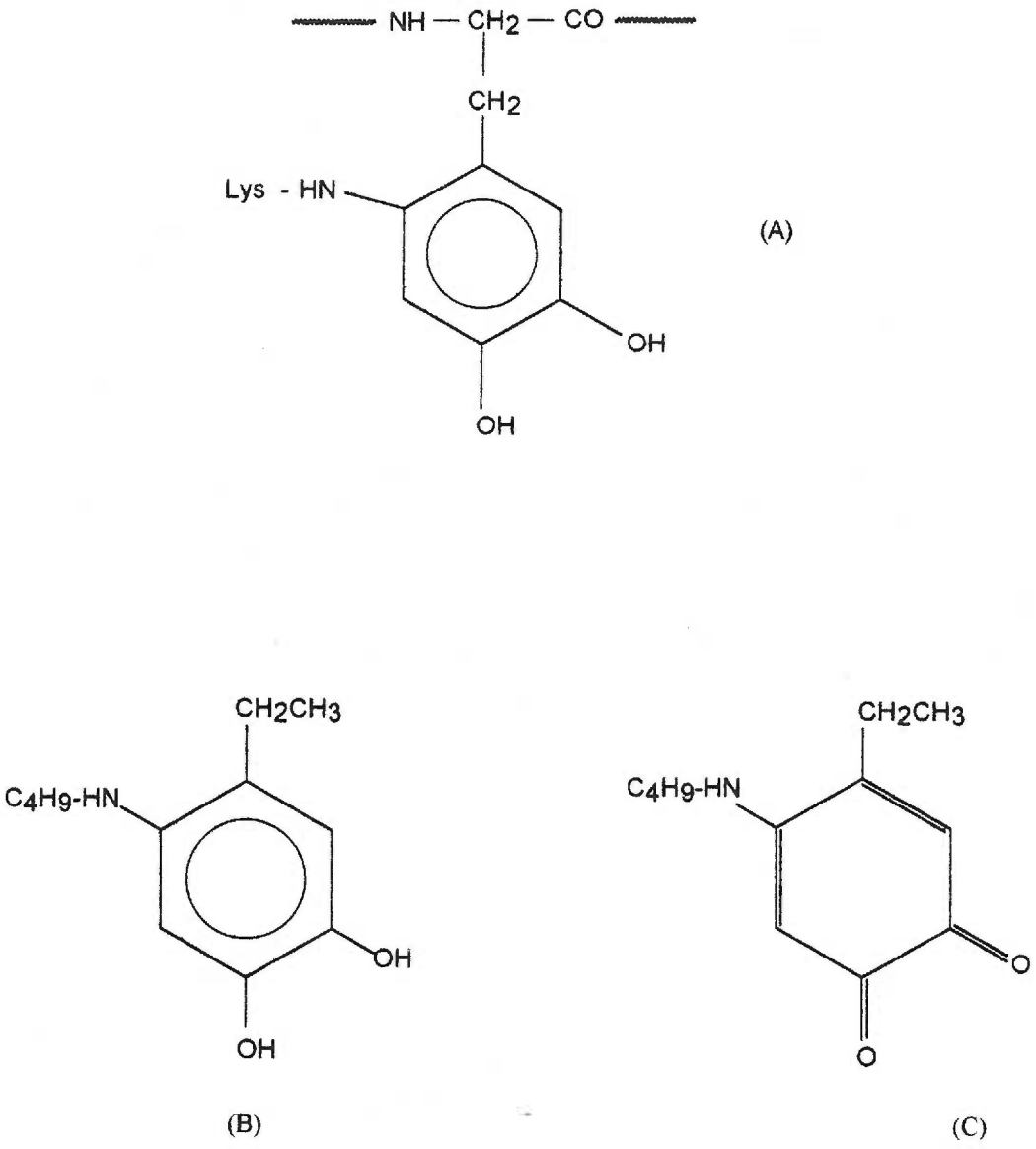


Figure 4 The quinone cofactor of lysyl oxidase : (A), this TOPA-like residue is covalently linked to theε-amino group of a lysyl residue on the protein backbone. The resonance Raman spectra of the cofactor compared well with the two model compounds : B and C. (Courtesy of J.Klinman (13))

distinct half reactions: the reductive and the oxidative half reaction. In the absence of oxygen, one mole of aldehyde is produced and released per mole of active site (31) showing that the first half of the reaction is independent of molecular oxygen. The oxygen is required for the second half of the reaction presumably wherein the aminophenol (or the reduced, expended form of the quinone) is oxidized to the iminoquinone, hydrolysis of which yields the oxidized enzyme and ammonia. The oxygen is reduced to hydrogen peroxide in the process.

Role of copper: stoichiometry and mechanism

The role of copper in this mechanism has yet to be ascertained. It has been demonstrated (32) that there is one tightly bound copper atom per 32 kDa monomer of lysyl oxidase. Nutritional studies have indicated that copper deficiency reduces cross linkage formation in vivo and thus leads to faulty connective tissue formation (33-35). It has been shown (32) that the copper-free enzyme does not catalyze the formation of p-hydroxybenzaldehyde in the presence or absence of oxygen even at levels stoichiometric with the active sites present in the native metalloenzyme. However, the copper-reconstituted apoenzyme regains the ability to catalytically oxidize p-hydroxybenzylamine. The inability of the metal-free enzyme to catalyze the first half-reaction of amine oxidation suggests a role for copper in this phase of lysyl oxidase catalysis. It has been suggested (36) that the copper stabilizes a catalytically competent conformation and/or may align the carbonyl cofactor and the amine substrate for

further processing.

1.4 ACQUIRED AND GENETIC DEFECTS OF COLLAGEN CROSSLINKING.

Defects of normal collagen crosslinking can be either genetic or acquired. A few of the many identified inborn connective tissue diseases known are Ehler Danlos Syndrome types VI (37,38), VII (39,40) and IX (41) and forms of cutis laxa (42). Type VI(A) Ehlers-Danlos patients have deficient hydroxylysine collagen due to deficiency in lysyl hydroxylase enzyme. Type VII Ehlers-Danlos patients have deficient conversion of procollagen to collagen due to deficiency in the procollagen peptidase enzyme. As a result there is reduced deposition of collagen as well as molecules retaining portions of the precursor specific peptides which are included in the fibers. Cutis laxa patients showed reduced lysyl oxidase activity (sometimes only 15-20% of the control values) and a parallel decrease in the aldehydic precursors for crosslinking. It is believed that either the lysyl oxidase itself is defective (42) or that there is an underlying disorder of copper metabolism. The latter seems to be the case in several strains of mice, some of which have low serum copper concentration as well as abnormal connective tissue with a defect in lysyl oxidase function (43).

Yet another human disease, X-linked Ehlers-Danlos Syndrome (Type V) has also been proposed to be due to lysyl oxidase deficiency (44).

As stated earlier, nutritional copper deficiency can also cause defects in collagen crosslinking (45). As a result of copper deficiency the lysyl oxidase enzyme is less active, and there is a decrease in the number of aldehyde functional groups formed. The physical manifestations of this defect include skeletal deformities and vascular aneurysms (46). These effects can be reversed by supplementing the diet with trace amounts of copper. Another less common crosslinking defect is lathyrism, which results from eating the peas of the *Lathyrus odoratus*, or sweet pea plant, that contains the toxic agent, beta-amino propionitrile (BAPN). BAPN inhibits the activity of lysyl oxidase (47). A decrease in lysyl oxidase activity results in a crosslinking deficiency. BAPN has been shown to affect the tensile properties of tissues. An increase in the BAPN dose increases the skin 'fragility' (48). Wirtschafter and Bentley (49) found that the aortas of lathyritic rats had a greater level of extractable collagen, and were more likely to develop aneurysms, due to drop in tensile strength. Also, BAPN has been shown to inhibit the increase in tensile strength of healing wounds (50). Once BAPN treatment ceases, the tensile strength of the wound increases.

1.5 IN-VIVO ENZYMATIC OXIDATIVE DEAMINATION AND THE NATURE OF CROSSLINKS FORMED.

Lysyl oxidase, is unable to enter the sterically hindered triple helix of

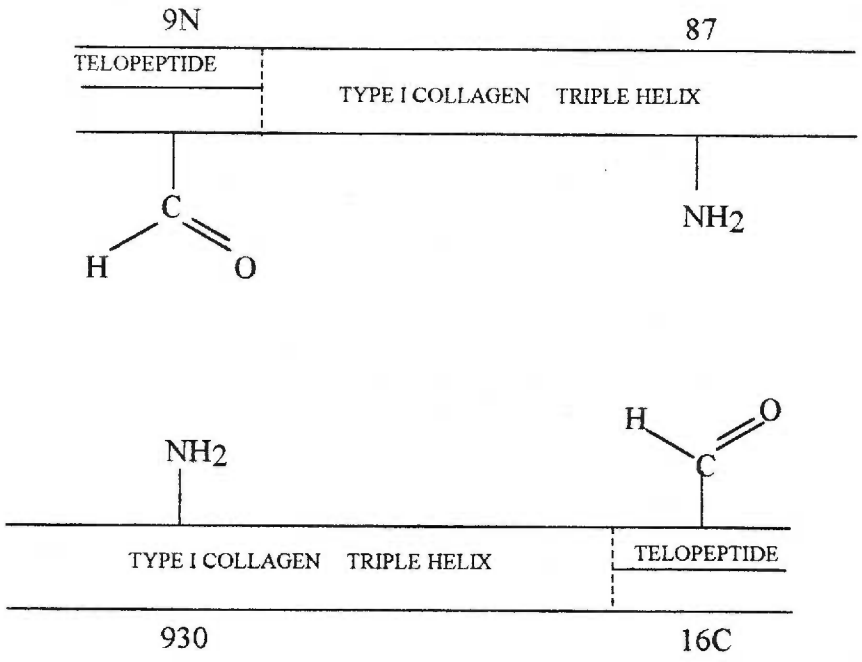


FIGURE 5 : The four sequence specific crosslinking residues of type I collagen. N and C designations represent the amino and carboxyl terminal telopeptides. The residues located within the telopeptide regions are shown in their oxidized form.

collagen. The lysyl and hydroxylysyl sites available to the enzyme for oxidation are restricted to these on the unhindered non-triple helical "telopeptide" regions of collagen. Figure 5 shows the precise location of the four hydroxylysines and lysines in the telopeptides of collagen. The two lysyl residues located within the telopeptides, namely the 9N-Lys and the 16C-Lys (51), are oxidized to their corresponding aldehydes. These aldehydic derivatives were given the trivial names allysine and hydroxyallysine and have been reviewed by Bentley (52). The aldehydes generated by lysyl oxidase may condense in a couple of ways: they may either condense with another aldehyde or may condense with an ϵ-amino group of lysine. Condensation between two aldehydes can occur between chains in the same triple helix, and is therefore predominantly an intra-molecular crosslink. This type of reaction is known as aldol condensation(53,54). Condensation between one aldehyde and one epsilon-amino group of a second lysine, hydroxylysine or a glycosylated hydroxylysine residue (52,55-57) to give Schiff bases occurs predominantly between residues in different triple helices and is therefore known as inter-molecular crosslinking. The aldimine crosslinks are identifiable following reduction with tritiated borohydride (58).

Depending upon which two residues combine, one of three aldimines can be created: dehydrolysinonorleucine, dehydrohydroxylysinonorleucine, or dehydrodihydroxylysinonorleucine. Dehydrolysinonorleucine (deLNL) originates through the condensation of an allysine residue with a lysine residue to form a Schiff base or aldimine bond. It is normally only a minor component in the

collagens because at least one of the precursor groups is hydroxylysine or hydroxyallysine.

A more common aldimine crosslink is dehydrohydroxylysinonorleucine (deHLNL), which is formed by condensation of an allysine residue with a residue of hydroxylysine. Alternatively, an hydroxyallysine residue may combine with a lysine to give dehydrolysinohydroxynorleucine (deLHNL). Yet another aldimine crosslink that is formed is dehydrodihydroxylysinonorleucine (deDHLNL), which is formed by condensation of hydroxyallysine residue with a residue of hydroxylysine. Amadori-rearrangement, a type of keto-enol tautomerism often seen with Schiff's base, occurs with deHLNL resulting in the formation of the more stable lysino-5-oxonorleucine (LONL) (Figures 6 and 7). A similar rearrangement of deDHLNL results in the formation of the more stable hydroxylysino-5-oxonorleucine (HLONL).

Neither the crosslink precursors, allysine and hydroxyallysine, nor their condensation products in collagen fibrils, are stable to dilute acid or alkali. However, reduction of the collagen preparations with reagents such as tritiated sodium borohydride stabilizes the crosslink precursors and their derivatives to hydrolysis and introduces a tritium atom, which facilitates their detection. Obviously, deHLNL, deLHNL and LONL would yield the same reduced product (except for the location of tritium). Resolution and isolation of the reduced components in hydrolysates is commonly achieved by conventional cation-exchange chromatography.

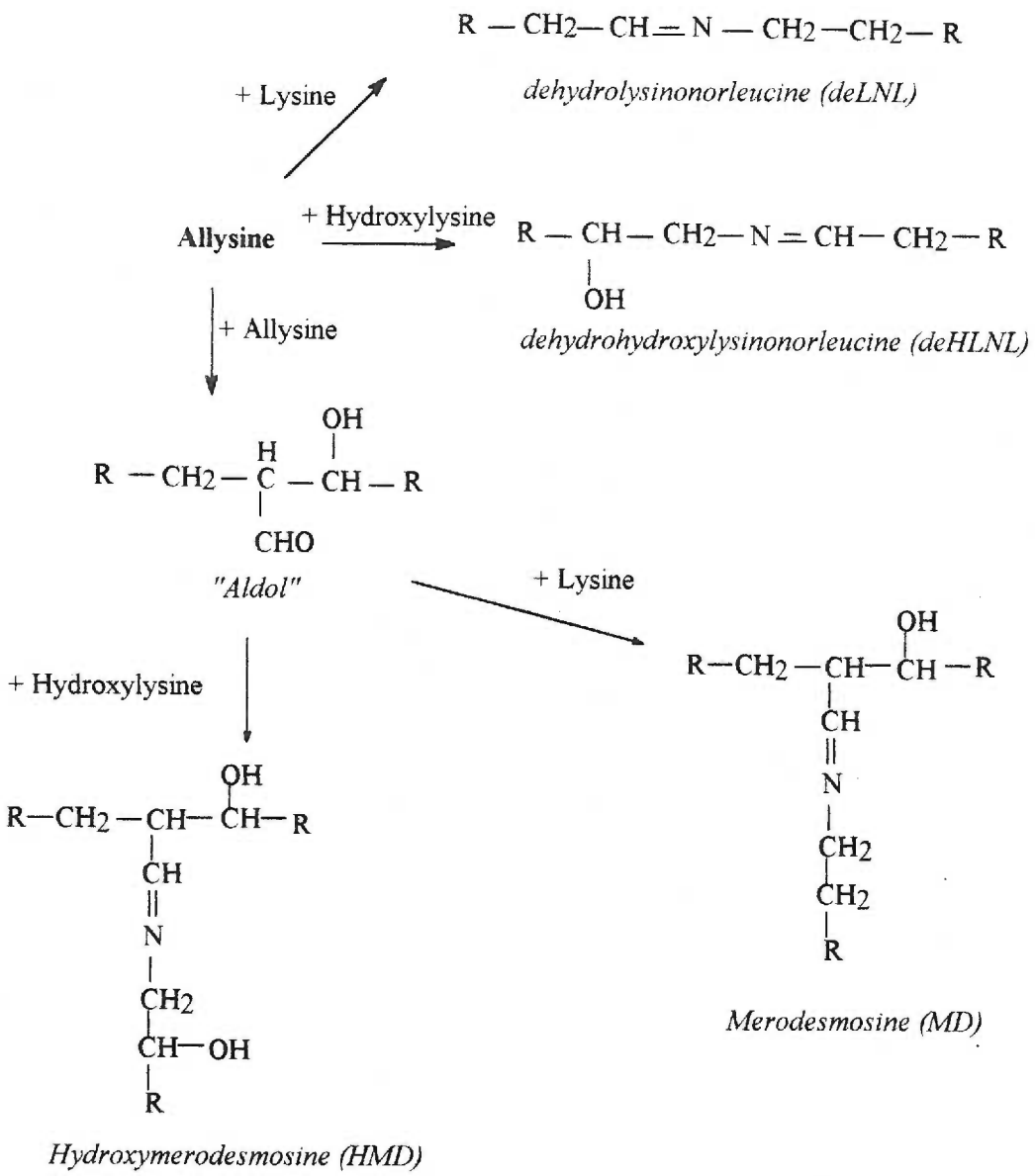


Figure 6: Summary of crosslinking reactions involving an allysine intermediate in collagen

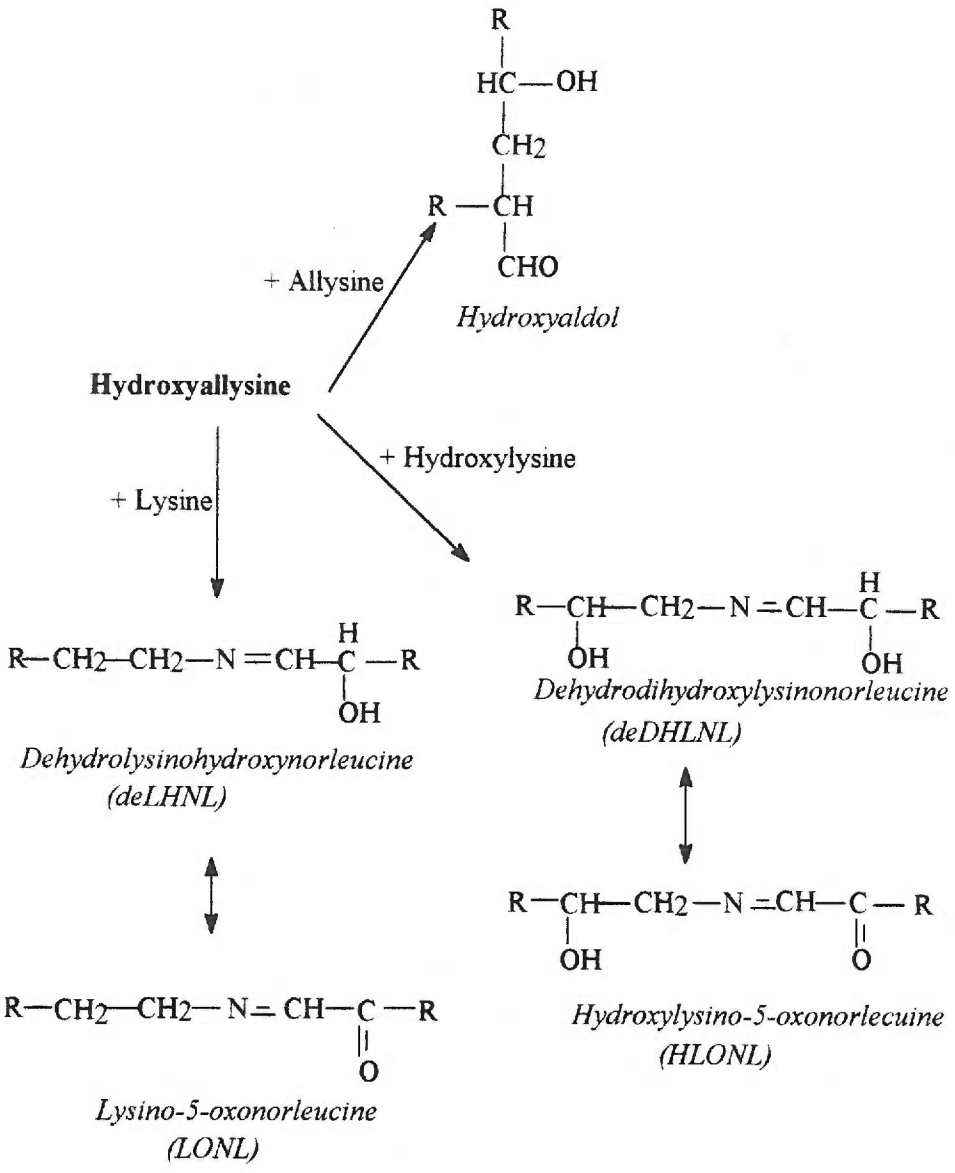


Figure 7 : Summary of cross-linking reactions involving a hydroxyallysine intermediate in collagen.

In general, deLNL is a minor component while deDHLNL is more prominent in hard tissues. The proportion of deHLNL and deDHLNL in soft tissue varies widely, depending on the tissue source and age. Robins et al. (59) found that the major reducible crosslink in embryonic bovine skin to be deDHLNL. This was replaced in late-gestation and early post-natal period by HLNL which increased during rapid growth then decreased until maturity. Volpin et al. (60) showed that in human dermis between the ages 2 days and 71 years, the deDHLNL disappeared shortly after birth while deHLNL was high during growth but diminished at maturity.

Bailey and Shimokomaki (61) sampled rat, bovine and human skin, tendon and cartilage and found that the amount of deHLNL decreased with age. Allain et al. (62) reported that in rat skin, deDHLNL was present at birth and had disappeared by three months, whereas deHLNL increased during this period and subsequently disappeared with further aging of the animal.

Summarising from the above paragraph, the level of the reducible crosslinks, measurable by borohydride reduction, decreases as an animal ages, while it is well known that mechanical stability of the tissue increases. This can be explained to be due to a molecular rearrangement of the crosslinks which results in increased stabilization. Several molecular models have been proposed as possible structural rearrangement products. Bailey et al (63) proposed that deHLNL, the predominant reducible crosslink in skin is oxidized as shown in the Figure 8, to form a peptide bond. By hydrolyzing in vivo labeled tissue, they were

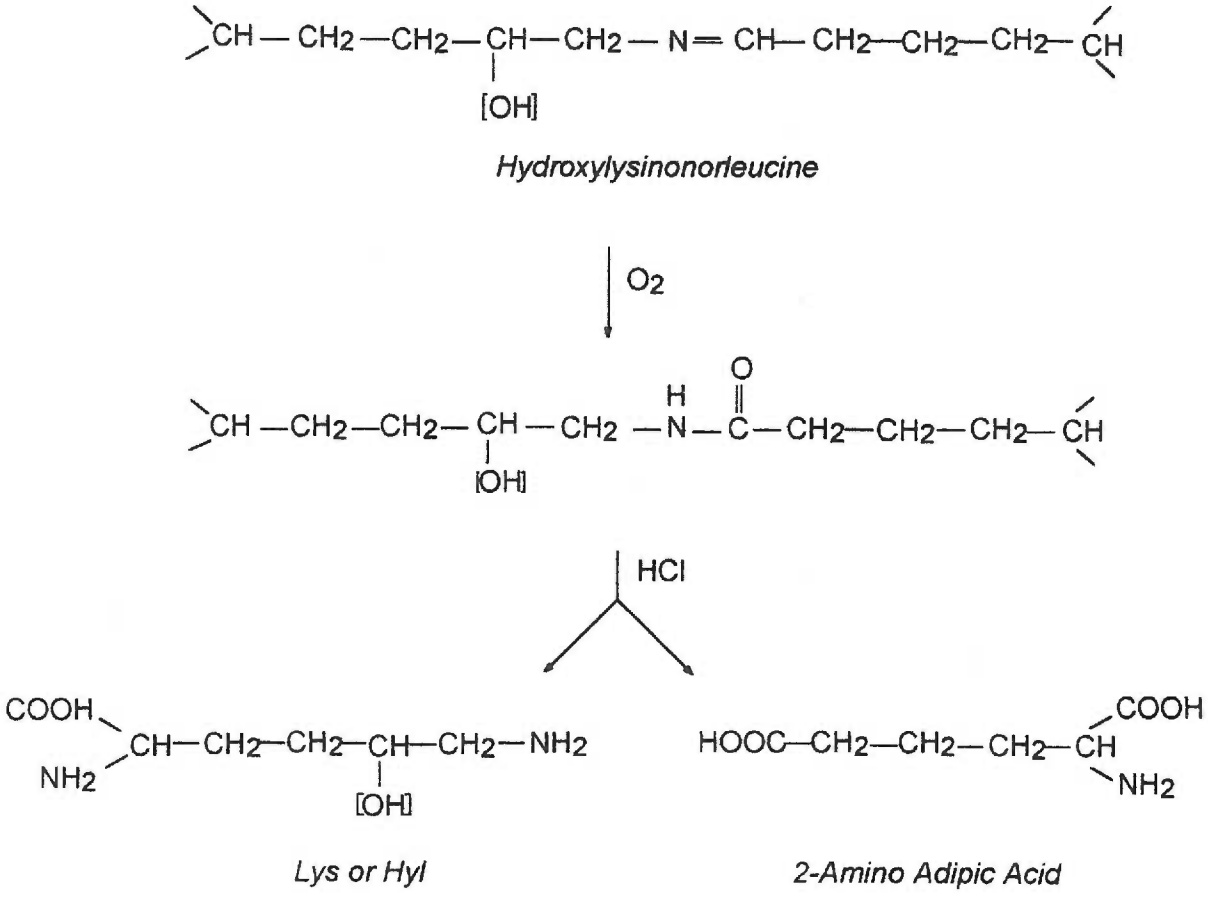


Figure 8 : A proposed mature collagen crosslink produced by oxidation of hydroxylysinonorleucine to form an amide linkage (51)

able to isolate amino adipic acid derived from the labeled lysine, providing indirect support of its significance. There is however no direct evidence for such an oxidation.

Apart from these divalent crosslinks, some multivalent crosslinks have been reported to be present in collagen fibrils. Some examples (Figure 6) are dehydrohydroxymerodesmosine (deHMD) (formed from an allysine aldol condensing with hydroxylysine). Another one is aldolhistidine (AHis) formed from Michael addition of allysine aldol with histidine imidazole-nitrogen. Yet another trimeric crosslink is hydroxyaldol-histidine (HAHis) formed by Schiff base condensation of hydroxyallysine with imidazole-nitrogen of histidine (64) and has been isolated from bovine skin without borohydride reduction. The only tetrameric crosslink reported in collagen is dehydrohistidinohydroxymerodesmosine (deHisHMD) formed from Michael addition of imidazole nitrogen of histidine across the ethylene double bond of deHMD (65). But the existence of many of these is controversial.

The most well known and well accepted mature crosslink of fibrous collagen (66) is the trivalent hydroxypyridinium crosslink reported originally by Fujimoto et al. (67-69). It has been proposed to be formed from two residues of HLONL which would then reform a hydroxylysyl residue as well as creating one residue of pyridinoline. An alternative proposal that has been made is that LONL reacts with a single residue of hydroxyallysine (70). This crosslink is highly fluorescent, UV radiation labile(68,71) and has not been identified in skin collagen

(72).

As mentioned earlier, the presence of these intra- and inter-molecular crosslinks can be demonstrated by treating intact tissue with tritium labelled potassium or sodium borohydride which reduces the double bond in the cross-link and results in the addition of the tritium label. When the tissue is subsequently hydrolyzed in acid this radioactively labelled and cross-linked compound survives and can be separated from the amino acids by use of a modified amino acid analyzer and quantified.

In the results presented in the experimental section, a similar technique is used for determining the nature of crosslinks formed in collagen when incubated with 3,4-Dihydroxyphenylalanine (DOPA).

1.6 INVITRO COLLAGEN CROSSLINKING: NON-ENZYMIC

1.6.1 Extraction of collagen for in-vitro reactions:

Long before the nature or chemistry of collagen crosslinks was known, Jackson and Bentley (73) proposed: "at any given time in developing connective tissue, there is a continuous spectrum of collagen aggregates of varying degrees of strength of crosslinkage, dependent upon the time that has elapsed since their constituent molecules were synthesized". They showed by using extraction

conditions in increasing order of ionic strength, they could extract increasing amount of collagen. Since then, it has been shown (59) that as an animal ages, the level of detectable reducible crosslinks decreases. It is believed that these crosslinks are replaced by more stable non-reducible crosslinks. The degree of insolubility (defined as the limited amount that can be extracted by a specific extraction condition) of collagen would dictate the extraction conditions to be used.

There are several methods by which collagen may be extracted from tissue. Miller et al. (74) have extracted recently laid down collagen using dilute acetic acid which hydrolyses immature reducible crosslinks (aldimines). Gustavson showed (75) that heating a collagen solution to temperatures approaching 100°C, in dilute acetic acid results in a thermal denaturation of collagen as well as hydrolysis of the aldimine crosslinks. Schmitt et al. (76) used pepsin treatment, which enzymatically cleaves within the non-helical telopeptide crosslinking region to extract collagen stabilized by mature collagen crosslinks. The type I collagen triple helix is, however, resistant to peptic cleavage. Pepsin treated collagen lacks the telopeptide ends, the locus of two of the four crosslinking sites. By removing these telopeptides, the two crosslinks between these four residues cannot form. As expected this should also effect the stability of the fibrils reconstituted in vitro from pepsin solubilized collagen.

In the experimental section, the first set is devoted to the determination of the comparative stability of the fibrils formed, when DOPA is incubated with

telopeptide-intact (acid soluble collagen; ASC) or the telopeptide-removed collagen (pepsin soluble collagen; PSC).

1.6.2 Need for in-vitro crosslinking of collagen:

As the chief structural protein of the body, collagen is uniquely designed to transmit tensile and compressive forces of great magnitude.

During extraction, collagen is dispersed in acid solution resulting in breaking of many of these crosslinks, and when this acid soluble collagen is reconstituted into a new form e.g. sutures, these crosslinks must be replaced in order to restore the unique properties originally in the fibers. The process of introducing artificial crosslinks in collagen is known as 'tanning'. The cross-linking which occurs during biological maturation of collagen can be simulated in vitro by several tanning agents.

The art of 'tanning' opened up new avenues for the use of collagen protein. One of the earliest of course was the leather industry. A more recent is the use of collagen as a biomaterial in the synthesis of prostheses and body implants. Table 1 summarizes the factors favoring the use of collagen as a biomaterial. Three major disadvantages of using collagen as a biomaterial are: antigenicity, lack of adequate wet tensile strength even though dry tensile strength of reconstituted collagen is excellent, and lack of resistance to degradation when implanted in tissue (77). The latter two disadvantages may be overcome by

'tanning collagen' *to the right extent*. This may be done by manipulating the extent and position of crosslinking. Factors controlling the extent of cross-linking are: type and concentration of the tanning agent. The problem of antigenicity of collagen may be overcome by using collagen from which the 'telopeptide' regions have been removed; for it is known that the antigenic determinants are located on these telopeptides. Antigenicity can be reduced by using pepsin-digested (atelopeptide) collagen. But atelopeptide collagen cannot crosslink, therefore 'tanning agents' that work in places other than the telopeptides have to be employed.

The side-chain functional groups most commonly involved in collagen crosslinking reactions are the carboxyl groups from aspartic and glutamic acid residues, the amino groups primarily from lysine and hydroxylysine residues, and side chain amide groups.

TABLE I (135)

FACTORS FAVORING THE USE OF COLLAGEN AS BIOMATERIAL

1.	Physical-mechanical:	High tensile strength Low extensibility Orientation of fibers
2.	Physical-chemical:	Controlled cross-linking by tanning agents; affects solubility, swelling, resorption Ion exchanger function Semipermeability of membranes
3.	Biological:	Low antigenicity Effect on wound healing Effect on blood coagulation

1.6.3 Free quinones as oxidants or 'tanning agents'

The in-vitro properties of quinones are as intriguing as their in-vivo reactions. For a long time, quinones have been known to have tanning effects on collagen, which at least some researchers have attributed to be due to oxidative properties of the quinones.

Lumiere and Seyewetz (78) showed that photographic films of gelatin may be made resistant to boiling water by solutions of polyphenol which had been exposed to air. Meunier and Queroix (79) believed that the quinone oxidized the protein. Fahrion (80) proposed that the superoxide-like make-up of the quinone and its functions as an oxidation agent were the primary property of the quinones, which were used as tanning agents for collagen. However Hilpert and Bruns (81) pointed out that the coupling of quinone on collagen proceeds so fast that there is little chance for the quinone to exert its oxidation potential. Moeller (82), opposed the chemical theory, claiming the polymerization products of the quinone to be the tanning agent. Thomas et al. (83) showed that following the removal of amino groups of lysyl residues by deamination, the affinity of collagen for quinone was markedly lowered, pointing to the amino group as the main site of collagen for quinone linkage.

More recently, Gade et al (84) showed that collagen fibrils reconstituted in the presence of the orthoquinone, DOPA at a concentration of 1×10^{-3} M were completely stabilized with respect to fibril dispersion after 24 hours of incubation

at 37°C. He showed that only about 10% of the gels dissolved when boiled in 0.01 N acetic acid at 100°C for 2 hours, and could not be extracted with cyanogen bromide unlike the control collagen fibrils. The wet strength of the gels increased to four times that of control collagen after three days of incubation at 37°C. He also showed that on reaction with DOPA, the actual number of lysyl residues detectable by HPLC amino acid analysis had decreased.

Gade (85) did not attribute these as being due to the oxidative properties of DOPA. Instead he proposed a three dimensional binding of DOPA to the lysyl residues in collagen. He proposed that once the DOPA was bound it underwent polymerization linking inter-collagen chains generating intercalated crosslink bridges. Gade found that 3,5-di-tert-butylcatechol treated collagen gel was more stable to hydrolysis in 0.5 M acetic acid than 4-tert-butyl catechol-treated gel, both of which were more resistant than DOPA treated collagen gels, when the o-quinones were all incubated at a constant concentration of 5×10^{-5} M. If a three dimensional network of crosslinked collagen via polymers of o-quinone were occurring then any quinone that is unable to polymerize easily, like tert-butyl catechol would be less effective.

In preliminary experiments, I observed that when collagen is incubated with polymerized DOPA, known as 'melanin', stabilization of collagen fibrils could not be detected. This makes the proposal of polymeric DOPA bound collagen very unlikely.

HYPOTHESIS

This thesis will describe the mechanism of crosslinking of collagen by the non-toxic ortho-quinone, 3,4-Dihydroxyphenylalanine (DOPA), and other o-quinones. I propose that ortho-quinones oxidatively deaminate the lysyl and hydroxylysyl residues in collagen converting them to allysyl and hydroxyallysyl residues, respectively. These aldehydes then condense with other nucleophiles to form Schiff bases or condense with other aldehydes to form aldol condensation products. The Schiff bases can be further stabilized. The thesis will demonstrate for the first time the ability of DOPA and other o-quinones to oxidatively deaminate free lysines as well as peptidyl-lysines in the absence of the enzyme which can catalyze this reaction.

IV. MATERIALS AND METHODS

A. Type I collagen extraction and purification

Type I collagen was extracted and purified by the method of Miller and Rhodes (74) with certain modifications to ensure complete removal of type III collagen contaminants. The collagen source was 4 month old fetal calf skin. The tissues were defatted by scraping and homogenized in phosphate buffered saline containing the enzyme inhibitors N-ethylmaleimide (1.23 g/l), phenyl methyl sulfonyl fluoride (0.174 g/l), and (ethylenedinitrilo)-tetraacetic acid (5.84 g/l).

The pellet was collected by centrifugation (2500 X g) in a Sorval RC-3 centrifuge, and washed two additional times in the same manner. The pellet was resuspended in 0.5 M acetic acid for 1 hour with shaking, centrifuged at 2500 X g to collect and discard the wash, and resuspended in either 0.5 M acetic acid.

The pepsin-digested collagen is prepared in an identical manner except that the pellet, collected after centrifugation, is resuspended in 0.5 M acetic acid containing pepsin (E.C. No. 3.4.4.1) at an approximate concentration of 2 g/l. The pepsin (Sigma Chemical Co.) was from porcine stomach mucosa and had been purified 60,000 fold. The media were allowed to shake overnight and then centrifuged at 2500 X g.

The fibrous collagens salt out at different salt concentrations when the pH of the medium is changed. Types I and III collagen are insoluble at 0.7 M NaCl

under acid conditions, whereas at neutral pH, type III collagen becomes insoluble at a much lower concentration (1.5 M to 1.8 M) than does type I collagen (2.5 M). Thus, both type III and type I collagen were precipitated at 0.7 M NaCl, and the precipitate collected by centrifugation at 5650 x g in a Beckman J-21 High Speed Centrifuge, and resuspended in 1.0 M NaCl, 50 mM tris-HCl (pH 7.5). The resulting solution containing both types I and III collagen was adjusted to a concentration of 2.0 M NaCl to precipitate all type III collagen which could be removed by centrifugation at 3900 x g. The supernatant was adjusted to a final concentration of 2.5 M NaCl, and the type I collagen precipitate was collected by centrifugation at 2950 x g. The type III collagen precipitation step was repeated and pellet resuspended in dilute acetic acid. Excess salt was removed by exhaustive dialysis against 5.0 mM acetic acid. The collagen concentration was measured by hydroxyproline assay using the auto analyzer method (see below), and and then the relative purity was analyzed by interrupted SDS-polyacrylamide gel electrophoresis (see below). Collagen solutions were stored in 5.0 mM acetic acid, at an approximate concentration of 3.0 mg/ml under liquid nitrogen.

The concentration of collagen solutions were determined by hydroxyproline assay and confirmed by amino-acid analysis.

B. Collagen gel solubility assay

Buffered acid-soluble collagen solutions were prepared as described in the appendix. Gels of collagen (telopeptide-intact and telopeptide-removed) incubated with and without DOPA were prepared. The methodology for pepsin treatment of collagen is also described in the appendix.

Three protocols were used to determine collagen gel resolubilization. In treatment 1, the sample was homogenized in a Virtis 45 homogenizer in 10 ml of 0.01 M hydrochloric acid, and then rotated on an Orbitron Rotator for 2 h at 4°C. In treatment 2, the sample was homogenized in 10 ml of 0.01 M HCl and then extracted at 37°C for 2 h. In treatment 3, the samples were homogenized in 10 ml of 0.01 M HCl and boiled for 2 hours. The collagen gels were then analyzed by the collagen gel resolubilization method described below:

At the end of each extraction, the samples were centrifuged at 40,000 x g using a Beckman J-21 high speed centrifuge. The supernatant and pellet were separated. An aliquot of the supernatant was then hydrolysed and analyzed for hydroxyproline by the Autoanalyzer method (described in the appendix).

C. Radioassay by liquid scintillation counting

Samples were radioassayed by liquid scintillation counting using a

Beckman L-250 Liquid Scintillation Counter. Samples were prepared in high efficiency, low quench scintillation cocktails: Optifluor and Hionic Fluor (Packard Instruments Co.). The latter was used when high ionic salts had been used in sample preparation. Background values were subtracted, and DPM values were determined from a channels ratio correlation curve.

D. Borohydride reduction of crosslinks:

A long column system for resolution of reduced cross-links as described by Mechanic (86) was employed.

1. Samples (either collagen or free N-acetyl-lysine) were reduced by the addition of sodium tritiated borohydride (Amersham, Radioactive) in 99% Dimethylformamide (Aldrich) to a final concentration of 3 mg/ml at 250 mCi/mole. Two drops of Antifoam A (Sigma 1:100 dilution) were also added. The predetermined volume of the borohydride suspension was added in three installments, at intervals of 3 minutes. The solutions were allowed to stir for 5 min after the last addition. This was carried out in a fume hood.

2. The reduced collagen samples were then dialyzed against distilled water, overnight, in the cold, to remove excess radioactive borohydride. The N-acetyl-L-lysine-DOPA samples were not dialyzed but acidified to pH~1 to evolve the excess unreacted sodium borohydride.

3. The samples were then lyophilized.

4. Measured amounts of the reduced lyophilized samples were transferred to a hydrolysis vial and hydrolyzed in 1.5 ml of 3N p-toluenesulfonic acid (Sigma) at 107°C for 24 h.

5. The vials were then cooled, filtered via a 0.4 μ filter and stored cold.

E. Chromatographic elution of the Borohydride reduced hydrolyzates (86) :

1. A column was packed to 58 cm with a spherical cation exchange resin (Type 150A, 31-41 μ, Beckman Instruments Inc., Palo Alto) which differs slightly from the one used by Mechanic et al.(86) in that they used a smaller diameter resin ~ 9-12 μ from Marks Instruments Co..

2. A 0.01 ml aliquot of the entire hydrolyzed sample was counted using Opti-fluor on a scintillation counter.

3. A volume of 0.7 ml of filtered hydrolyzate was applied to the column. A 0.2 ml amino acid "spike" consisting of glycine, hydroxyproline, phenylalanine, aspartic acid, serine, proline, alanine, hydroxylysine and histidine in concentrations of 1 mg/ml was added to aid in the localization of constituents.

4. Elution was performed with a complex gradient generated with a 5-chambered gradient device and a Mark Instruments pump, using two buffers. The starting buffer (pH 3.15) was 0.2 M sodium citrate and 0.4 M citric acid containing

6% isopropanol and the limiting buffer was 0.4 M sodium citrate (pH 9.1). The column was eluted at a flow rate of 68-71 ml/h. and fractions collected at a rate of 1.75 ml/fraction. About 280 tubes were collected

5. From each fraction, aliquots of 0.16 ml were pipetted into 7 ml scintillation vials in addition to a 5 ml volume of scintillation fluor. The samples were equilibrated for at least 4 hours in the counter, and then counted.

6. Aliquots of 0.2 ml were mixed with 0.2 ml ninhydrin reagent and the color developed by heating in a water bath at 100°C for 10 minutes. The positions of marker amino acids were recorded on the radioactivity elution profile.

F. REMOVAL OF CITRATE WITH DESALTING COLUMN

1. A column was packed to 14 cm with a strongly acidic cationic exchanger (Dowex-50W [HCR-W2]).

2. The column was initially equilibrated with water, run with 4 bed volumes of 10% HCl, washed with 2 bed volumes of water and regenerated with 4 bed volumes of 10% NaOH/$NaHCO_3$. The column was thoroughly washed with water to remove all traces of NaOH (neutral to pH paper) before loading sample.

3. Calibration was carried out by loading 0.5 ml of threonine (since structurally it was the closest to hydroxynorleucine) at a concentration of 1 mg/ml in citrate buffer. Fractions were collected with Pharmacia Frac-100 fraction collector at a

rate of 1.5 ml/min per tube.

4. The column was eluted with distilled water for 7 min. and then eluted with 10% HCl. Threonine eluted between 10-17 minutes; i.e. 3 minutes after the eluant was changed to 10% HCl. The citrate salts were found in the first 3 tubes only (as measured by conductivity), the eluant was distilled water.

5. After column regeneration, a 0.25 ml aliquot of the radioactive sample was applied to the column and column was eluted first with water for 7 min. to elute all the citrate salts. Then after 7 minutes the column was eluted with 10% HCl and fractions were collected

6. Radioactivity in the fractions were measured and the samples containing radioactivity were pooled together. The pooled samples were lyophilized and subjected to Fast Atomic Bombardment (FAB).

G. HPLC ANALYSIS

HPLC system

The Gradient Liquid Chromatograph is a modular dual pump system comprising a Beckman Model 421 microprocessor based, programmable LC system controller, two Beckman model 110B Solvent Delivery Modules (pumps), a model 210 sample injection valve, a dual-chamber dynamic mixer (Dynamax, Rainin Co.), a 4.6 m.m. I.D. stainless steel analytical column (C-18, Beckman,

ultrasphere, 5 μ dia, 4.6 mm X 25 cm.) and an HP 3396A Integrator (Hewlett-Packard). The detector used was a Waters 410 Differential Refractometer.

Theoretical basis for Refractometer operation:

The refractometer consists of two rectangular silicon photodiodes mounted on a single chip. If the liquid in the sample side of the cell has the same refractive index as that in the reference cell, the photodiodes will produce equal signals. If the liquids in the two sides of cell have different refractive indices, the photodiode show different signals. The signals from the photodiodes are processed and amplified by the analog board, then converted to an output signal and sent to the external recorder.

A typical hplc run

1. Calibration curve was obtained by injecting steadily increasing concentrations of L-Lysine into the column. The fractions were collected using a fraction collector (Frac-100, Pharmacia Fine chemicals).
2. The column was eluted with an isocratic solvent system containing water + 0.1% TFA and flow rate was 0.5 ml/min.
3. Using the same protocol, N-α-acetyl-L-lysine was also eluted on the column.
4. 0.25 ml of sample (with total radioactivity of 11575) was injected into the C-18 column. The fractions were collected at 0.5 ml/tube. 0.2 ml of every even number tube was diluted with 10 ml of Opti fluor and counted.

H. Sample preparation for mass-spectral analysis:

7 ml volume of N-*α*-acetyl-L-lysine (Sigma) at a concentration of 25 mg/ml in phosphate buffered saline (PBS) was incubated in triplicates at 37°C for 72h, in the presence of 1 mM DOPA with and without 0.1 mM copper(II)chloride (Sigma) or Ferric chloride (Aldrich). The two controls were N-*α*-acetyl-L-lysine at 25 mg/ml and 1mM DOPA in PBS; incubated at 37°C for 72h. The samples were then reduced with sodium borohydride as indicated above. The samples were further acidified with hydrochloric acid to pH~1. 20 mls of the acidified samples were poured onto Chem-elut columns (Varian Chemical Co.). The solution was allowed to equilibrate for exactly 5 minutes. The columns were washed three times with 7 ml of ethyl acetate (Omnisolve, VWR Scientific) in order to extract N-*α*-acetylallysine and other ethyl acetate soluble substances out of the bulk of the unreacted starting material. The ethyl acetate extracts were rotary evaporated, redissolved in a small volume of ethyl acetate. The small volumes were then evaporated to dryness under a steady flow of nitrogen.

I. Trimethylsilyl derivatization for GC-MS analysis:

The dried ethyl acetate extracts were treated with 0.074 ml of N,O-bis-[Trimethylsilyl]trifluoroacetamide + 1% Trimethylchlorosilane (BSTFA + 1% TMCS)

(Pierce) at 85°C for 20 min. to produce trimethylsilyl derivatives.

J. Gas Chromatography-Mass spectroscopy (GC-MS):

GC-MS was performed on a Hewlett Packard 5890 gas chromatograph equipped with 30 m X 0.25 mm DB-1 column (J and W Scientific) interfaced to a Mass Selective detector. After an initial 4 min hold at 100°C the column was programmed to 250°C at a rate of 6°/min, then ballistically ramped to 280°C and held for 11 min for bake-out. The injection port was kept at 200°C and the MS interface at 250°C.

K. N-Acetylation of Pipecolic acid:

5 mg of Pipecolic acid (Sigma) was treated with 0.3 ml of an equivolume mixture of acetic anhydride (Sigma) and acetic acid (Sigma) at 80°C for 1 h. The mixture was evaporated under nitrogen at 45°C to dryness and converted to the trimethylsilyl derivative prior to mass spectral analysis.

L. Interrupted SDS-Polyacrylamide Gel Electrophoresis (131)

A 14 X 12 cm 6.5% running gel slab with a 4% stacking gel was prepared from a 30% acrylamide (0.7% bis) stock solution in a bio-rad SE500 Vertical Slab Gel Unit. The conditions of the running gel were as described by Laemli (132), with modifications. In short, the running gel consisted of 0.375 M Tris (pH 8.8), 0.1% SDS and 0.5 M urea. The stacking gel differed from the running gel in the Tris concentration (0.125M) and pH (6.8). 0.15 mg loads of purified collagen in 0.03 M Tris-HCl, 0.1% SDS, 0.0025% Bromophenol Blue, and 20% Glycerol, were layered in 1 cm sample wells. The gel slab unit was immersed in a running buffer consisting of 0.025 M Tris-HCl (pH 8.8), 0.195 M glycine, and 0.1% SDS. The pulse rate was raised from 100 to 200 pps over 30 minutes using a Ortec 4100 Constant Pulsed Power Supply. The pulse rate was maintained at 200 pps for an additional 10 minutes. The electrical current was then interrupted, buffer removed from the sample wells, and 0.01 ml of 10% β-mercaptoethanol (BME) in running buffer layered in the wells. The BME was allowed to penetrate the gel for 1 hour, after which time the electrical current was re-applied and maintained throughout the remainder of the run at 200 pps. The gel slab was stained with Coomassie Brilliant Blue R-250 stain (133).

M. Hydroxyproline analysis (134)

Collagen samples were hydrolyzed in constant boiling 6.0 N HCl at 108°C for 18 hours. The hydrolyzates were dried under nitrogen and resuspended in double distilled-deionized water. Hydroxyproline standards were prepared from a solution at concentrations of 0.001, 0.002, 0.004, 0.006 and 0.008 mmole/ml, and were used to prepare a standard curve. Hydroxyproline content was measured by the Technicon autoanalyzer method. In this procedure each hydrolyzed sample is mixed with an excess of citrate acetate buffered (pH 6.0) chloramine-T-hydrate (2.8 g/L) and incubated at room temperature for 18 minutes. The product of this reaction was then mixed with 3.0 M perchloric acid which deactivates excess chloramine T over a six minute room temperature incubation period. Finally, the reaction was completed by mixing with 5% para-dimethylaminobenzaldehyde, and incubating at 60°C for 24 minutes. The production of a chromophore was measured at 550 nm using a Technicon 15 mm Colorimeter. The hydroxyproline content of the test samples are from the standard curve.

N. The preparation of buffered type I collagen solutions

Type I collagen stock solutions were diluted to 1.47 mg/ml concentration with phosphate buffered saline. The final buffer salt concentrations were 0.135 M NaCl, 2.5 mM acetate, and 30 mM phosphate (pH 7.5). All solutions were prepared at 4°C.

V. EXPERIMENTAL

In the experiments presented in this section, the effect of ortho-quinones on free and bound lysines will be presented. Both collagen and N-α-acetyl-lysine were used as substrates. Collagen has a peptidyl-bound lysine whereas N-α-acetyl-lysine is the free acetylated amino-acid. Throughout the thesis, the term "collagen" means type I collagen.

It is well known (87,88) that ortho-quinones interact via nucleophilic addition with both cysteine and lysine. Therefore it was important to eliminate any cysteines. Type I collagen contains no cysteine residues within its primary sequence, whereas type III collagen has one cysteine at the C-terminal end. Extreme care was taken to eliminate type III collagen contaminants from the bovine collagen extracts and this has been described in the appendix. In these studies, both telopeptide-intact (acid soluble) and telopeptide-cleaved (atelopeptide, pepsin-treated) collagen have been used.

PART I. : CROSSLINK ANALYSIS OF DOPA TREATED COLLAGEN AND N-α-ACETYL-LYSINE

1. EFFECT OF TELOPEPTIDE ON DOPA-TREATED COLLAGEN GEL SOLUBILITY

The first experiment is aimed at determining the importance of the telopeptide. This is demonstrated by measuring the relative stability of the fibrils formed from telopeptide-intact collagen versus that of the telopeptide-lacking collagen with and without DOPA.

Acetic acid soluble collagen has at the N and C terminal ends of the collagen helix, non-helical telopeptides which are known to provide lysyl residues necessary for the formation of aldimine crosslinks (51). If reconstituted and incubated at 37°C for extended periods of time, the fibrils gradually become resistant to dispersion when cooled due to the reformation of aldimine crosslinks between fibrils (Figure 9). The result is a "stabilization" of the collagen gel. On the other hand, pepsin treated collagen gels completely disperse upon cooling, no matter how long the gels have been incubated at 37°C. As pointed out above, the loss of telopeptide results in the loss of two of the four critical crosslinking residues, and thus the ability to form aldimine crosslinks. Collagen gel stabilization is not possible.

In the following experiment,the relative solubility of DOPA stabilized acid-

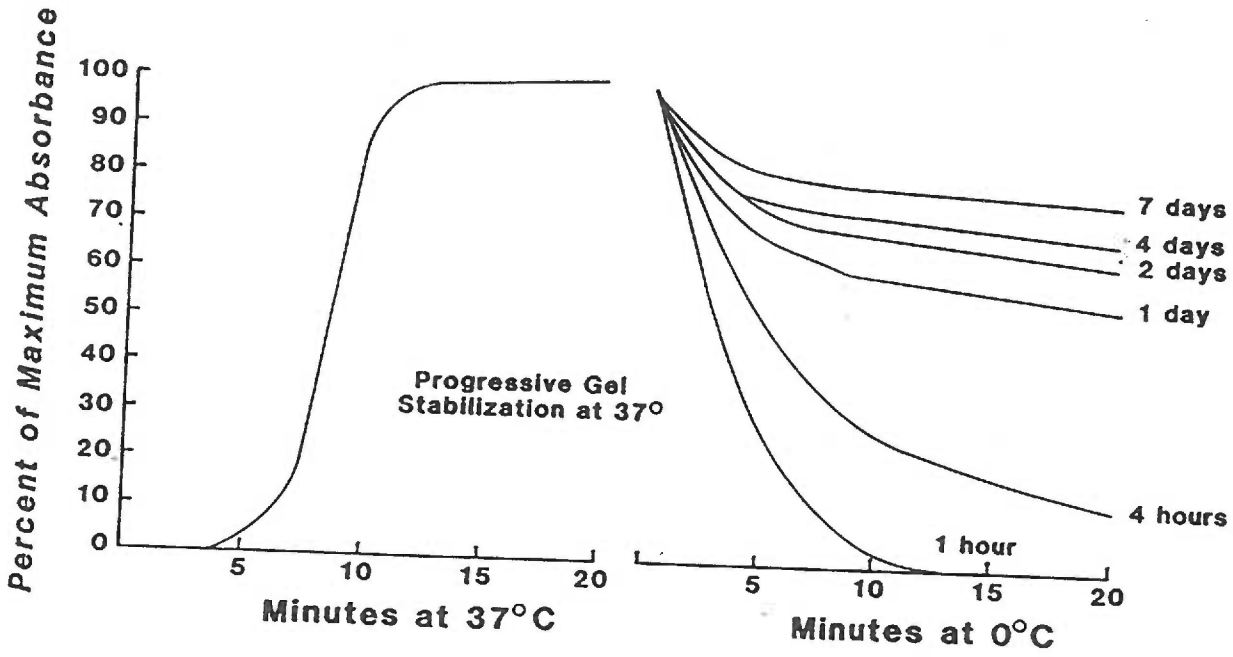


Figure 9 : Collagen gel stabilization at 37˚C and destabilization at 0˚C. Acetic acid soluble collagen gels are progressively stabilized with increasing periods of time at 37˚C due to the formation of aldimine crosslinks.
Reproduced from Bentley and Fellman (100)

soluble (telopeptide intact) and pepsin-treated (telopeptide removed) collagen was determined.

Experimental

Three 2 ml volumes of buffered collagen (telopeptide-intact and telopeptide-removed) solutions were incubated with 0.1 mM DOPA at room temperature, in 10 mm diameter Spectrapor dialysis tubing (M.W. cut-off = 12 kD - 14 kD) for 24 hours. The controls were the collagen solutions without DOPA. The sample bags were dialyzed and the gels were subjected to three different extraction protocols (Methods section).

An aliquot of the supernatant was then hydrolysed and analyzed for hydroxyproline by the Autoanalyzer method (described in the methods section).

Results

The solubility data for the DOPA-collagen and control collagen gels under the three methods of solubilization is shown in Figure 10. Solubility expressed in terms of the amount of collagen solubilized.

The pepsin soluble and acid soluble collagen gels were deeply pigmented after incubation with DOPA. The DOPA treated gels were less soluble than the control collagen gels, especially under relatively mild extraction conditions at 4°C.

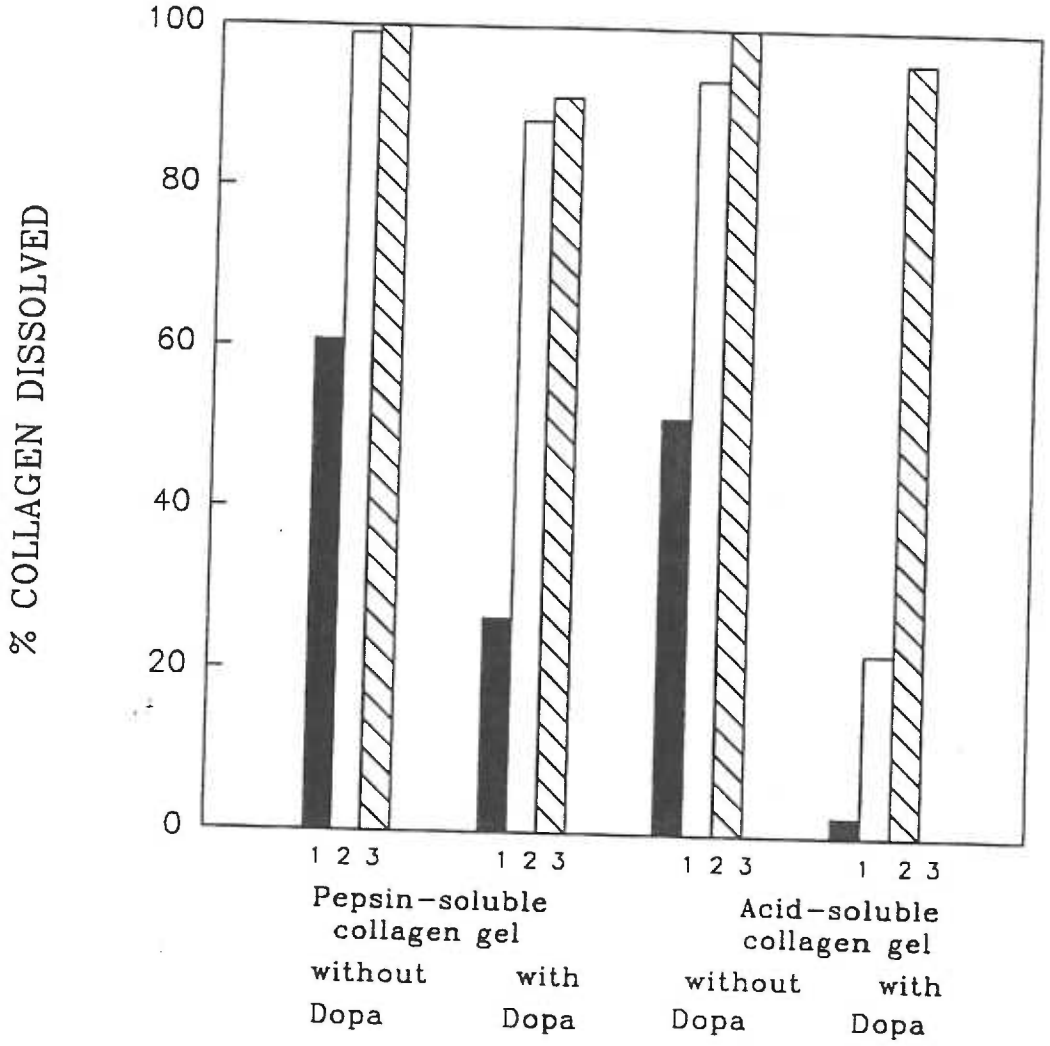


Figure 10. Solubility of collagen incubated with DOPA as determined by hydroxyproline estimation. Telopeptide-removed pepsin-digested collagen and telopeptide-intact acid-soluble collagen were incubated with and without 10^{-3}M DOPA at 37 °C for 72 h and were extracted with dilute acids. The extraction conditions are as follows:

1. Gel extracted with 0.01 M HCl at 4 °C for 2 h.
2. Gel extracted with 0.01 M HCl at 37 °C for 2 h.
3. Gel boiled in 0.01 M HCl for 2 h.

Also, DOPA treated telopeptide-intact collagen gels were dramatically less soluble than those of the pepsin-digested atelopeptide DOPA-collagen gels.

Using a typical collagen extraction protocol of dilute hydrochloric acid at 4°C, the DOPA-acid soluble collagen gel was almost completely insoluble. Thus, about 30% of the DOPA-pepsin treated collagen dissolved using this protocol, whereas about 60% and 50%, respectively, of the pepsin-digested and acid-soluble collagen controls were recovered under the same condition.

Warming at 37°C in HCl for 2 hours resolubilized most of the control collagen gels: about 90% of the acid soluble collagen and almost the whole of the pepsin-soluble collagen gel. DOPA treated collagen were however more resistant to dissolution, the acid soluble-DOPA treated collagen retained about 80% of its fibrils, only 20% of its fibrils were solubilized. Pepsin soluble-DOPA treated collagen was however less resistant to dissolution and about 85% dissolved.

Boiling in HCL for 2 hours was the most disruptive method used and it dissolved all of the control collagen. Even DOPA-treatment could not greatly stabilize the fibrils, however stabilization of about 5-10% was observed in the telopeptide-intact collagen.

Discussion

On treatment with DOPA, both acid-soluble and pepsin-treated collagen gels were more resistant to degelling. A comparison between gel solubilization

and crosslink formation has been made earlier by Bello et al. (89) who showed that acetic acid soluble collagen gels become increasingly stable with increasing periods of incubation at 37°C due to aldimine crosslink formation. Later Gade (85) showed that collagen incubated with DOPA resulted in the stabilization of collagen fibrils, he also showed that this gel could not be solubilized completely on boiling in acetic acid. Gade, however, showed that on boiling the telopeptide-intact collagen-DOPA gels in 0.01 N acetic acid only about 20% dissolved. It is proposed that boiling in 0.01 N HCl is more drastic than 0.01 N acetic acid because 0.01 N HCl may be capable of partially hydrolyzing the protein.

The difference in stability between acid-soluble collagen and pepsin soluble collagen may at least in part if not entirely be due to the participation of lysyl residues in the telopeptide region in some kind of crosslinking reaction. Digesting collagen with pepsin results in the removal of up to 75% of the telopeptides from either ends.

Conclusion

DOPA is better at crosslinking acid-soluble (telopeptide-intact) collagen than pepsin-soluble (telopeptide-removed) collagen, therefore the telopeptide region is important in the crosslinking region.

2. BOROHYDRIDE REDUCTION ANALYSIS OF COLLAGEN-DOPA ADDUCTS

In the last experiment DOPA was shown to insolubilize the telopeptide-intact collagen fibrils more than atelopeptide collagen. The next experiment is designed to determine the chemical nature of these stabilized fibrils and the residue(s) in telopeptide region that might be responsible for the difference.

It is hypothesized that DOPA oxidatively deaminates the lysyl/hydroxylysyl residues in collagen, which may further react via Aldol or Schiff base condensation. This experiment is an attempt to reduce these condensation products with a tritiated reducing agent (NaB^3H_4) and isolate the radioactive fractions.

Experimental

Collagen gels were prepared by incubating 20 mg of collagen (telopeptide-intact and telopeptide-removed) in 3.5 ml of phosphate buffered saline, with and without 0.1 mM DOPA at 37°C for 72 h. The controls were collagen solutions incubated in the absence of DOPA, under similar conditions. The DOPA-collagen and control collagen gels were homogenized using a Virtis 45 homogenizer. The samples were then reduced with tritiated sodium borohydride, dialyzed and acid hydrolyzed. The hydrolyzates were eluted by column chromatography as described in Methods section.

Results

Elution profiles of acid hydrolyzates of borohydride reduced atelopeptide control and DOPA-treated collagen and those of telopeptide intact control and DOPA-treated collagen are shown in Figure 11 and 12, respectively. The positions of the amino acids in relation to the radioactive peaks as indicated by ninhydrin, are shown in each of the profiles. As in other studies (86,90-94), the peak identified as DHNL falls between aspartic acid and serine, the peak for HNL falls between proline and glycine, and the peak for HLNL lies between hydroxylysine and histidine. The peak between phenylalanine and hydroxylysine is approximately at the position of DHLNL but is farther from HLNL than other published results (86,93,94). As in other studies (86,95,96), DHNL elutes as a double peak.

As seen in Figure 11, the pepsin digested collagen control (A) contains a negligible amount of DHNL as compared to the DOPA-treated collagen gel (B). The addition of the oxidant, DOPA, has resulted in a 3.5X increase in the radioactive peak corresponding to DHNL, the reduced form of hydroxyallysine. The other reduced aldehydic precursor, HNL (from allysine) is seen to emerge as a separate peak slower than DHNL, eluting at the correct position relative to the amino acids.

Figure 12 gives the elution profile of radioactively labelled reducible components obtained from the acid hydrolyzates of reduced acid-soluble collagen control gel (A) and the acid-soluble collagen gel incubated with DOPA (B). Once

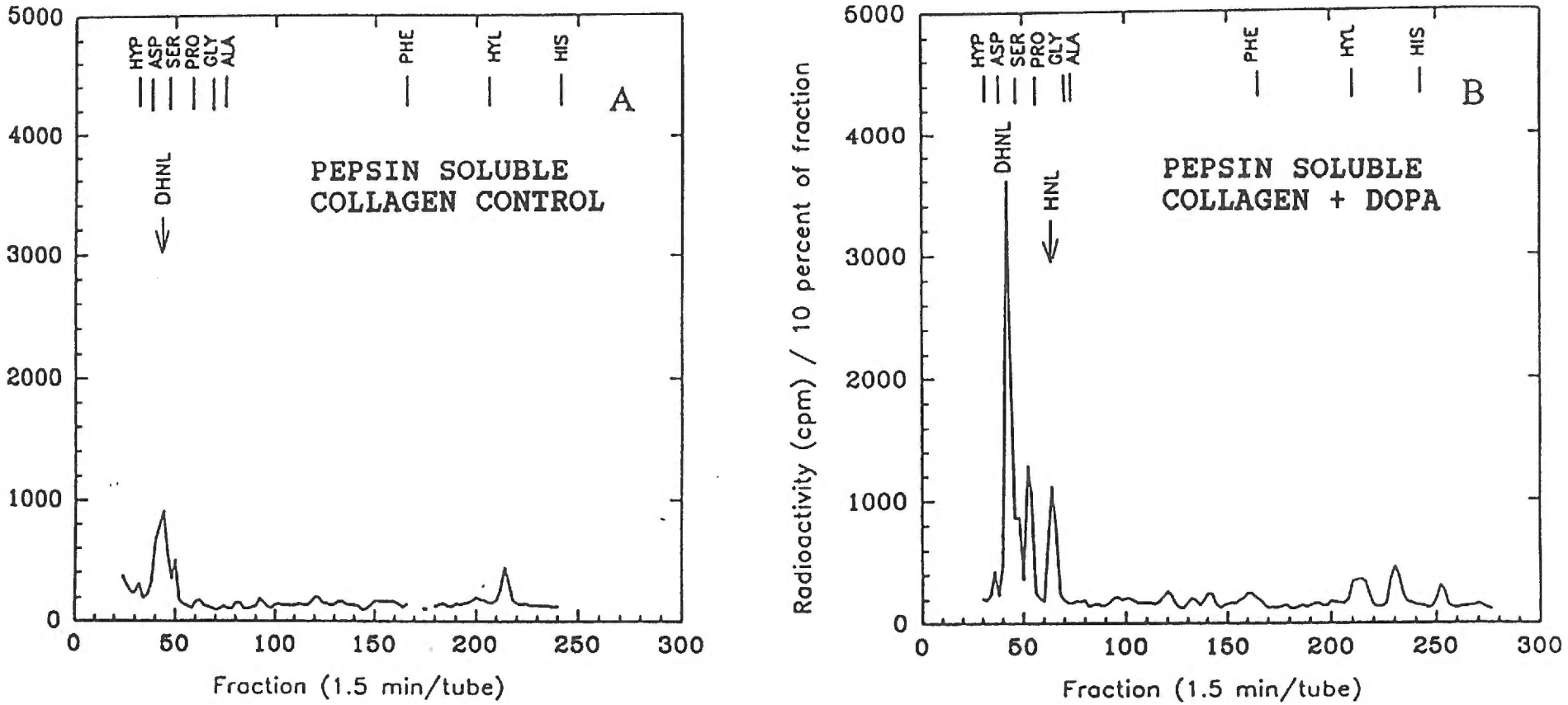
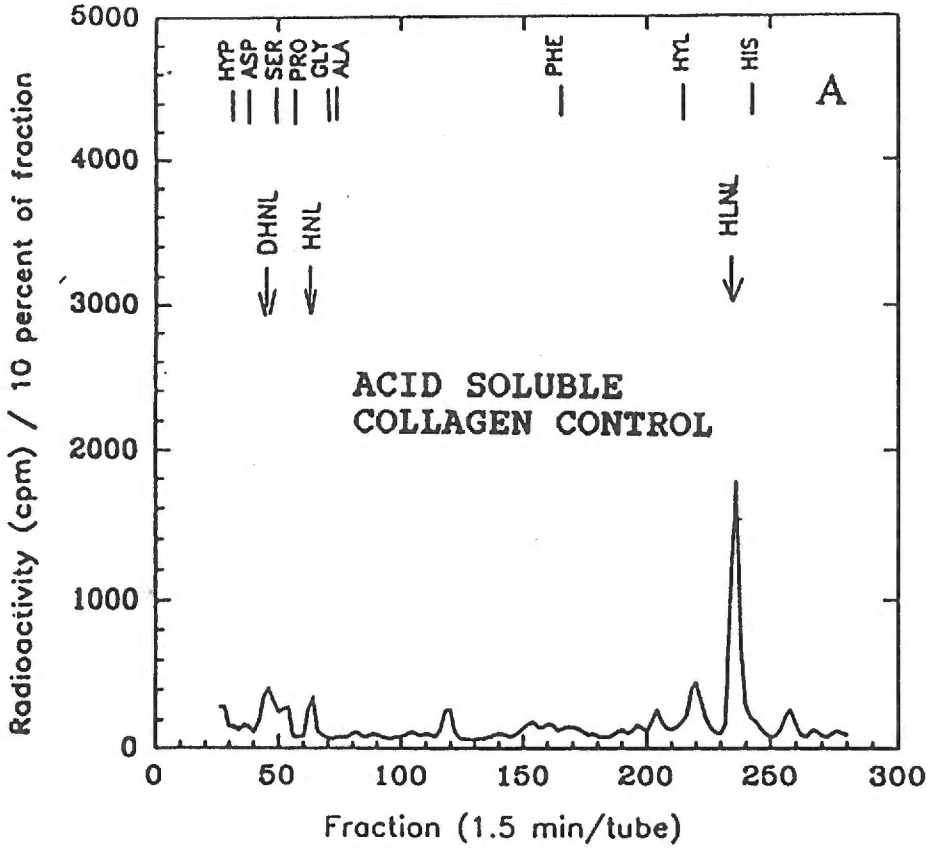


FIG 11 Elution profile of radioactively labelled reducible components obtained from the acid hydrolyzates of reduced pepsin-digested collagen control gel (A) and pepsin-digested collagen gel incubated with DOPA (B). The reduction was carried out with tritiated sodium borohydride.



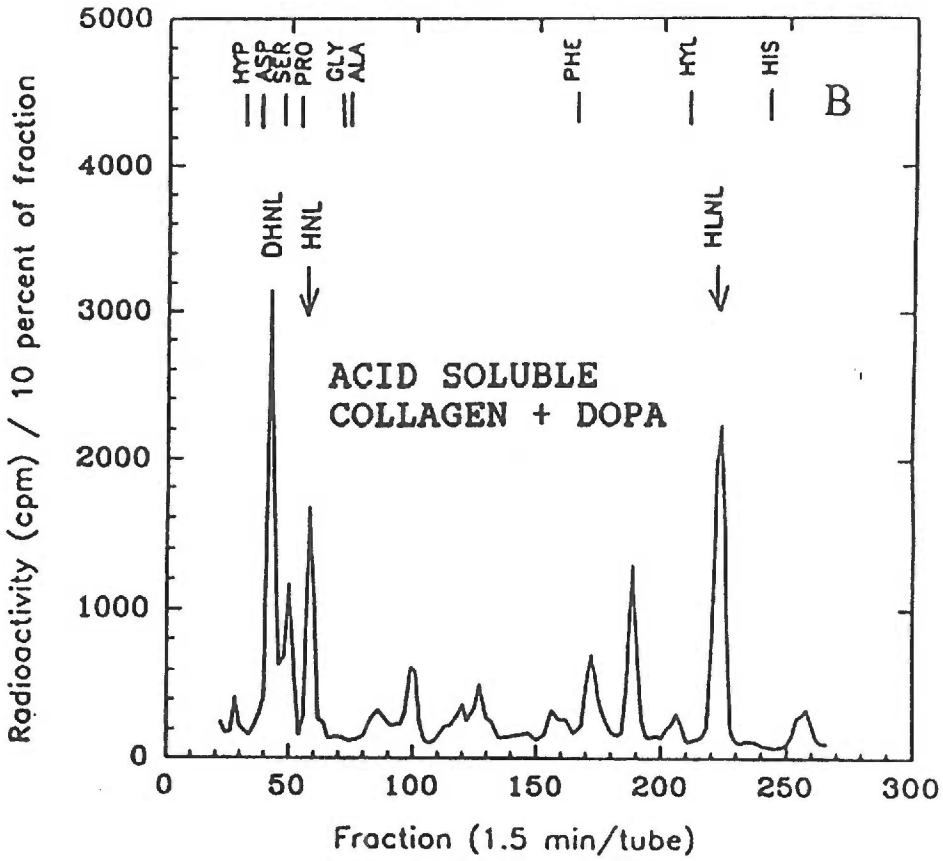


FIG 12 Elution profile of radioactively labelled reducible components obtained from the acid hydrolyzates of reduced acid-soluble collagen control gel (A) and acid-soluble collagen gel incubated with DOPA (B). The reduction was carried out with tritiated sodium borohydride.

again, it was observed that addition of DOPA, dramatically increased the amount of crosslink precursors DHNL and HNL in the elution profile. A single radioactive peak obtained around fraction 240, eluting between hydroxylysine and histidine, in case of the acid-soluble control may correspond to one of the crosslinks, probably HLNL. It is not unusual to observe this peak in acid-soluble collagen, because collagen prior to extraction from tissues has naturally occurring crosslinks. These transient, reducible crosslinks are broken on acid extraction, generating the aldehydic precursors, which when warmed to 37°C in phosphate buffered saline will reform. Four specific residues (Lys 9N, Lys 16C, Hyl 87, Hyl 930) have been identified to be involved in such natural Schiff base crosslinks (97-99).

Discussion

Elution profiles of acid-soluble collagen and pepsin-treated collagen incubated with DOPA imply that DOPA is capable of oxidatively deaminating peptidyl lysines in collagen. In relation to the control sample of acid-soluble collagen gel which was not treated with DOPA, an increase in the amount of radioactivity in the peak corresponding to the crosslink HLNL was observed in the case of DOPA-treated acid-soluble collagen gel. This may be due to the participation of some additional residues of the telopeptide. This could be the reason why the acid-soluble DOPA collagen gel is less soluble than those formed

with pepsin-digested DOPA-collagen gel.

The peak eluting between phenylalanine and hydroxylysine in Figure 12B approximates the position of DHLNL but is farther from HLNL than in other published results (86,93,94). The nature of the species eluting at this position is uncertain. It is again emphasized that the mesh size of the resin used in these experiments are slightly larger than those employed by the other authors (86,93,94) which may account for a difference in relative elution positions.

The appearance of a major peak corresponding to the elution position of DHNL (Figure 11B and 12B) is puzzling since there are considerably more lysyl than hydroxylysyl residues in collagen. It is possible that the hydroxylysyl residues in the triple helical region are more accessible to DOPA than the lysyl residues.

Conclusion

Chromatographic elution profile of borohydride reduced DOPA- collagen gels showed the presence of peaks which are believed to be due to oxidative deamination products of lysines and hydroxylysines.

3. BOROHYDRIDE REDUCTION ANALYSIS OF N-α-ACETYL-LYSINE: DOPA SAMPLES.

Introduction

The previous experiments showed that DOPA is able to crosslink collagen probably via an oxidative deamination mechanism generating reactive aldehydes that further condense to form the Schiff base products. This same reaction is also known to be catalyzed by the enzyme lysyl oxidase, although only at very specific lysines/hydroxylysines. It is known that lysyl oxidase has a tendency to copurify with extracted collagen and elastin. It has been shown that this insoluble form of the enzyme is catalytically functional (35). The insoluble enzyme could be inactivated by heat or by acid. The protocol employed for the acid extraction of collagen (appendix) should have effectively inactivated the enzyme. It is, however, not known whether the presence of ortho-quinones or an alkaline media could reactivate the insoluble enzyme. By using a substrate other than collagen or elastin, like N-α-acetyl-L-lysine, it should be possible to eliminate the participation of lysyl oxidase. It would also shed light on whether DOPA is capable of oxidatively deaminating all lysines or whether a specific spatial configuration is essential.

The choice of N-α-acetyl-L-lysine is important because it has a protected α-amino group, thus any reaction with amine would have to occur at the ε-amino group. Also, the α-amino group protection would prevent the undesirable side

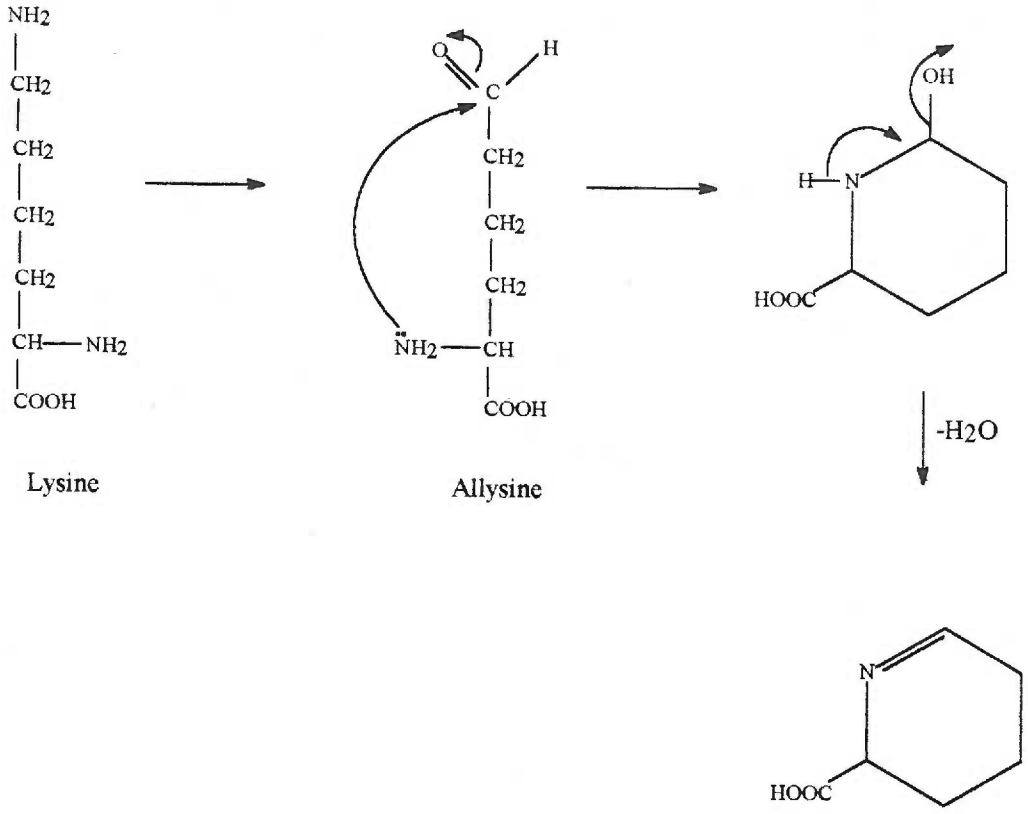


Figure 13: An nucleophilic attack of the unprotected nitrogen results in an internal cyclization, which is accompanied by dehydration to give an unsaturated cyclic product.

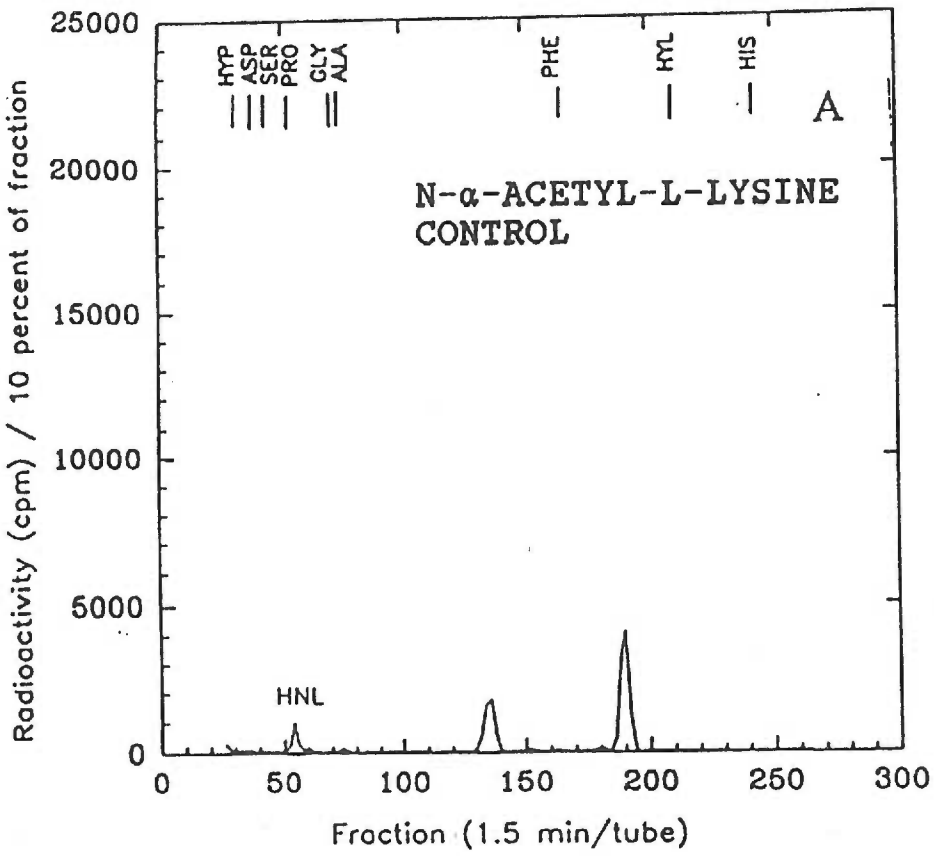
reaction (Figure 13) of internal cyclization. Allysine which is not α-N protected could form a very stable six-membered ring closure product as a result of the nucleophilic attack of the allysine amide nitrogen upon the aldehyde group (99), which will not be opened by any nucleophile.

Experimental:

N-α-acetyl-L-lysine at a final concentration of 6.3 mg/ml was incubated with 1 mM DOPA in PBS at 37°C for 72h. The control was N-α-acetyl-L-lysine at the same concentration without DOPA incubated under similar conditions. The solutions were reduced with tritiated sodium borohydride (details in Methods section) and acidified to pH~1 with conc. HCl to evolve any unreacted sodium borotritide as tritium gas. The solution was then lyophilized, hydrolyzed in pTSA and eluted on a cation exchanger. Column chromatography was performed by the procedure in the Methods section.

Results:

Figure 14 shows the elution profile of radioactively labelled components obtained from acid hydrolysates of borohydride reduced N-α-acetyl-L-lysine (A) and N-α-acetyl-L-lysine incubated with DOPA (B). The N-α-acetyl-L-lysine incubated with DOPA shows a strong radioactive peak at the correct position



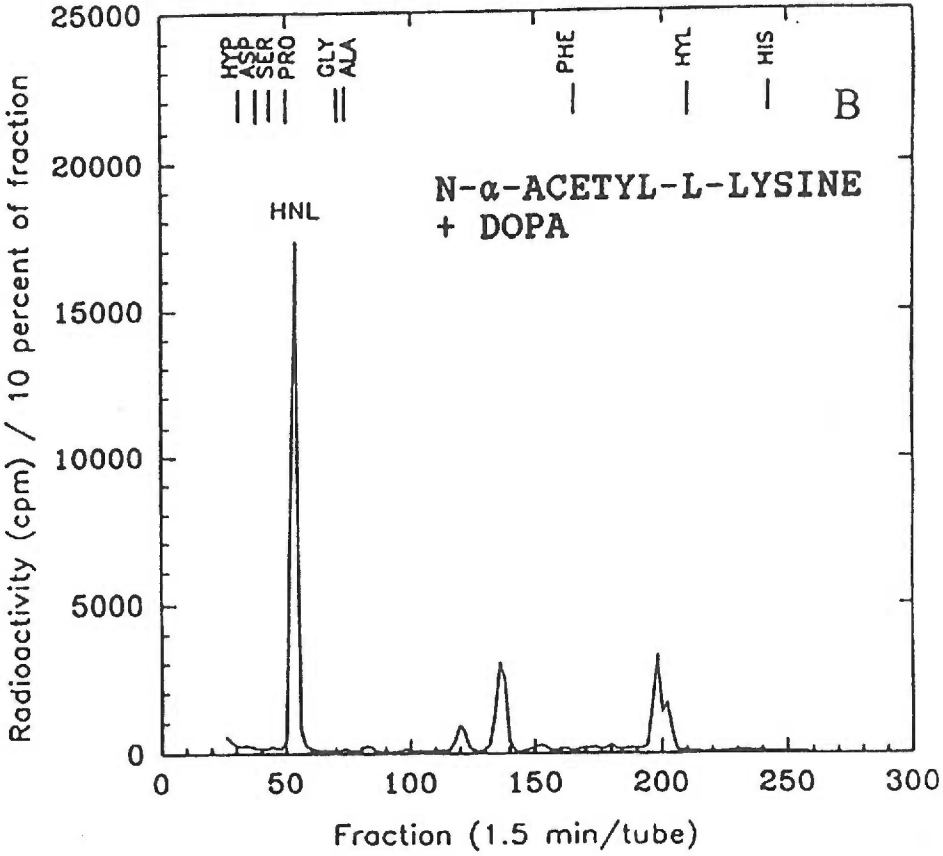


FIG 14 Elution profile of radioactively labelled components obtained from the reduced N-α-acetyl-L-lysine solution (A) and N-α-acetyl-L-lysine incubated with DOPA (B). The reduction was carried out with tritiated sodium borohydride.

relative to amino acid markers corresponding to hydroxynorleucine, HNL (the reduced form of allysine) which is negligible in the control sample. A radioactive peak at fraction 190 is also seen in Fig 12 B. The nature of the peak is not certain. But this peak elutes at the approximate position of DHLNL (see previous section). Because DHLNL can be generated only from hydroxylysine, the presence of this peak in the N-acetyllysine and DOPA sample is good evidence that this peak seen in Figures 12B, 14A and 14 B is not DHLNL. The other radioactive peaks at fraction 135 is seen in Figure 14B but since they also appear in the control (Figure 14 A) their occurrence is unrelated to the action of DOPA. There appears to be no peak in the region where the crosslinks elute.

Discussion:

Detection of a borohydride reduced peak at the region where hydroxynorleucine elutes with respect to other amino acids in Figure 14, in the presence of DOPA strongly supports the hypothesis of oxidative deamination. But failure to detect dimeric crosslinks in the same profile is unexpected.

A possible explanation for the absence of peaks corresponding to the dimers, could be that the Schiff base that is formed is highly unstable. The Schiff base may either dissociate into different fractions or may be converted to more complex crosslinks via addition of more nucleophiles. The former is a more likely explanation, in view of the fact that no measurable (above the baseline)

radioactivity was detected in the region where the Schiff bases elute, but had they been converted to more complex structures, there would have been some critical level of the dimer present. Assuming that the latter was happening then the Schiff base that is formed should have some finite lifespan in solution before it is converted to the more complex crosslinks. Thus, at any instance, it should be possible to reduce a few unreacted Schiff's condensation product and isolate a radioactive peak.

However, if the Schiff's condensation product was unstable, it's lifetime in solution would be very short and negligible, so that upon treatment with borohydride no reduced dimer would be formed and isolated.

The above observation is consistent with the proposal based on the mass spectral analysis that the dimer is highly unstable, and that an internal cyclisation results in the simultaneous elimination of a N-α-acetyl lysine molecule (see Part II)

Conclusion

DOPA appears to oxidatively deaminate the ϵ-amino group of lysine converting it to allysine. Evidence for this is the emergence of a radioactive peak which earlier work has shown to be due to reduced allysine (HNL). Structural identification of this peak is necessary for full confirmation.

4. IDENTIFICATION OF THE PEAK ELUTING BETWEEN HYDROXYLYSINE AND HISTIDINE: IS IT REALLY THE REDUCED ALLYSINE?

The next experiment was designed to achieve two goals. Objective 1 was to conclusively identify the chemical nature of the peak labelled as 'HNL' in Figure 14B. Objective 2 was to design a simple assay which would measure the degree of oxidation occurring.

Thus far, it has been shown that DOPA stabilizes collagen fibrils and probably forms crosslinks. But a more intriguing point is that DOPA appears capable of oxidizing both free and bound lysines.

Identifying the peak of interest as HNL is based on two facts: one, it is radioactive; and two, it elutes at the same region relative to marker amino acids earlier identified by other researchers.

The following experiments were proposed to be done with the radioactively tagged "HNL" from Figure 14B. The first step would be to purify this compound of interest away from the large amount of citrate salts. Once the compound is removed from the citrate salts and dissolved in volatile solvents, it could be lyophilized and subjected to 'Fast atomic bombardment'. This would prove the structure conclusively. FAB is especially advantageous when very little substrate is present and no GC-MS can be performed.

A second objective was to separate the 'HNL' on a HPLC system. In the present case, we have used a refractometer as a detector for the identification of

the 'HNL'. The advantage of using a refractometer is that no pre- or post-column derivatization is necessary. A water-TFA buffer system has been used as eluant. It was postulated that the 'HNL' which should have a finite refractive index would appear as a distinct signal on the chromatograph at a specific retention time. The fraction under this signal could be tested for radioactivity, since the 'HNL' is labeled. Once, it is proved that the radioactive peak coincides with a distinct refraction peak on a chromatograph, then any future experiments could be performed with cold sodium borohydride and this would then be a more cost effective and faster assay to measure the extent of the oxidative reaction.

Experimental:

1. The contents of fraction tube numbers 52-58 (Figure 14B) that contains the 'HNL' were pooled together, and lyophilized.
2. The residue was redissolved in water (0.5 ml) and split into two aliquots of 0.25 ml each.
3. One fraction was eluted from a short Dowex (cation exchanger) desalting column (see methods section for details) and fractions were collected. The tubes containing radioactivity were pooled together. The first five tubes were measured for citrate buffer salts by a conductivity monitor. The tube containing the major amount of radioactivity was lyophilized and FAB was attempted on it.
4. The second 0.25 ml fraction of the tube from step 2 was lyophilized and redissolved in 0.25 ml of water-TFA (0.1%) buffer and was loaded on an HPLC

C-18 column. Fractions were collected and monitored for radioactivity and absorbance on the refractrometric scale.

Results: ***Part I - Desalting column:***

The initial calibration studies showed that citrate salts can be separated from aminoacids by using a desalting column. The citrate salts were found in the first 3 tubes only ~ retention times 1-3 min. (as measured by conductivity), the eluant being distilled water. Threonine, as a representative amino-acid, eluted between 10-17 minutes; i.e. 3 minutes after the eluant was changed to 10% HCl.

The radioactive sample of interest 'HNL' also seemed to effectively separate from the citrate salts. The conductivity of the first three tubes were very high as compared to the rest, indicating that they contained the citrate salts. The radioactivity was isolated as a sharp peak in fractions 18-20. These fractions were pooled and subjected to Fast Atomic Bombardment (FAB). No signal was obtained that could explain the nature of the 'peak'. However, FAB showed strong signals that pertained to concatomers of citric acid. Thus the sample still had citrate salts which would mask any signals from the radioactively labeled 'HNL' if present.

Conclusion

The reduced compounds are present in very small amounts, therefore several purification steps were necessary to estimate these products. Such

extensive purification seriously limit the amount of yield.

Results: ***Part II - HPLC run***

Calibration studies were carried out with lysine and N-acetyl-lysine by injecting increasing concentrations into the HPLC system. Using this protocol, lysine was seen to elute at 6.5 min and N-*α*-acetyl-L-lysine eluted at retention time of 13 min.

The sample containing the 'HNL' eluted between 7-11 minutes, detected as being radioactive. There was a sharp radioactive peak at 8 minutes, but there was no corresponding signal on the refractive index scale, even though the sensitivity settings used were very high (see methods section).

Conclusion:

It is concluded that the concentration of Hydroxynorleucine is too low to be observed using a refractometer detector. An option would be using larger batches of starting material and concentrating the tubes that may contain Hydroxynorleucine, but this will greatly concentrate the buffer salts and any other impurities, making the identification of a single peak corresponding of HNL extremely unlikely.

All attempts at deducing the structure of the radioactive fraction with FAB technique failed. Our attempts to devise a simple assay for HNL also was unsuccessful.

PART II

1. MASS SPECTRAL STRUCTURE DETECTION OF THE ALLYSINE AND ITS DERIVATIVES

Introduction

In this experiment the chemical nature of 'HNL' is confirmed. The earlier attempt at purification and identification of the radioactively labelled fraction as 'HNL' failed due to the presence of insufficient amounts of substrate. In this section, an alternative method of concentrating the substrate is discussed.

Oxidative deamination products of N-α-acetyl lysine by DOPA should be extracted out of the bulk of the unreacted material and into ethyl acetate, after acidification to pH~1-2. Only uncharged molecules are extracted into ethyl acetate. N-α-acetyl lysine has an unprotected ϵ-amino group, which at pH~1 would carry a positive charge. Hydroxynorleucine, on the other hand, would bear no charge at pH~1. The reduced Schiff base, at an acid pH, may form a quarternary ammonium salt and thus may acquire a positive charge. It would thus not be extracted into ethyl acetate. Because the reduced or non-reduced N-α-acetyl allysine would be extracted into ethyl acetate, it should be possible to detect it by GC-MS.

A recent publication by Dooley (19) indicates that the mechanistic role of copper in amine oxidase is to perform a one-electron transfer from the enzyme bound, reduced quinone to oxygen which may be important in the regeneration

of the reoxidized catalyst (Figure 2, Introduction). It could be argued that the role of copper with free quinones is similar. Copper with ortho-quinone in the absence of lysyl oxidase would propagate oxidative deamination, and the reduced quinone could be regenerated by the copper, thus minimizing any wastage of the catalyst. In the following experiment DOPA is incubated with N-*α*-acetyl-lysine in the presence of copper, because a maximum yield of the oxidation products was desired.

Experimental:

A 7 ml volume of N-*α*-acetyl-L-lysine at a concentration of 25 mg/ml in PBS was incubated at 37°C for 72h, in the presence of 1 mM DOPA with and without 0.1 mM copper(II)chloride. The two controls were N-*α*-acetyl-L-lysine at 25 mg/ml in PBS and 1mM DOPA in PBS; incubated at 37°C for 72h. The samples were then reduced with sodium borohydride. The samples were acidified to pH ~1 and extracted with ethyl acetate and evaporated to dryness as specified in the Methods section.

Trimethylsilyl derivatisation:

The dried ethyl acetate extracts were treated with 0.074 ml of BSTFA + 1% TMCS (N,O-bis-[Trimethylsilyl]trifluoroacetamide + 1% Trimethylchlorosilane) (Pierce) at 85°C for 20 min. to produce trimethylsilyl derivatives. GC-MS was performed as

explained in the Methods section.

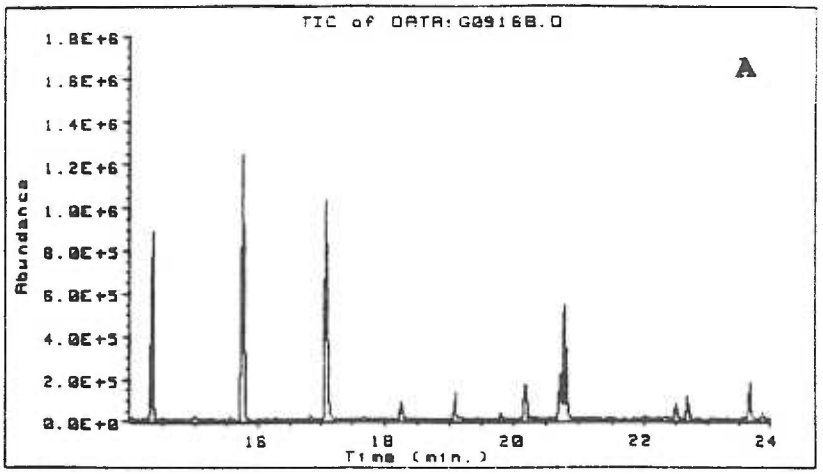
The GC chromatographic elution of the trimethylsilylated derivative of the ethyl acetate soluble fractions of N-*α*-acetyl-L-lysine-DOPA samples along with the relevant controls were compared. Samples were labelled as follows:

SAMPLE No.	Concentration of		
	N-*α*-acetyllysine (mg/ml)	DOPA (mM)	Copper (mM)
A (control)	0	1	0
B (control)	25	0	0
C	25	1	0
D	25	1	0.1

Results:

Comparing the chromatograms (Figures 15 A,B,C and D) it can be seen that only in sample D and in trace quantities in sample C (see below), was there a peak at retention time 14.417 min. This is not present in any of the controls (A and B). The rest of the chromatographic peaks are common to samples B-D.

The mass spectral fragmentation pattern of the species eluting at retention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1.4E+6
1.2E+6
1.0E+6
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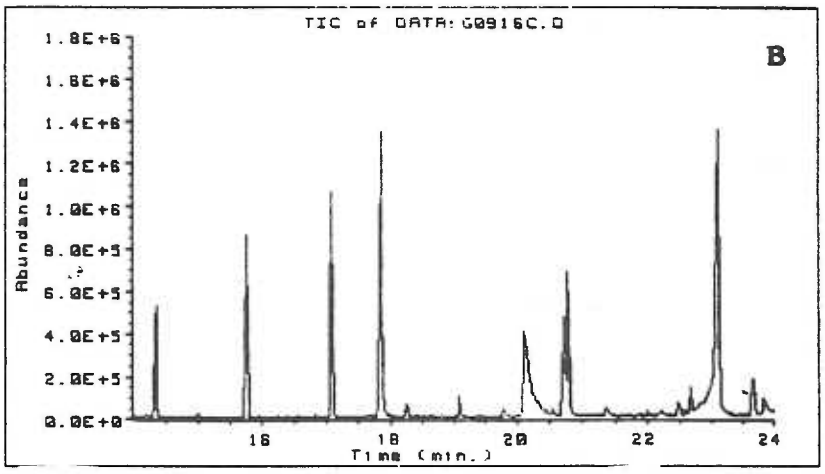
6.0E+5
4.0E+5
2.0E+5
0.0E+0
16
18
20
22
24
Time (min.)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1.2E+6
1.0E+6
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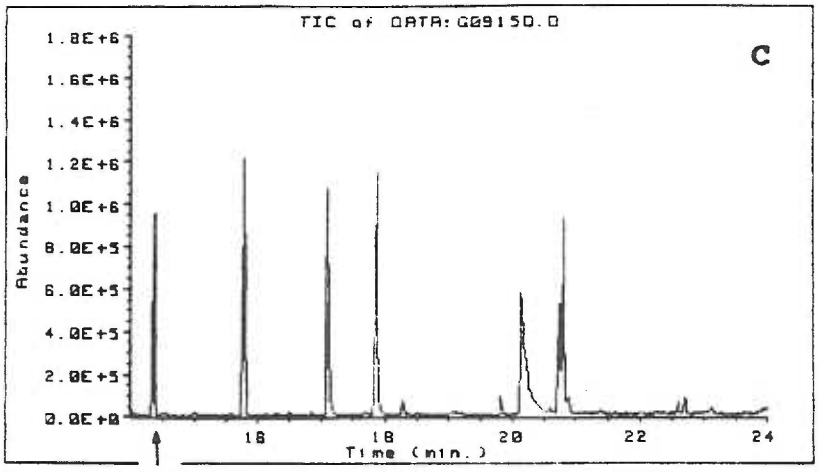
4.0E+5
2.0E+5
0.0E+0
16
18
20
22
24
Time (min.)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1.2E+6
1.0E+6
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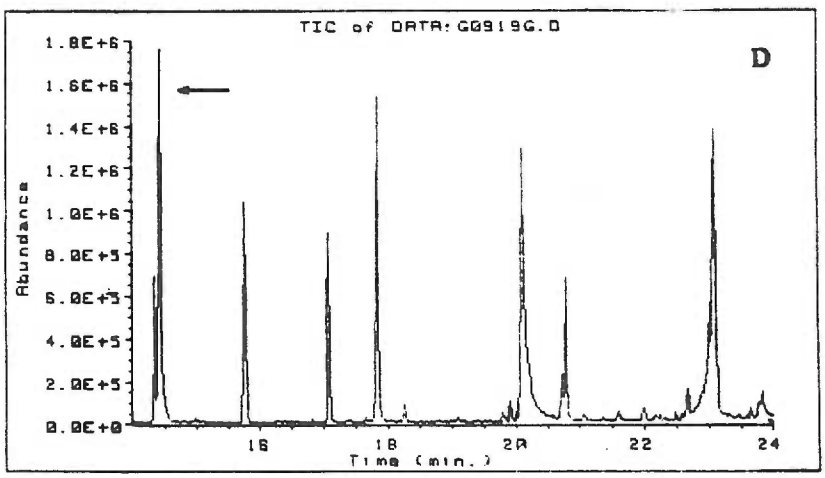
4.0E+5
2.0E+5
0.0E+0
16
18
20
22
24
Time (min.)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16
18
20
22
24
Time (min.)

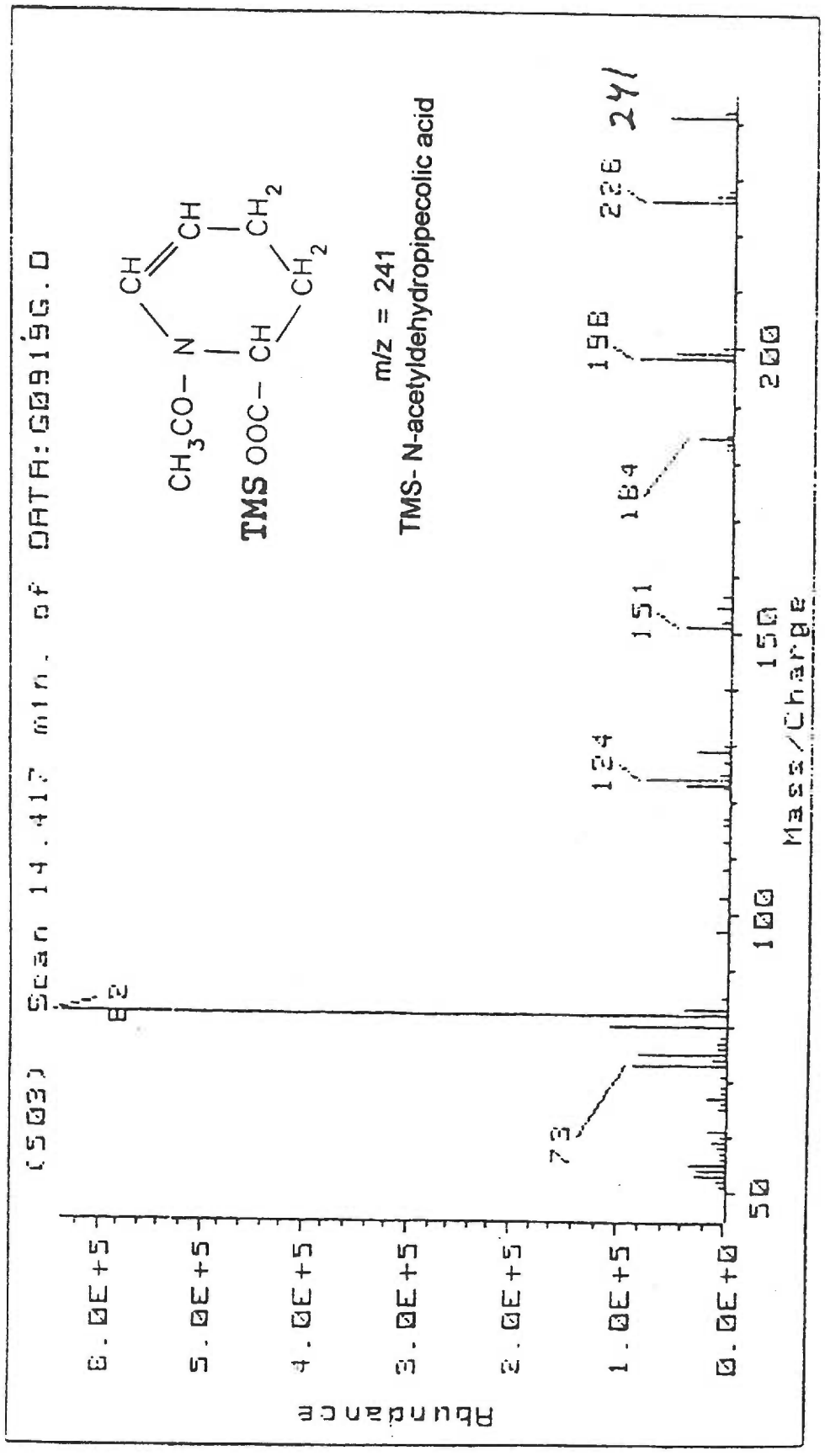


Figure 16 (i) : Mass spectral fragmentation pattern for the cyclic compound proposed to be trimethylsilylated N-acetyl dehydropipecolic acid.

time 14.417 min. is shown in Figure 16(i). The spectrum shows a proposed molecular ion at m/z 241, thus indicating a molecular weight of 241. Also seen is the (M-15) fragment at m/z 226, corresponding to the well known loss of a methyl group from trimethylsilyl derivatives. The majority of the prominent fragments can be directly related to the proposed structure by the following cleavages (Figure 16 ii)

Value of m/z	(M-x) signal	comments
241	(M) signal	molecular ion
226	(M-15)	loss of TMS methyl group
198	(M-43)	loss of (-CO-CH_3)
184	(M-57)	loss of (N-CO-CH_3)
151	(M-90)	loss of (TMS-OH)
124	(M-117)	loss of (COOTMS)
82	(M-159)	loss of side chains from the ring

The signal at 124 is due to the (M-117) fragment; a characteristic fragment for all α-amino acids. The very strong signal at m/z 82 is due to the dehydropyridine structure obtained after ionization of the two side chains: CO-CH_3 and COOTMS. The signal at 73 is typical of a trimethylsilyl group.

The same major fragments were also detected at the same retention time

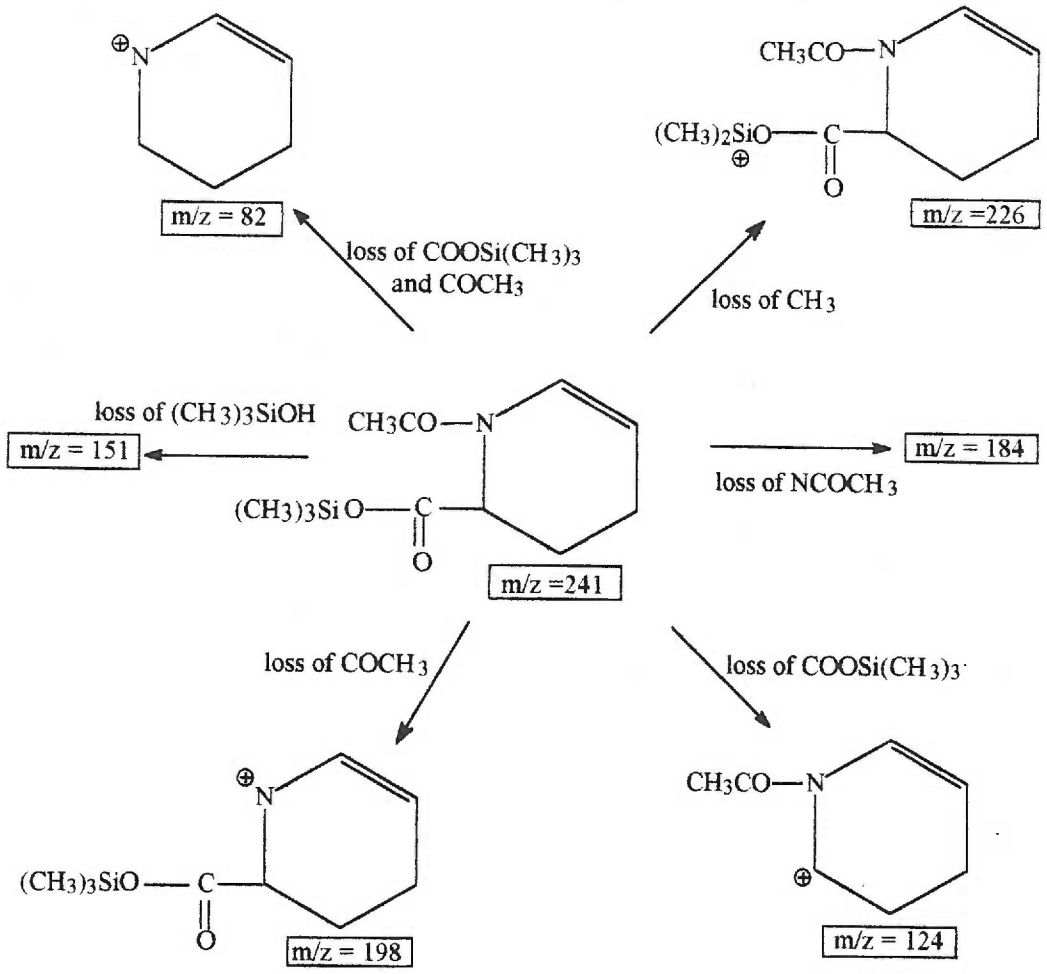


Figure 16 (ii) : Electron impact ionization of trimethylsilylated N-acetyl dehydropipecolic acid would result in the generation of the above proposed positively charged ions based on available fragmentation data for trimethylsilyl derivatives.

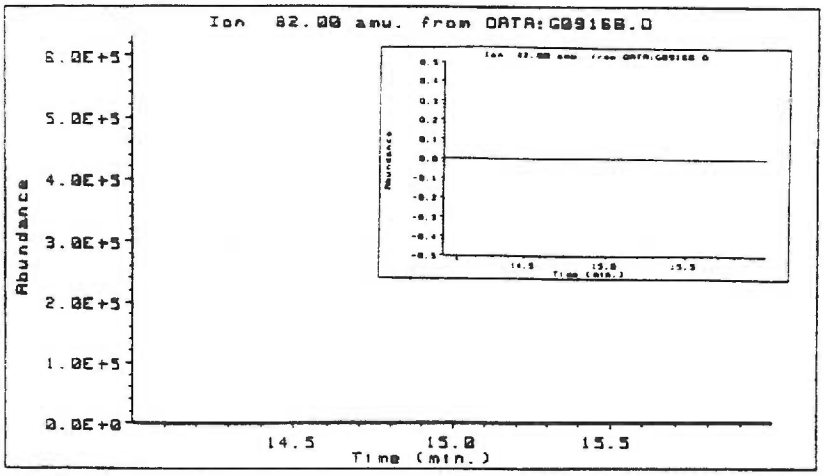
in sample C, consisting of N-α-acetyl-L-lysine with DOPA in the absence of copper, but this time in trace amounts. Figure 17 shows the extracted ion chromatograms for ion 82 of the cyclic compound in the reduced derivatized samples A-D, and as can be seen in sample C traces of 82 ion is present.

It was also confirmed that the peak at retention time 14.417 min was obtained in the samples C and D, which had not been reduced with sodium borohydride. This is consistent with the fact that N-acetyldehydropipecolic acid is not reducible with sodium borohydride. Sodium borohydride does not reduce carbon-carbon double bonds (101).

Additional evidence that the spectrum is concordant with the proposed structure is obtained using a known standard of N-acetylated pipecolic acid. Pipecolic acid was acetylated as explained in the materials section and derivatized with BSTFA + 1%TMCS. Its mass spectrum (Figure 18) yielded an identical fragmentation pattern except that all the signals corresponded to two mass units more because the proposed ring structure is saturated. This was consistent with the proposal of an N-acetylated dehydro-pipecolic acid as the chemical structure for the unknown common to samples C and D.

Discussion:

It was essential to demonstrate the structure of the proposed aldehyde or any of its derivatives, in order to convincingly prove that ortho-quinones causes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4.0E+5
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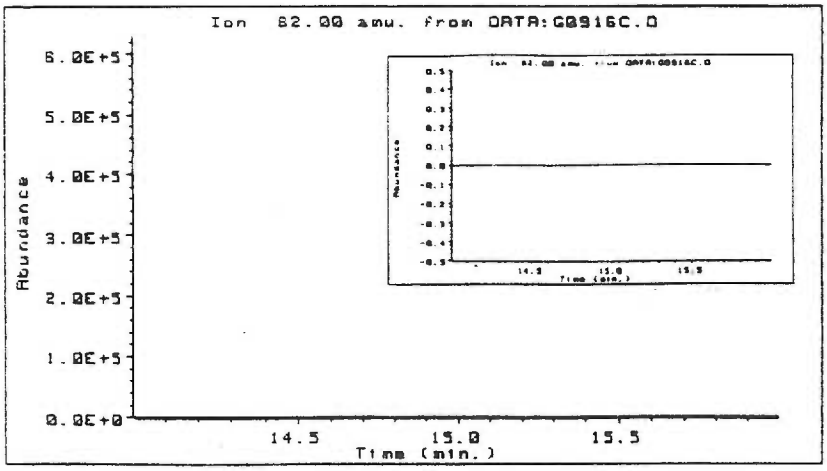
1.0E+5
0.0E+0
14.5
15.0
15.5
Time (min.)

A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Abundance
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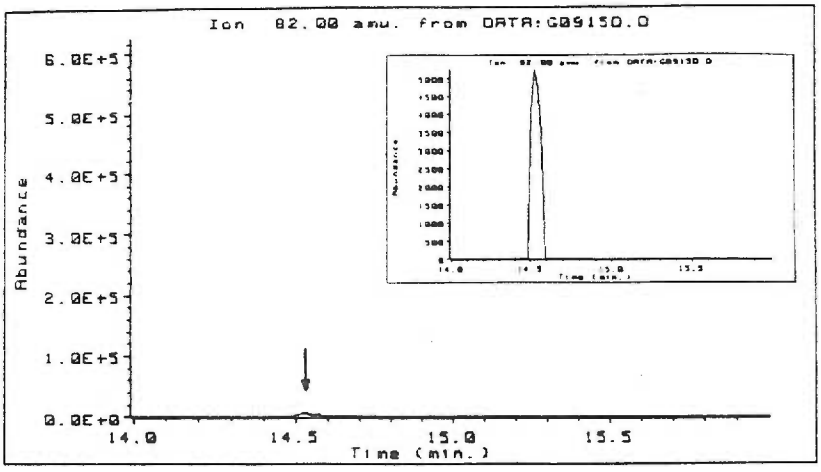
15.0
15.5
Time (min.)

B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Abundance
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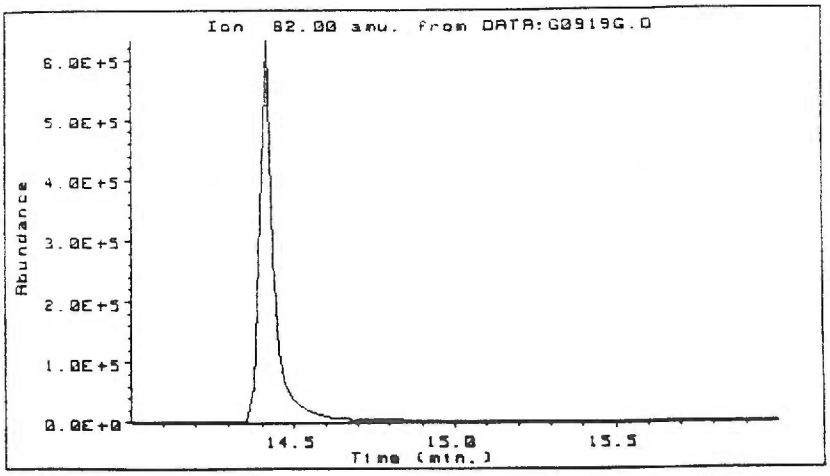
2.0E+5
1.0E+5
0.0E+0
14.0
14.5
15.0
15.5
Time (min.)

C


Ion 82.00 amu. from DATA:G0919G.D
Abundance
6.0E+5
5.0E+5
4.0E+5
3.0E+5
2.0E+5
1.0E+5
0.0E+0
14.5
15.0
15.5
Time (min.)

D

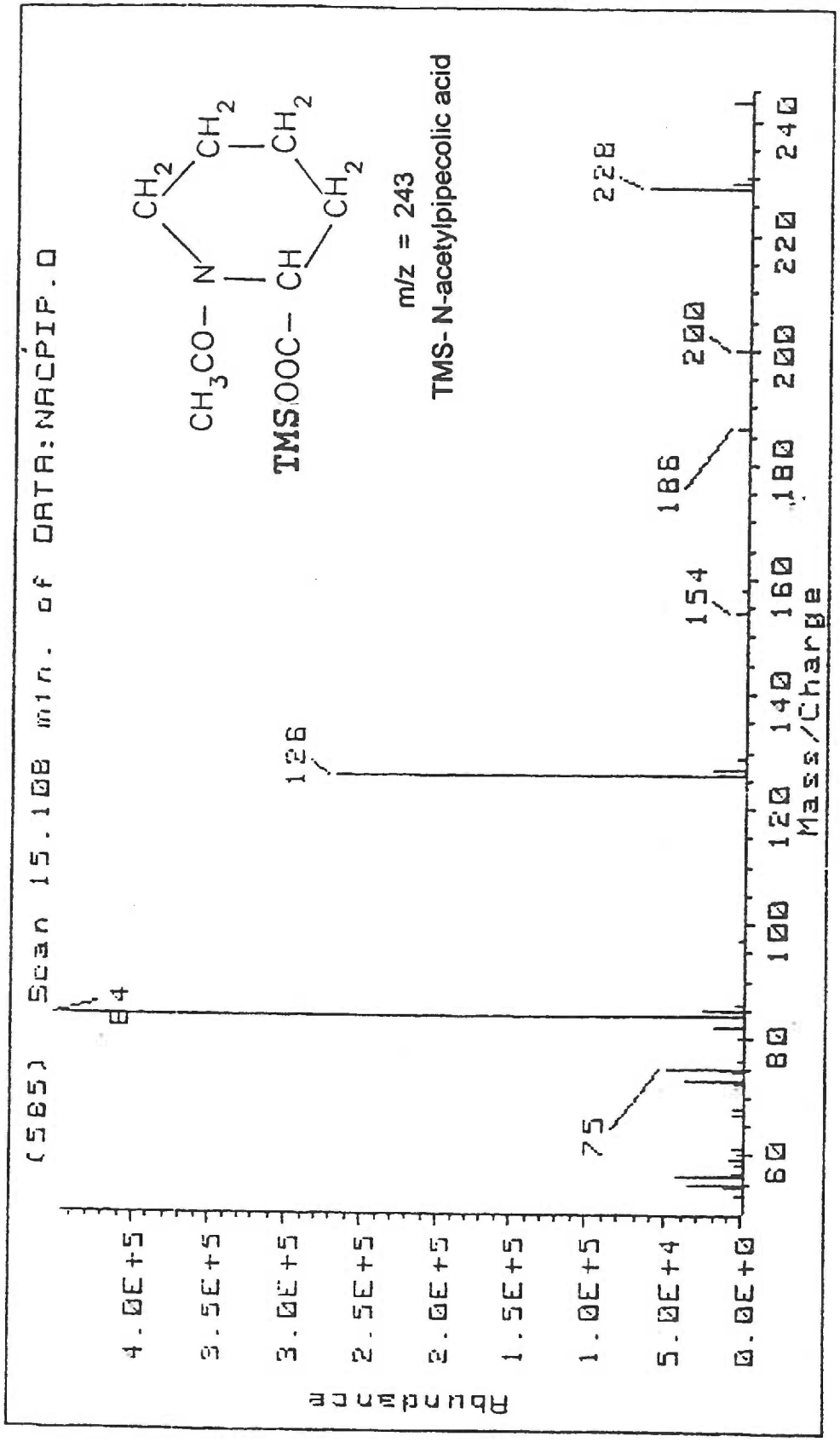


Figure 18 : Mass spectral fragmentation pattern for trimethylsilylated N-acetyl pipecolic acid

oxidative deamination of lysines. This would then be a far more confirmatory proof of the proposed reaction than any HPLC profile of radioactive fractions, based on techniques standardized years ago. However, detection of relatively smaller amount of aldehyde present with a huge bulk of N-***α***-acetyl-L-lysine is not an easy task. It would be essential to concentrate the aldehydic portions before attempting any detection. We theorized that by employing the means of ethyl acetate extraction at an acid pH, we would be able to effectively separate the hydroxynorleucine and other unknown ethyl acetate soluble species from the bulk of the unreacted N-acetyllysine. GC-MS studies were done on the ethyl acetate soluble fractions.

Mass spectral studies of the ethyl acetate soluble fraction is consistent with the proposed structure shown in Figure 16(i). We propose that N-***α***-acetyl-L-lysine is oxidatively deaminated to N-***α***-acetylallysine. The aldehyde group in N-***α***-acetylallysine is highly reactive and would immediately condense with a neighboring N-***α***-acetyl lysine to yield a Schiff base. However, the stability of this dimer largely depends on the nonavailability of any lone pair of electrons on allysine nitrogen. The acyl protection is thus not enough to hinder internal cyclization once the Schiff base is formed. The proposed scheme is shown in Figure 19. This explains why no crosslinks were detected in the elution profiles of N-***α***-acetyl-L-lysine. Such an internal cyclization resulting in the destabilization of the Schiff base crosslink is presumably impossible in collagen because of the secondary structure of the protein. Molecular modelling studies by Veis et al.

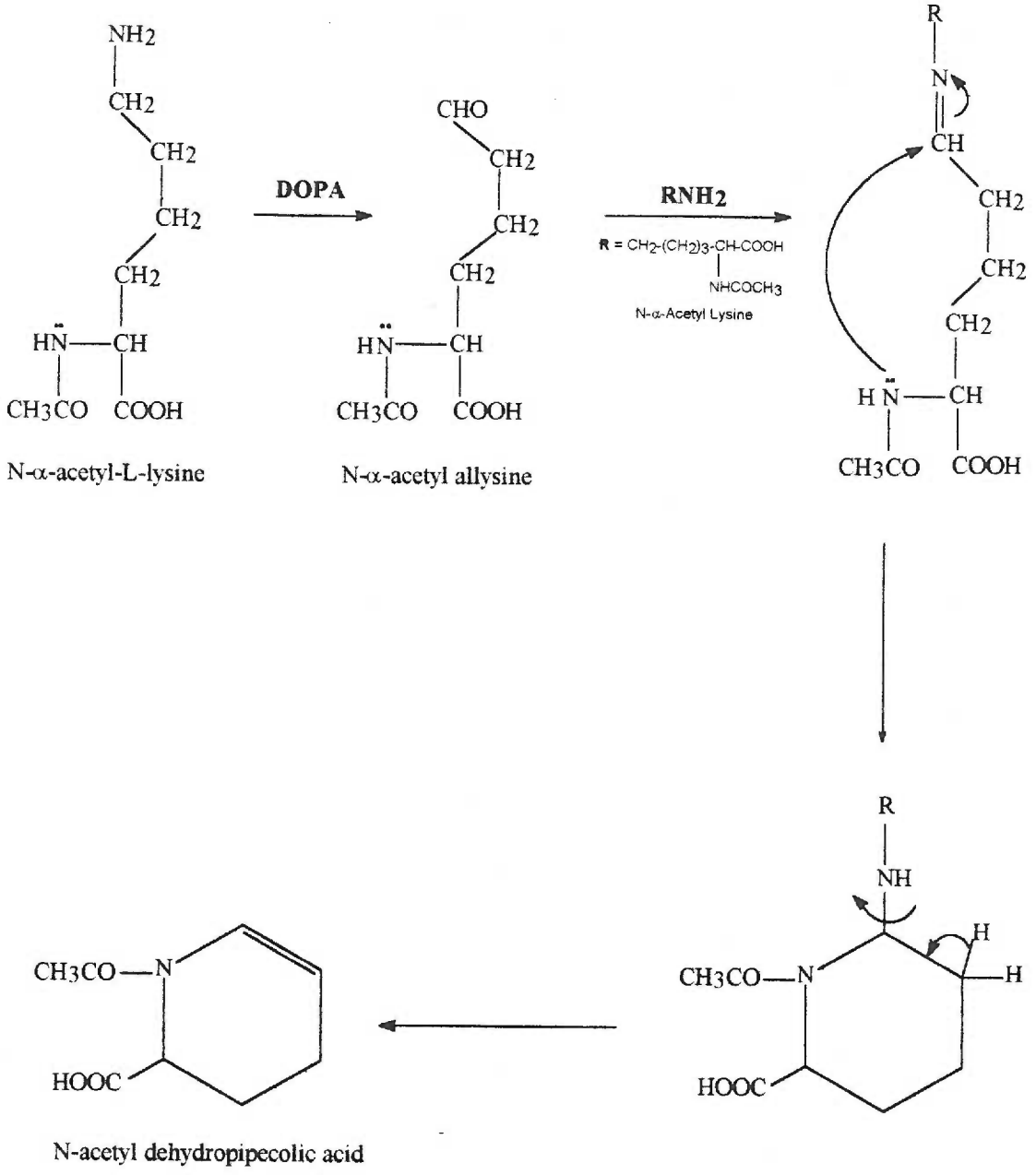


Figure 19 : Proposed scheme for the formation of the cyclic product, N-acetyl dehydropipecolic acid during the oxidative deamination reaction of DOPA on N- α-acetyl Lysine.

(102) and Miller and Jones (103) have shown a preference for the telopeptide chain to form an antiparallel β-sheet, any six-membered ring would destroy the secondary structure.

An extensive study on the probable mechanism of collagen crosslinking via the 'allysine' molecule has been described by Dolz and Heidemann (99) using synthetic compounds. In their paper, they described the chemical synthesis of allysine derivatives starting from 3,4-dihydro-2H-pyran. They showed that the reactivity of the aldehyde function is very high. They observed an unexpected nucleophilic attack of the allysine amide nitrogen upon the aldehyde group forming a ring-closure product (Figure 20), the structure of which they have identified by ^{1}H-n.m.r., ^{13}C-n.m.r. and mass spectroscopy. The cyclic product which they describe as derivatives of 5,6-dehydropipecolic acid exhibited very similar chromatographic behavior as the corresponding aldehyde compounds, but they were able to separate the two efficiently by HPLC systems or HPTLC plates. It was not possible to inhibit the undesired reaction completely by adding 2,4-dinitro phenylhydrazine or other aldehyde-trapping reagents. They observed that hydrazone formation was only moderate, and the velocity of the ring closure reaction increased. They also demonstrated that the ring could not be opened even by heating in water or by mild hydrolysis, indicating the very high stability of the six-membered ring.

In the above experiment a similar cyclic derivative of dehydropipecolic acid is described which is formed when N-α-acetyl-L-lysine is reacted with the ortho-

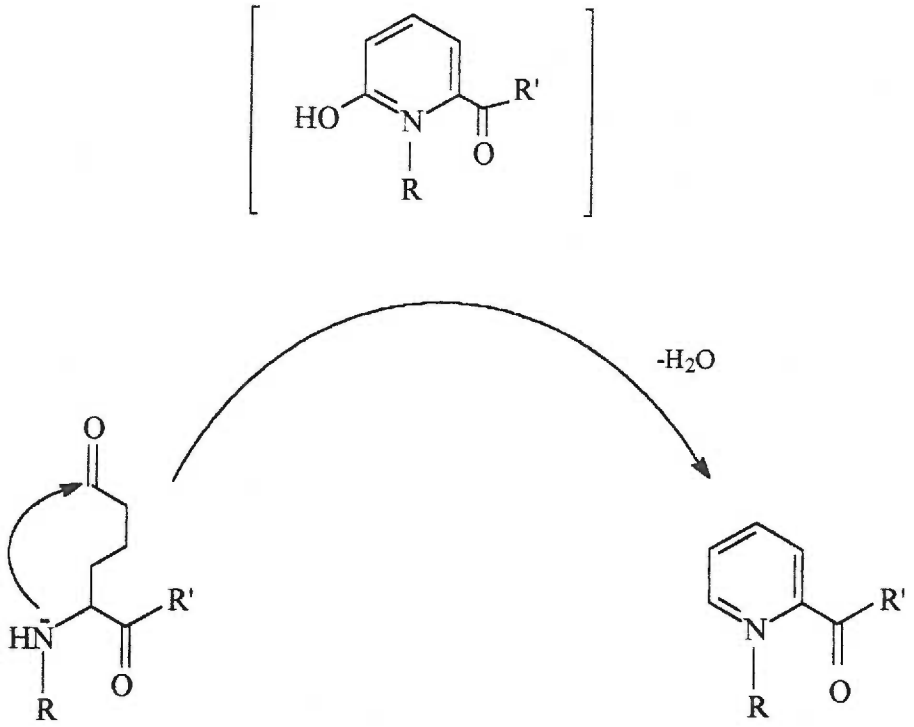


Figure 20: Proposed pathway for the ring closure reaction. R & R' symbolize any residue. The velocity of this reaction is mainly dependent on R [From Dolz and Heideman (99)]

quinone , DOPA.

It should be noted that even though the uncyclized, reduced 'Hydroxynorleucine', was detected by radioactive studies illustrated in Part I, the GC-MS studies failed to identify it. It is because the hydroxynorleucine present in the uncyclized form in the reaction is in the order of a few picograms and GC-MS studies using the available facility required the substrate to be in a few nanograms.

Conclusion:

DOPA oxidatively deaminated free and peptidyl lysines. In the case of collagen, this oxidized lysine (allysine) undergoes Schiff base condensation to form dimers. But with N-*α*-acetyl-L-Lysine, the allysine formed cyclizes before it can form any further products. Because this cyclic compound is very stable, it can be quantitated to give a relative measure of the oxidative reaction of N-*α*-acetyl-L-lysine and analogs.

2. DETECTION OF THE ALDEHYDE USING AN ANALOG OF LYSINE: 6-AMINOCAPROIC ACID

Introduction

In the last experiment it was shown that DOPA can oxidatively deaminate the ϵ-amino group of lysines, but the reduced aldehyde could not be isolated because of the ease with which cyclization took place. In this experiment, by employing a different substrate, the reduced aldehyde is identified.

Initially, all attempts to isolate the aldehyde before it underwent a Schiff base condensation failed. Instead I isolated a cyclic compound formed because the Schiff base that resulted was highly unstable and spontaneously undergoes a cyclization reaction accompanied with an elimination of a nucleophile, to give the dehydropipecolic acid derivative. It was surprising that I was not able to isolate the aldehyde by mass spectroscopy. A possible explanation could be that the aldehyde is not in sufficient concentration to enable its detection by MS.

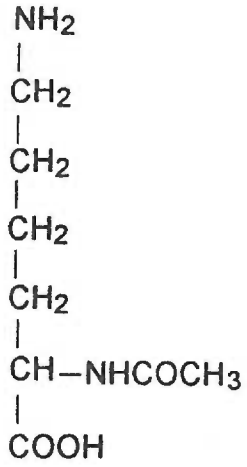
We contemplated different ways of trapping this reactive aldehyde even before it had a chance to undergo condensation or the cyclisation reaction. It was proposed that this could be done in a couple of ways. One way would be to use highly reactive nucleophiles to trap the aldehyde generated. An example would be the use of a 2,4-dinitrophenylhydrazine to trap the intermediate aldehyde. The phenylhydrazone derivative of the aldehyde would be highly stable and could be easily identified by mass spectroscopy or some other spectroscopic method. But

as pointed out by the earlier work of Dolz and Heideman (99), even derivatizing the aldehyde was not sufficient to avoid cyclization.

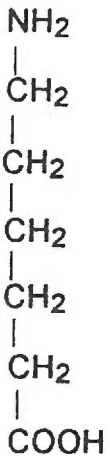
Another way to avoid cyclization would be through the use of a starting material which would be an analog of lysine but which would lack the α-amino group, so that internal cyclization would not be possible. The aldehyde would thus form the dimer, the Schiff base, but not the cyclic compound. Also, since the chemical nature of the lysine analog would be very different, the aldehyde may have a somewhat lesser reactivity allowing the identification of the aldehyde. We chose to employ this second strategy and the analog that we employed in place of lysine was 6-amino caproic acid (Figure 21). The following experiment describes the steps in detail.

Experimental:

7 ml volumes of 6-aminocaproic acid at a concentration of 25 mg/ml in PBS were incubated in triplicate at 37°C for 72h, in the presence of 1 mM DOPA with and without 0.1 mM copper(II)chloride. The two controls were 6-aminocaproic acid at 25 mg/ml in 7 mls of PBS and 1mM DOPA in PBS; incubated at 37°C for 72h. The samples were then reduced with sodium borohydride as described in the Methods section and acidified with hydrochloric acid to pH~1 and pooled together. 20 mls of the acidified pooled samples were extracted with three consecutive 7 ml volumes of ethyl acetate using Chem-elut columns (details in Methods section),



N-α-ACETYL-L-LYSINE



6-AMINOCAPROIC ACID

Figure 21 : Chemical structure of N-α-acetyl lysine and its analog 6-aminocaproic acid.

in order to extract N-*α*-acetylallysine and other ethyl acetate soluble substances out of the bulk of the unreacted starting material. The ethyl acetate extracts were rotary evaporated, redissolved in a small volume of ethyl acetate. The small volumes were then evaporated to dryness and derivatized with BSTFA + 1%TMCS, prior to GC-MS analysis (For details see Methods section). Since the samples were borohydride reduced prior to acidification and extraction, considerable amounts of boric acid was extracted into ethyl acetate. It was important to remove the excess boric acid from the mixture before GC analysis. This was done by derivatizing the dried ethyl acetate extract with 0.15 ml of BSTFA-TMCS, and evaporating it off. Derivatized boric acid is volatile. The remaining extract was redissolved in more BSTFA-TMCS (0.075 ml) and analyzed by GC-MS.

Samples that were prepared and incubated for 72 h before extraction and derivatization were labelled as:.

Sample No.	Concentration of		
	6-aminocaproic acid (mg/ml)	DOPA (mM)	Copper (mM)
A (control)	0	1	0
B (control)	25	0	0
C	25	1	0
D	25	1	0.1

Results:

The GC chromatographic profile of the derivatized ethyl acetate soluble fractions of DOPA and DOPA/Cu catalyzed 6-amino caproic acid samples along with the relevant controls were compared.

The chromatograms (Figures 22 A and B) represent the controls DOPA and 6-aminocaproic acid, respectively. Chromatograms (Figure 22 C and D) are the GC-elution profile for 6-aminocaproic acid incubated with DOPA with and without 0.1 mM copper.

In the Figure 22B, which is the control 6-amino-caproic acid, there was a tiny peak at ~12.4 min. and a huge peak at 14.0 min. In the 6-aminocaproic acid incubated with DOPA (Figure 22C), there was the same peak at ~12.4 min. and

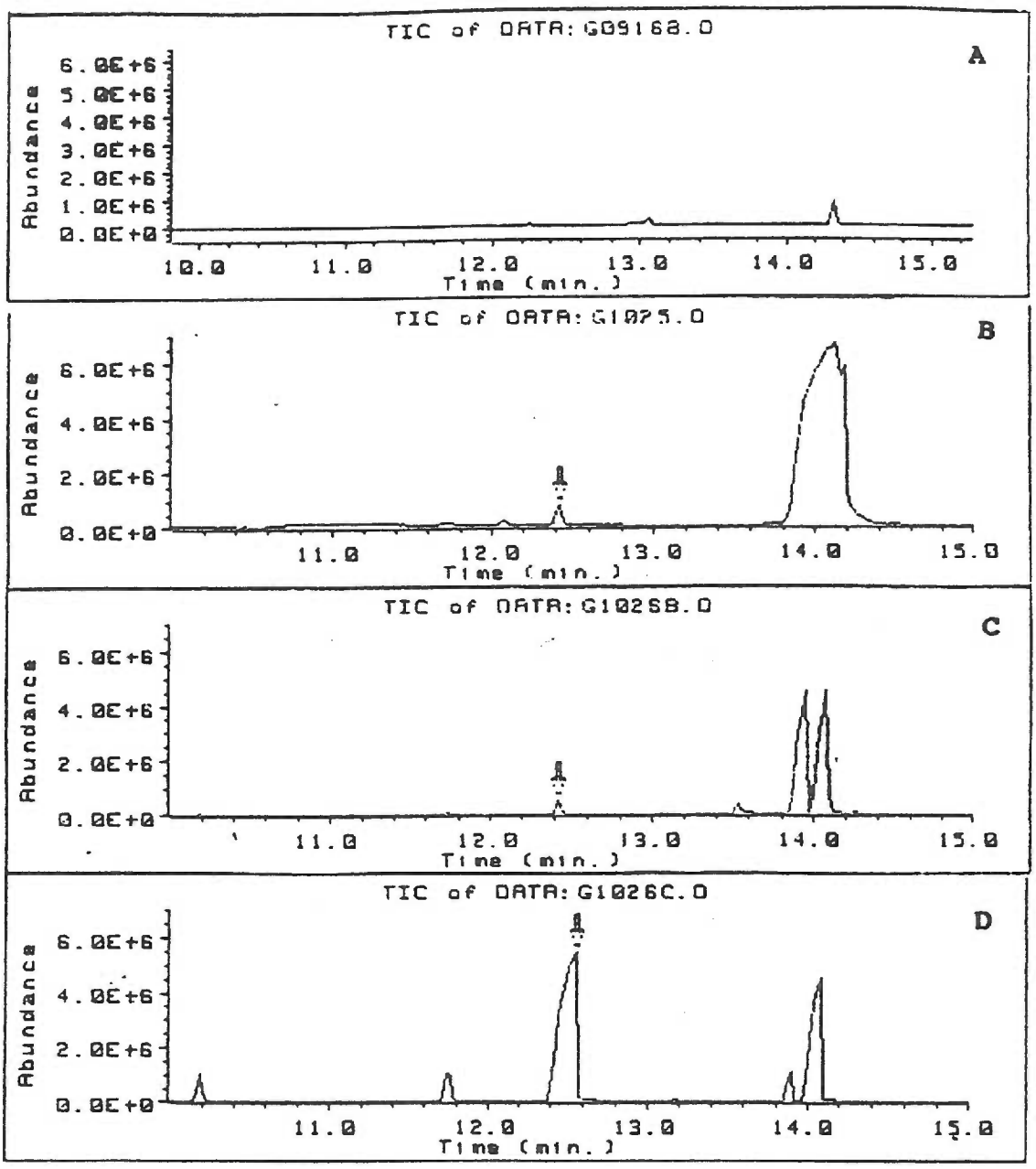


Figure 22: Gas chromatographic profile of trimethylsilylated derivatives of borohydride reduced samples of (A) DOPA (control); (B) 6-aminocaproic acid (control); (C) 6-aminocaproic acid with only DOPA and (D) 6-aminocaproic acid with DOPA and copper in phosphate buffered saline incubated for 37°C for 72h.

a doublet of equal intensities at 13.9 and 14.1 min., respectively, in place of the huge single peak seen in Fig 22B. In Figure 22D, which was the 6-aminocaproic acid incubated with DOPA and copper, the peak at ~12.4 min. was greatly enhanced, further the signal at 13.9 min was greatly diminished and that at 14.1 min. remained the same.

From standard runs, it was known that the peak at 13.9 min. was the derivative of unreacted 6-aminocaproic acid and the one at 14.1 min. was the derivative of adipic acid. The emergence of adipic acid in the mixture is initially puzzling, but it is well known that in the preparation of Nylon from 6-aminocaproic acid, there is a tremendous amount of adipic acid formed as a byproduct (104). It is thus believed that adipic acid is formed by another non-competing reaction that is taking place. The fact that the presence of copper greatly decreases the amount of starting material but has no effect on the amount of adipic acid, is additional evidence that it is formed in a non-competing reaction.

The mass spectrum of the species eluting at retention time ~12.4 min. is shown in Figure 23 A,B,C,D. The spectrum shows a proposed (M-15) peak at 261 and the proposed molecular ion has the m/z value of 276. This molecular weight of 276 agrees with that of the ditrimethylsilyl derivative of 6-hydroxycaproic acid.

A slightly varied program was tried to estimate the presence of any dimers. Because it is possible that the Schiff base nitrogen would be protonated at pH~1, the dimers might be charged and would thus not be extracted into ethyl acetate. For this reason, the non-ethyl acetate extracted sample (prior to extraction) was

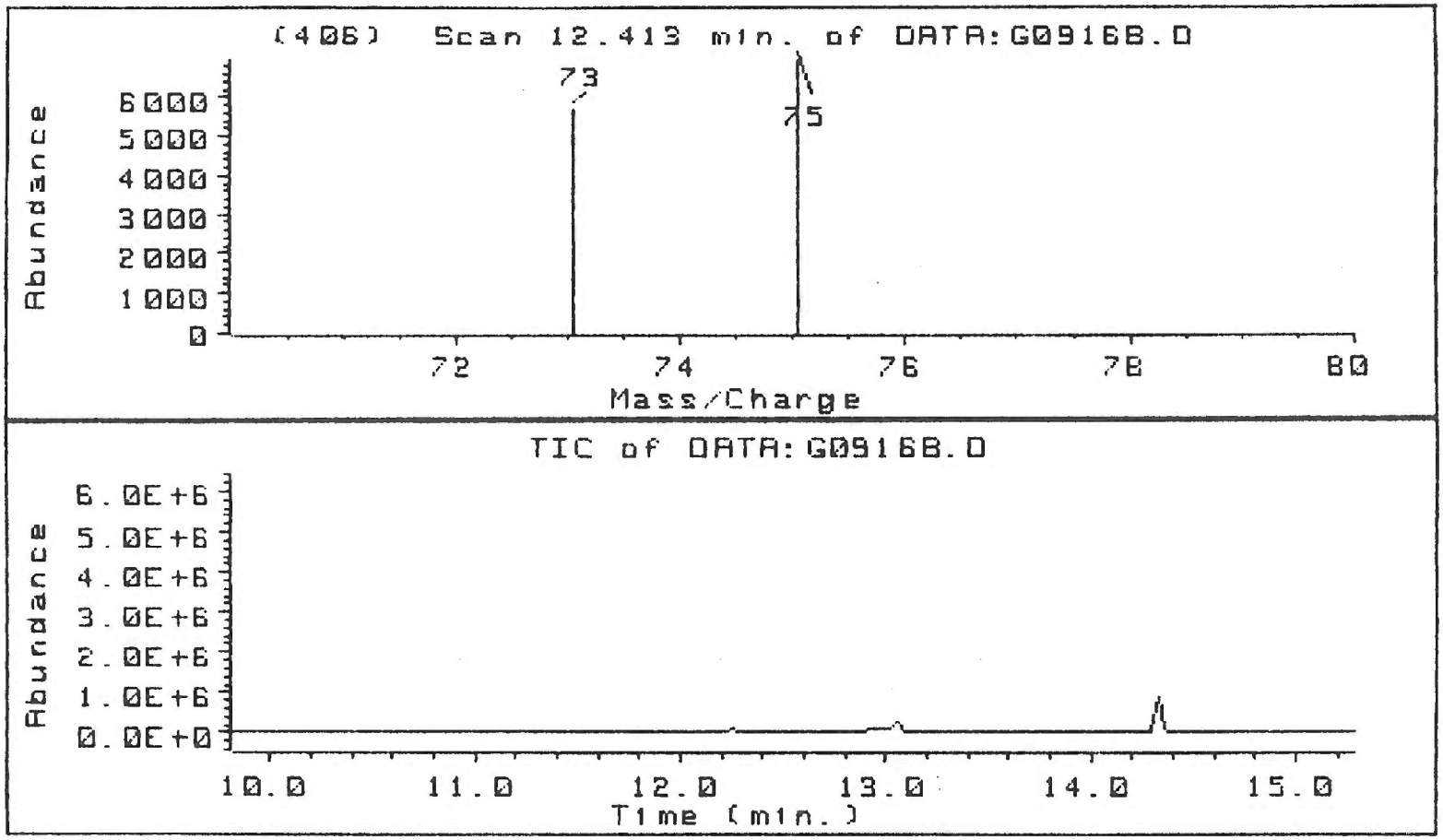


Figure 23A: The top panel shows the mass spectrum of the species eluting at retention time 12.413 min. The fragment 75 is a column artifact.Bottom panel shows the relevant portion of the GC-profile for the control sample A (DOPA only).

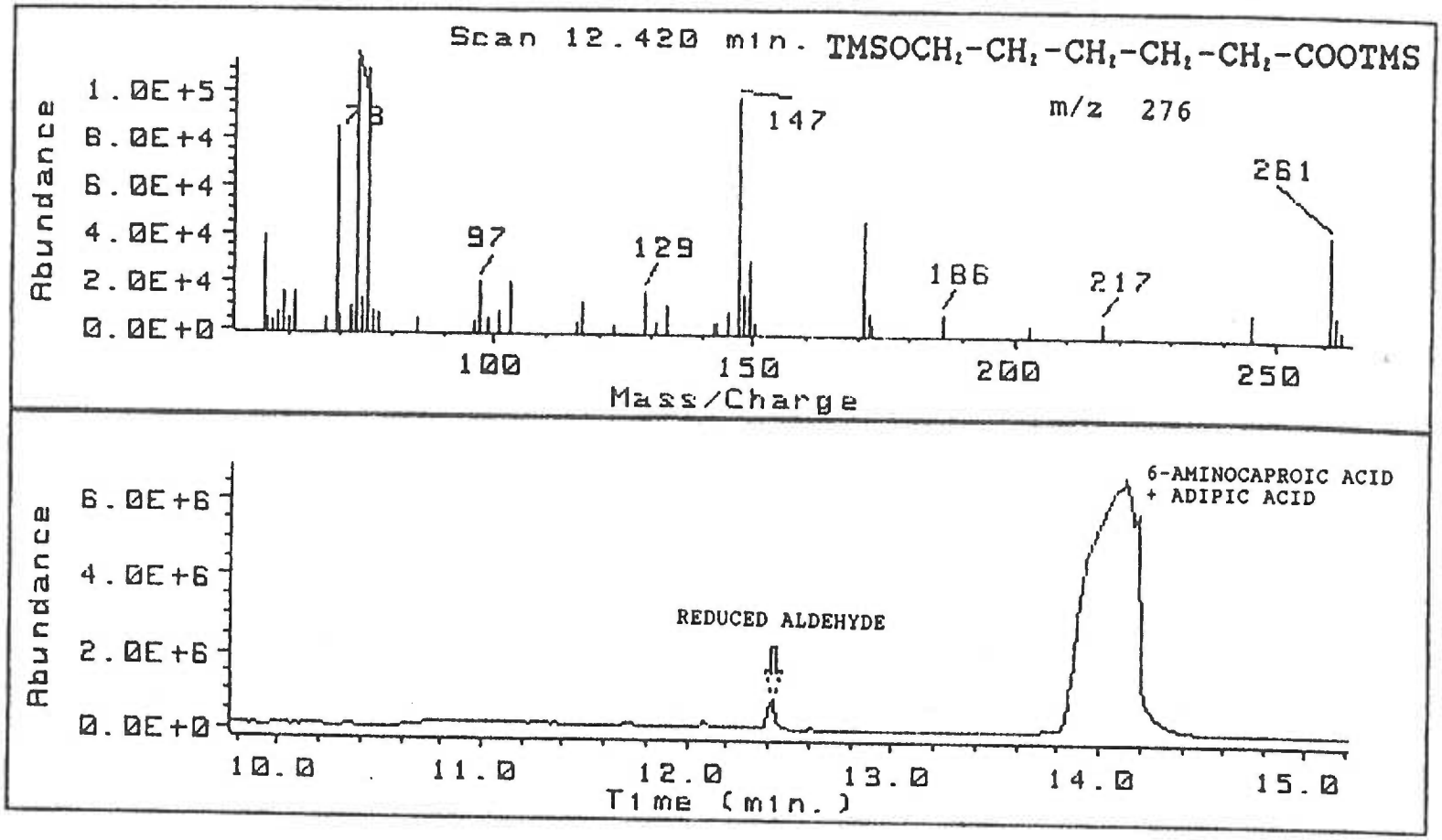


Figure 23B: The top panel shows the mass spectrum of the proposed reduced aldehyde of 6-aminocaproic acid (TMS derivatized) eluting at retention time 12.420 min. Bottom panel shows the relevant portion of the GC-profile for this control 6-aminocaproic acid sample B.

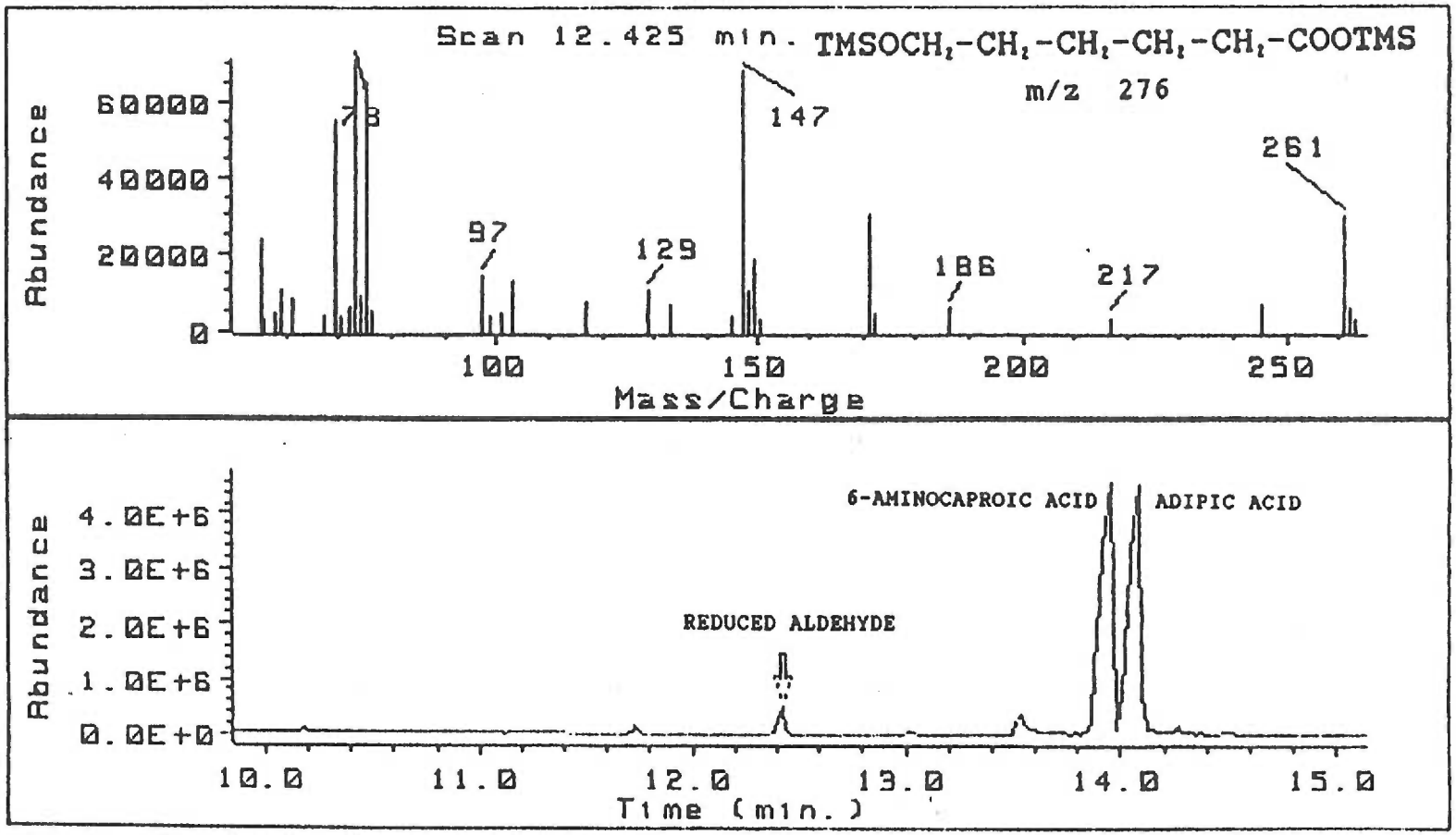


Figure 23C: The top panel shows the mass spectrum of the proposed reduced aldehyde of 6-aminocaproic acid (TMS derivatized) eluting at retention time 12.425 min. Bottom panel shows the relevant portion of the GC-profile for the sample C (6-aminocaproic acid with DOPA).

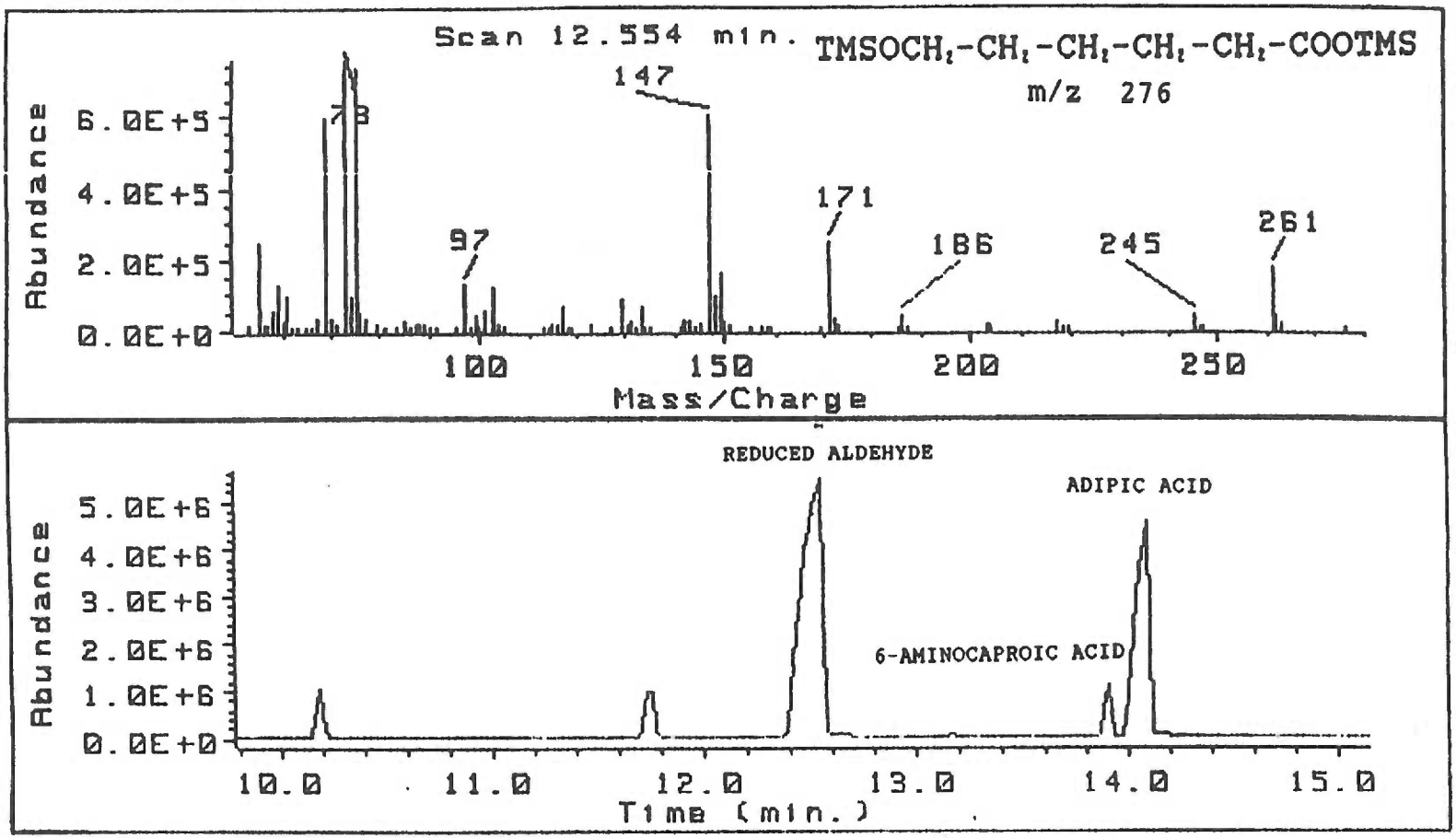


Figure 23D: The top panel shows the mass spectrum of the proposed reduced aldehyde of 6-aminocaproic acid (TMS derivatized) eluting at retention time 12.554 min. Bottom panel shows the relevant portion of the GC-profile for the sample D (6-aminocaproic acid with DOPA and copper).

tested by GC-MS. The GC-MS program for isolation of the dimers started at elution time 6 min. and four different peaks were obtained at retention times of 16.672 min., 17.6 min., 19.0 min. and 27.433 (Figure 24) were obtained. The scan at 16.672 min. gave a (M-15) peak at 243 and a probable molecular mass of 258. Scan at 17.6 min. gave a (M-15) ion at 216 and a molecular ion at 231. Scanning at 19 min, a (M-15) peak of 332 and proposed molecular mass of 347 was obtained, which corresponded well with the tri-trimethylsilyl (TMS-3) derivative of 6-aminocaproic acid. The scan of the last peak at 27.433 min gave a peak of 355, but no higher masses were seen when the sample was run at higher concentration. None of the three peaks at 17.6, 19.0 or 27.433 min. could be attributed to any as yet recognized structure.

Discussion :

6-aminocaproic acid, with an I.U.P.A.C. name of 6-aminohexanoic acid, is a structural analog of lysine which is missing the α-NH_2 group. The absence of the α-NH_2 group prevents the internal cyclization reaction, thus increasing the possibility of detecting either the monomeric aldehyde or the Schiff base dimer.

Using 6-aminocaproic acid, the corresponding reduced aldehyde could be detected but the dimer could not be identified. These results showed that upon adding DOPA to 6-aminocaproic acid even though there was no large increase

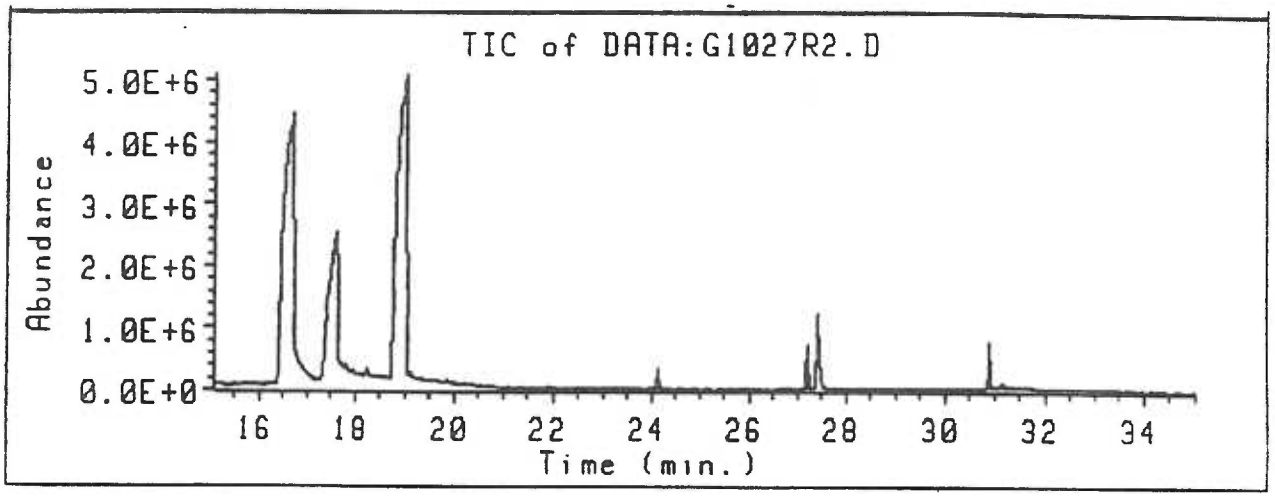
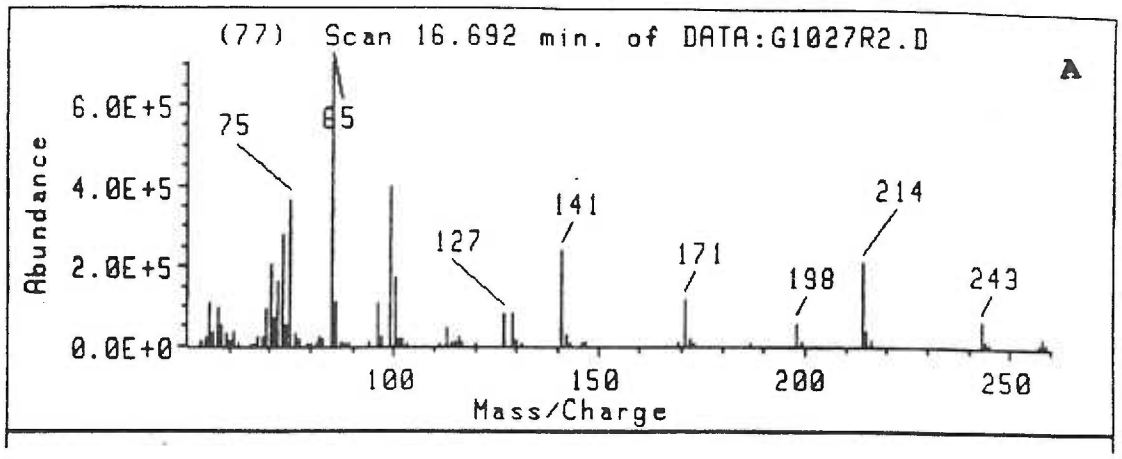
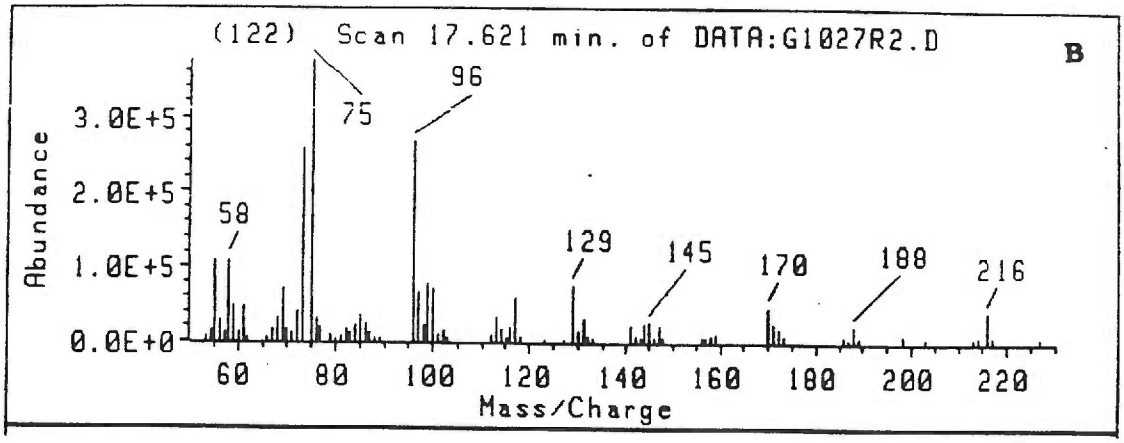
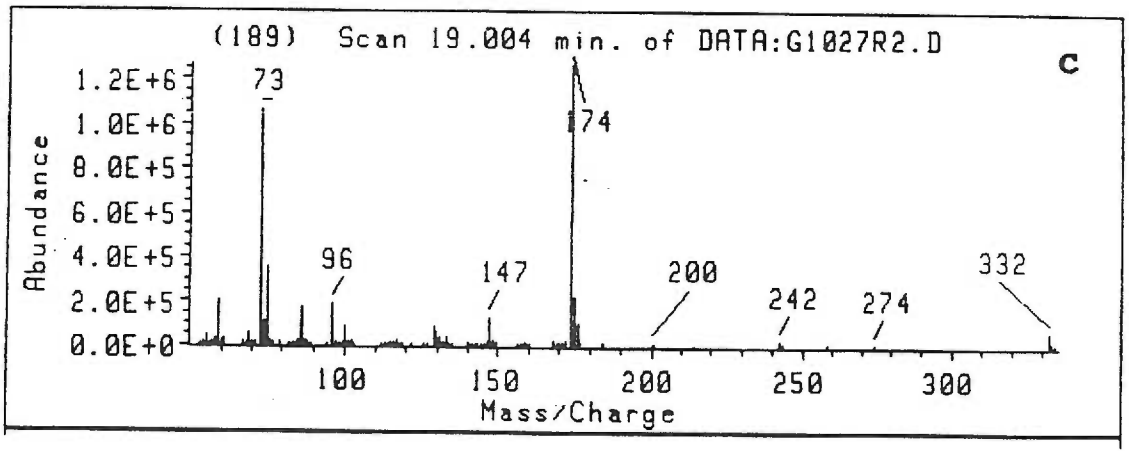


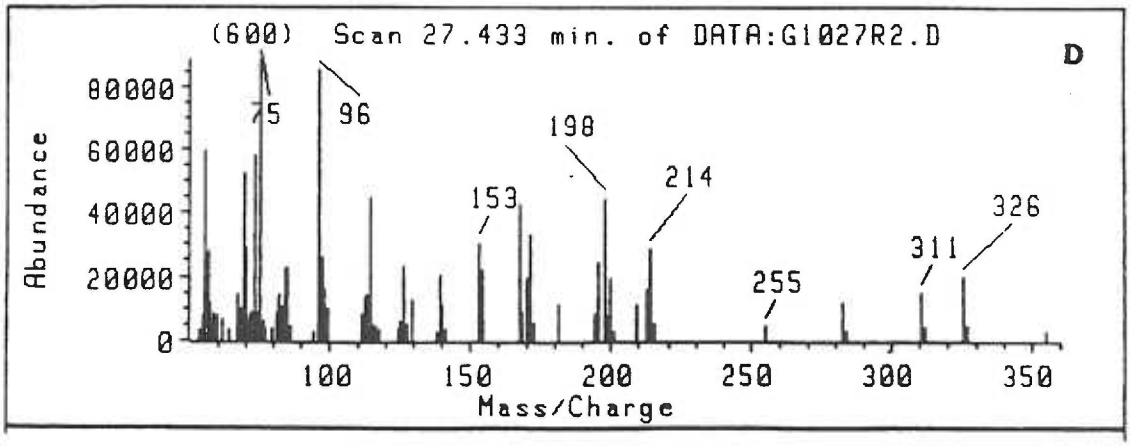
Figure 24 (i) : Gas chromatographic elution profile of the Sample D shows the presence of four distinct peaks at the retention times of 16.672, 17.600, 19.000 and 27.433 min.

Next Page:
Figure 24 (ii) : The mass spectra of the species eluting at retention times of 16.672 [A]; 17.600 [B]; 19.000 [C] and 27.433 [D] min.


(77) Scan 16.692 min. of DATA:G1027R2.D
A
Abundance
6.0E+5
4.0E+5
2.0E+5
0.0E+0
75
85
127
141
171
198
214
243
100
150
200
250
Mass/Charge


(122) Scan 17.621 min. of DATA:G1027R2.D
B
Abundance
3.0E+5
2.0E+5
1.0E+5
0.0E+0
58
75
96
129
145
170
188
216
60
80
100
120
140
160
180
200
220
Mass/Charge


(189) Scan 19.004 min. of DATA:G1027R2.D
C
Abundance
1.2E+6
1.0E+6
8.0E+5
6.0E+5
4.0E+5
2.0E+5
0.0E+0
73
74
96
147
200
242
274
332
100
150
200
250
300
Mass/Charge


(600) Scan 27.433 min. of DATA:G1027R2.D
D
Abundance
80000
60000
40000
20000
0
75
96
153
198
214
255
311
326
100
150
200
250
300
350
Mass/Charge

in the aldehyde formed (compared with the control), the amount of the unreacted starting material was greatly reduced. This suggested that DOPA was acting as a catalyst and that the 6-aldehyde of caproic acid was formed but was further converted into some other species, probably the dimer. The fact that the content of adipic is not much altered lends support to the hypothesis that the oxidative deamination by DOPA proceeds independent of the formation of adipic acid.

Adding copper to 6-aminocaproic acid incubated with DOPA greatly enhances the yield of the reduced aldehyde and is accompanied by a large decrease in the amount of the starting material. This suggests that DOPA does act as a catalyst, the action of copper being simply to enhance the regeneration of the DOPA. This mechanism is dealt with in detail in a later section of this thesis. Again, the fact that the amount of adipic acid found is equal to that seen in the absence of copper, shows that neither DOPA nor copper has any effect on this unknown side-reaction. The fact that we see a huge amount of aldehyde in the presence of DOPA with copper could be because the rate of formation of the aldehyde is greater than the rate of formation of the dimer. There is thus an accumulation of unreacted aldehyde, which in the case of N-*α*-acetyl-L-lysine is highly reactive, probably due to the presence of the zwitterion at one end, which makes the aldehydic group all the more acidic. The same end in 6-aminocaproic acid is highly acidic due to the presence of only a -COOH group at the position, the aldehydic group is then less acidic than the carboxylic group at the other end, the overall pKa of the molecule is thus towards a lower range, making the species

less reactive at pH~7.4.

Conclusion

6-Aminocaproic can be oxidatively deaminated by DOPA to its corresponding aldehyde and this aldehyde was found to be stable enough for its detection by mass spectroscopy after borohydride reduction to the corresponding alcohol.

PART III

EFFECT OF VARIOUS FACTORS ON OXIDATIVE DEAMINATION BY DOPA AND OTHER ORTHO-QUINONES.

1. EFFECT OF COPPER(II) CONCENTRATION

Introduction:

The last two experiments proved that DOPA, an orthoquinone, is able to catalyze the oxidative deamination of lysines to allysines. It was fortunate that deamination of N-*α*-acetyl-lysine yielded a cyclic compound, for this is a stable end product. It has been shown by Dolz and Heidemann (99) that such a derivative is highly stable and that the ring cannot be opened by boiling or other harsher treatments. Further, since no aldehyde could be seen in the samples treated with DOPA in the presence of copper, it was proposed that most of the aldehyde is converted to the cyclic product. The quantitation of the cyclic compound would thus be an appropriate measure of the extent of oxidative deamination.

Earlier, I had made an unsuccessful attempt to devise an HPLC assay to measure the extent of oxidative reaction. Because, the cyclic compound formed should be directly proportional to the extent of the reaction and because of the ease and accuracy with which it could be quantitated by GC-MS, we used it as an assay to study the effect of various factors on oxidative deamination.

It is fortunate that the dehydropipecolic cyclic compound is the end-product, rather than an intermediate in the oxidative deamination reaction. In the case of 6-aminocaproic acid, the aldehyde identified is an intermediate. This may react to form the dimer or other end-products. Therefore, the aldehyde formed from the 6-aminocaproic acid is not a good measure of oxidative deamination.

The following experiment was done to study the effect of varying the concentration of copper on the oxidative deamination. The importance of copper and other divalent metals have been explained earlier. Most amine oxidases, including lysyl oxidase, are copper requiring enzymes. Earlier, Kagan et al. (140) showed that incubating quinones with copper could boost the oxidation reaction on elastin, to about five times the amount without copper. Kagan et al incubated elastin (which had been labelled with [4,5-^{3}H]L-lysine) with copper and an ortho-quinone and reduced with cold borohydride. Their oxidized product retained the tritium on lysyl carbon-5 and so they could identify and quantitate it by chromatography.

Experimental:

7 ml volume of N-α-acetyl-L-Lysine at a concentration of 25 mg/ml in PBS were incubated in triplicates at 37°C for 72h, in the presence of 1 mM DOPA with varying concentrations of copper(II)chloride. The two controls were N-α-acetyl-L-Lysine at a concentration of 25 mg/ml and 1mM of DOPA in PBS, both incubated

at 37°C for 72h. The concentrations of copper incubated were 0.01, 0.05, 0.1, 0.5 and 1 mM.

At the end of 72 h, the samples were acidified to pH~1 with conc. HCl. The triplicate samples were pooled together and a final volume of 20 mls. of the pooled acidified sample was extracted with three consecutive volumes of 7 mls ethyl acetate, using Chem-elut columns (Methods section), in order to extract the cyclic dehydropipecolic acid out of the bulk of unreacted, ethyl acetate insoluble materials. The ethyl acetate extracts were rotary evaporated, redissolved in a small volume of ethyl acetate and then evaporated to dryness under nitrogen prior to BSTFA derivatization and GC-MS analysis (Methods Section).

The GC-chromatographic profiles of the trimethylsilylated derivatives of the ethyl acetate soluble fractions of N-α-acetyl-L-Lysine-DOPA-copper samples along with the relevant controls were obtained. Samples were prepared and labelled as follows:

Sample No.	Concentrations in phosphate buffered saline		
	N-α-acetyl-L-lysine (mg/ml)	DOPA (mM)	Copper (II) chloride (mM)
A(control)	0	1	0
B (control)	25	0	0
C	25	1	0
D	25	1	0.01
E	25	1	0.05
F	25	1	0.1
G	25	1	0.5
H	25	1	1

All the samples were incubated at 37°C for 72 h, prior to acidification.

Results:

The relevant ion chromatograms for the cyclic dehydropipecolic acid derivative fragment of mass 82 were taken. The area under the peak at ~14.417 min corresponding to 82.00 a.m.u. was compared. This peak was absent in the

two controls A and B.

The complete mass spectrum of the peak at retention time ~14.417 min. in samples C to G was identical to the one shown in Figure 15D. Ion fragment 82 a.m.u is the most abundant ion in the mass spectrum. This maximizes the sensitivity as well as the specificity of the relative measurements being made.

The plot of the varying amounts of the cyclic compound formed as the y-axis, against that of the corresponding amounts of copper (II) used as the x-axis, is shown in Figure 25. The plot shows a hyperbolic curve, showing about 90% completion of the total reaction, at a concentration of 0.3 mM of copper (II) used.

Discussion :

The amount of detectable 82 a.m.u.under the peak at retention time ~ 14.417 min. was used as a measure of the cyclic compound formed. This is more accurate than measuring the total ion current at retention time~14.417 min.

The 82 a.m.u. ion is the base peak of the ion-chromatogram corresponding to the cyclic dehydropipecolic acid derivative. A 'base peak' is the most abundant ion in a ion-chromatogram. The accuracy of measurement is greatly magnified because ion 82 is both an abundant and structure specific fragment in the mass spectrum of the cyclic compound. By comparison ion 73, which is common to all trimethylsilyl derivatives, is generally abundant but not structure specific, thus

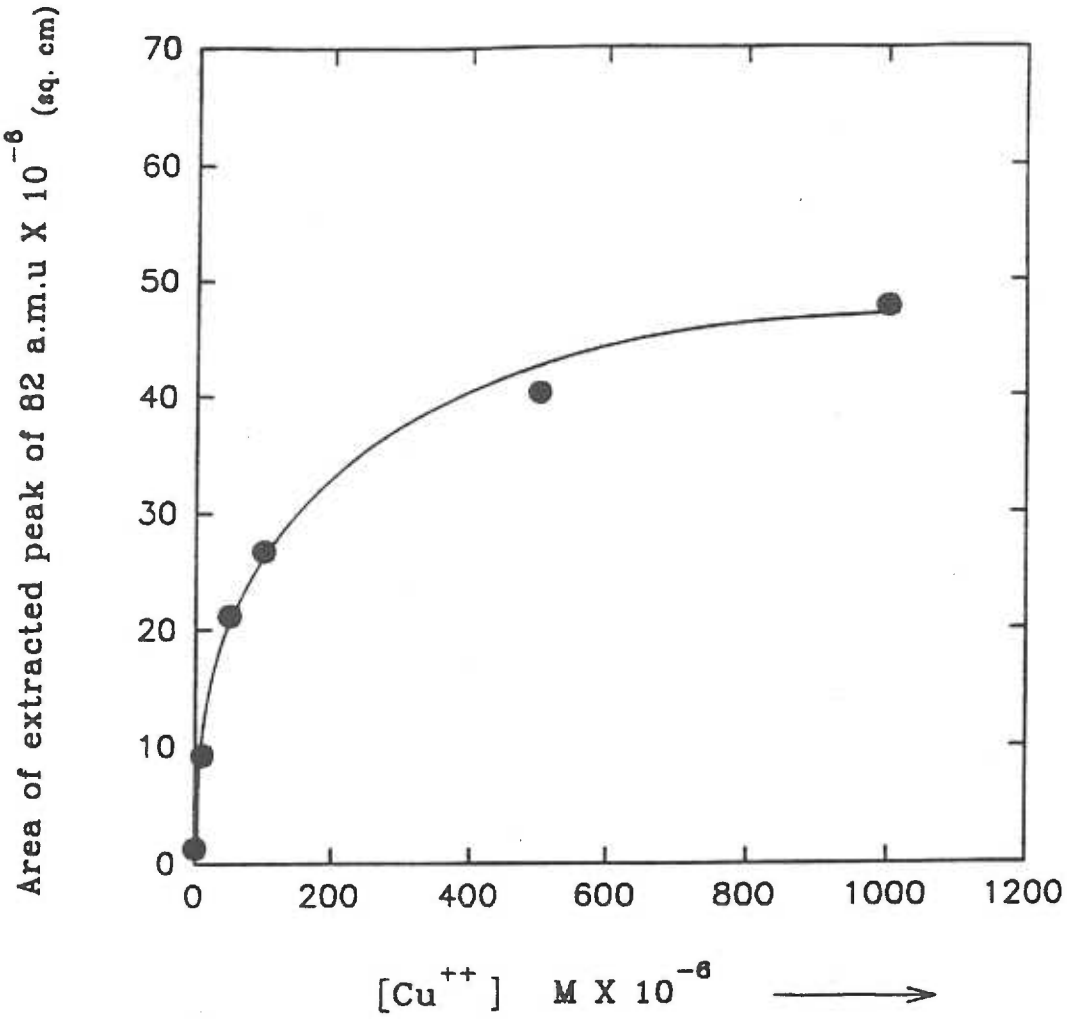


Figure 25 : Effect of different concentrations of copper on the formation of N-acetyl dehydropipecolic acid by oxidative deamination when 0.133 M N-acetyl lysine is incubated with 1 mM DOPA and copper at 37°C for 72 h.

leaving the possibility that compounds coeluting with or near the cyclic compound could contribute to the peak area if ion 73 or the total ion current were monitored. See Figure 26 for a graphical comparison.

The function of copper in the oxidative deamination reaction is proposed to be regeneration of the catalyst, the o-quinone. It is known that nutritional copper deficiency reduces collagen cross-link formation in vivo and thus leads to a faulty connective tissue formation (45). It has been proposed (19) that for lysyl oxidase and for other amine oxidases, copper(II) and other divalent metal ions function in the reoxidation of the substrate-reduced carbonyl cofactor to its corresponding oxidized form. The substrate reduced carbonyl cofactor is the 'aminoquinol' which is formed when the quinone cofactor accepts the amino group from the substrate that is oxidatively deaminated. The function of copper (II) in the present system of free N-*α*-acetyl-L-Lysine and DOPA may be compared to that in amine oxidases. The similarity between the two cases is in the regeneration of the catalyst, the aminoquinol of the o-quinone that is formed is functionally incapable of performing any catalytic activity, unless it is reoxidized to its o-quinone form. The divalent metal helps in the removal of ammonia, thus oxidizing the aminoquinol and in the process being itself reduced to the lower oxidation state, in this case to the copper (I) form. Molecular oxygen then reoxidizes the copper (I) to copper (II) form, which then can oxidize another molecule of aminoquinol. The proposed mechanism is illustrated in Figure 27.

The essential difference between the case of an amine oxidase and the

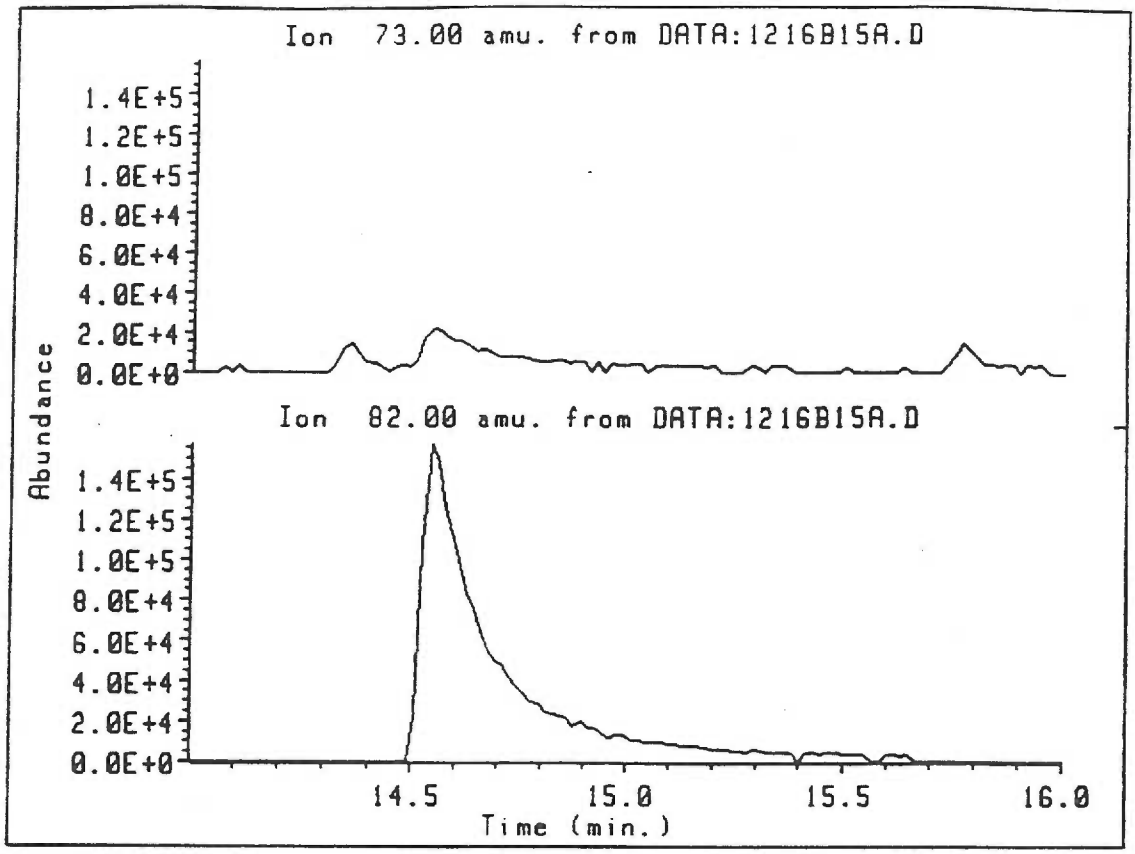


Figure 26: The ion chromatograms for ions 73 and 82, respectively, of the cyclic compound over the region of interest in a typical reaction mixture. The comparative profiles exhibit the structure-specificity and higher abundance of ion 82.

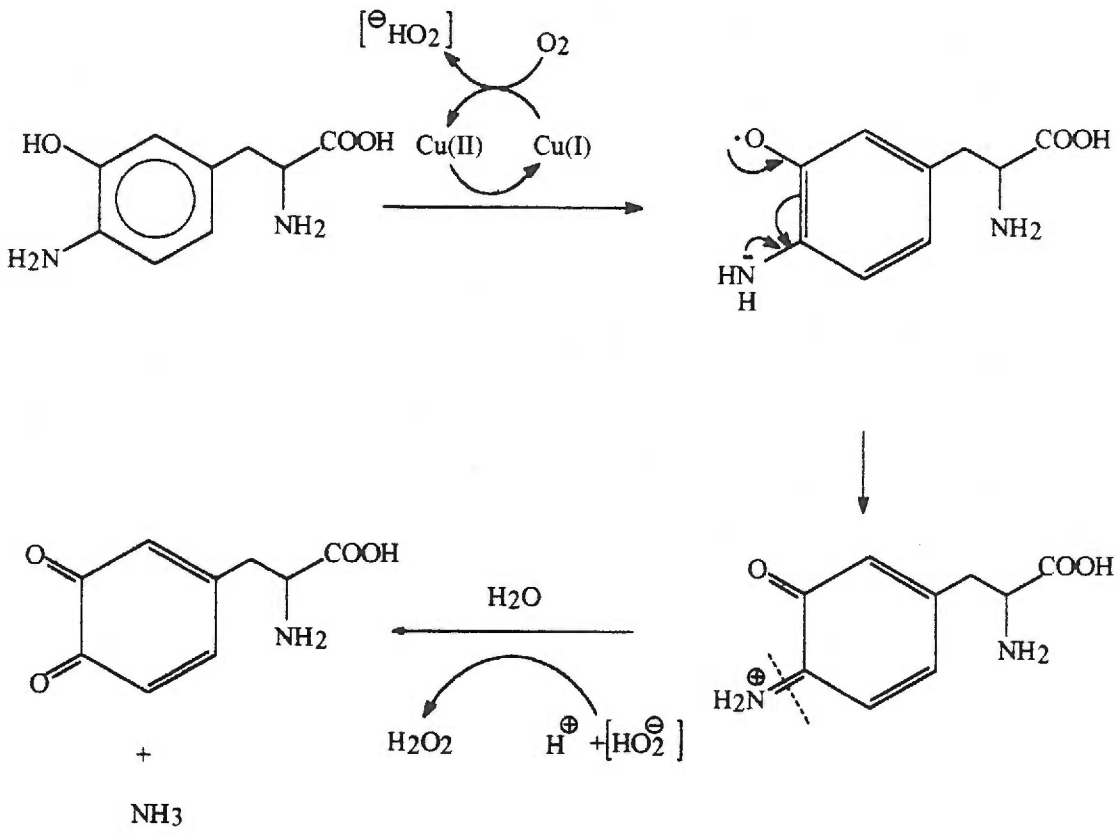


Figure 27 : Proposed mechanism for the participation of copper in the oxidative reaction. Copper acts as a catalyst, donating an electron to oxygen, and converting the aminoquinol via a semiquinone intermediate to the oxidized DOPAquinone. Ammonia and hydrogen peroxide are produced as byproducts.

present system of free N-α-acetyl-L-Lysine and DOPA is that the ortho-quinone, DOPA is freely available in the medium and is not bound to the peptide backbone. Thus, DOPA is continuously auto-oxidized to higher-molecular weight polymers known as melanin. Once, DOPA is converted to melanin it becomes insoluble and loses its ability to function as a catalyst. The extent of oxidative reaction is thus dependent on the amount of unpolymerized DOPA. What little amount is available for oxidative reaction is soon converted to the non-reusable aminoquinol form. When copper(II) is added to the system a continuous cycle of conversion to aminoquinol followed by its oxidation to the ortho-quinone form occurs, side by side with the competing polymerization reaction. There is thus a small portion of DOPA which is retrievable and reusable for the oxidative reaction, albeit that the concentration is continually decreasing due to the competing polymerization reaction. DOPA is a catalyst and copper (II) enhances the ability of DOPA to perform as a catalyst, in spite of the accompanying catalyst-depleting reaction of melanization. It will be shown in the later section that by using an ortho-quinone that is incapable of polymerization (pyrroloquinoline quinone), much larger amounts of the oxidative products can be formed.

The plot between increasing copper concentration and amount of cyclic compound formed is hyperbolic. This is a good indication that by increasing the amount of copper(II) the amount of DOPA that can be regenerated is also increased, thus the amount of cyclic compound formed also increases steadily until a critical concentration of copper(II) is reached beyond which there is no

further increase.

Conclusion:

Incubating substrate with 1mM DOPA together with 0.3 mM copper (II), about 90% of the total reaction is achieved.

EFFECT OF IRON(III) CONCENTRATION

Introduction

In the next experiment, the effect of varying concentration of iron(III) on the oxidative deamination is studied. Iron is a divalent or trivalent metal. It is not clear why all amine oxidases require copper, and not iron. Iron centers do exist in other quinoproteins, for eg. at the redox sites of hemoproteins and other proteins that are crucial for electron transfer processes.

Experimental:

The procedure employed was identical to that discussed in the previous experiment. Iron(III)chloride was substituted for the copper (II) compound and the concentrations of iron(III)chloride used in the reaction were 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mM.

The GC-chromatographic profiles of the trimethylsilylated derivatives of the ethyl acetate soluble fractions of N-*α*-acetyl-L-Lysine-DOPA-iron samples along with the relevant controls were compared. Samples were prepared and labelled as shown in the following Table:

Sample No.	Concentrations in phosphate buffered saline		
	N-*α*-acetyl-L-lysine (mg/ml)	DOPA (mM)	Ferric (III) chloride (mM)
A (control)	0	1	0
B (control)	25	0	0
C	25	1	0.05
D	25	1	0.1
E	25	1	0.25
F	25	1	0.5
G	25	1	0.75
H	25	1	1

All the samples were incubated at 37°C for 72 h, prior to acidification.

Results:

The chromatograms of the samples were compared. The area under the peak at ~14.415 min corresponding to 82.00 a.m.u. was compared. This peak was absent in the two controls A and B.

As with the case of copper, the plot (Figure 28) between varying concentration of iron(III) and the area of peak at retention time~ 14.415 min. is hyperbolic. It is consistent with the fact that with increasing concentration of iron the amount of oxidative products formed increases, until an excess of ferric ions results, any increase after that amount does not result in any further increase in the concentration of oxidized products formed.

Figure 29 shows the relative extent of reaction products formed using various concentrations of iron and copper.

Discussion:

The comparative plot of the effect of copper and iron on the extent of oxidative deamination (Figure 29) shows that copper is more effective than iron.

The standard reduction potentials, E° values, at 298.15 K (25°C), and at a pressure of 1 atm, of copper(II) and iron (III) are given below:

$Cu^{2+} + e^- \text{-----> } Cu^+ \qquad E°/V = 0.153$

$Fe^{3+} + e^- \text{-----> } Fe^{2+} \qquad E°/V = 0.771$

This means that both Fe(III) and Cu(II) are capable of accepting electrons more easily than hydrogen. Fe(III) accepts the single electron from aminoquinol more easily generating a semiquinone. However, not only is Cu(II) a reasonably good acceptor of electron from the reduced substrate (in the process being reduced to Cu[I]) but it is also good at losing an electron and converting to its re-

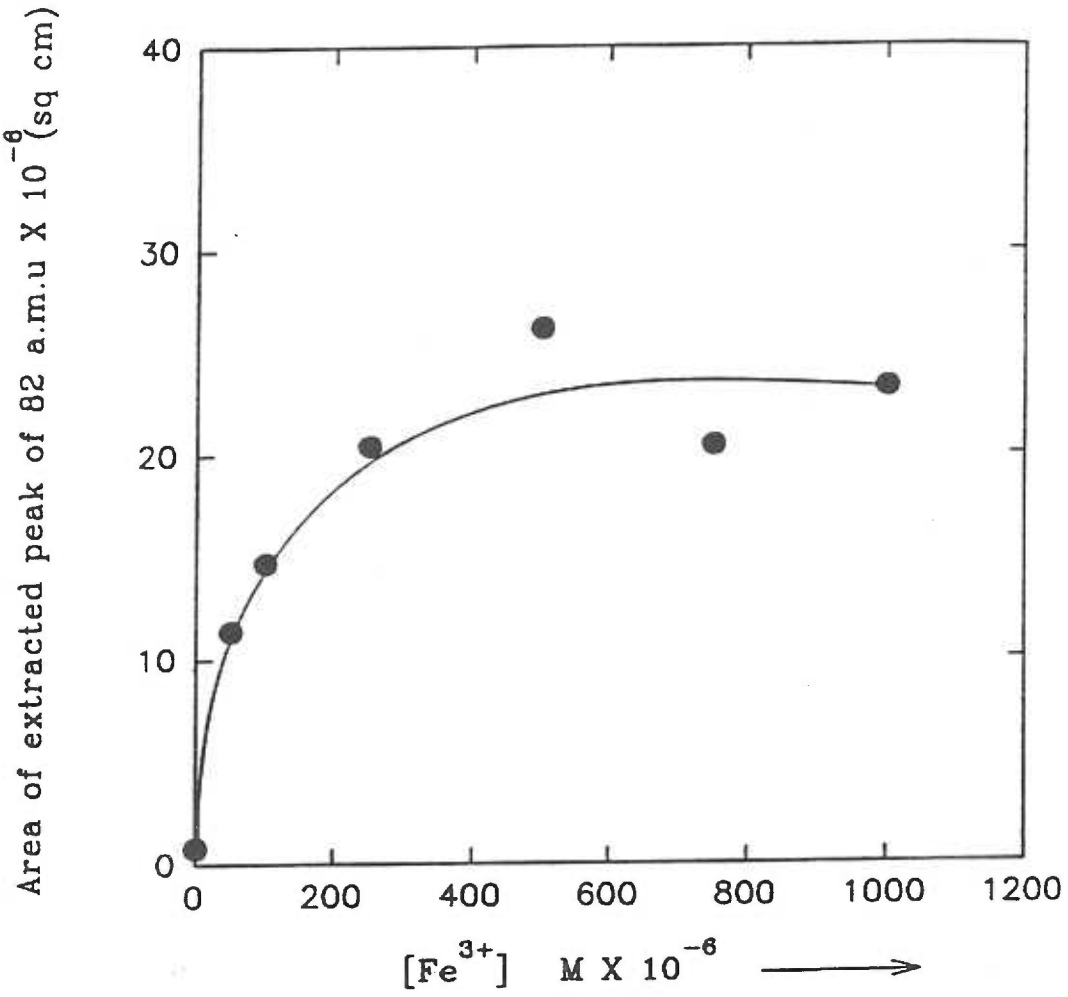


Figure 28 : Effect of different concentrations of iron on the formation of N-acetyl dehydropipecolic acid by oxidative deamination when 0.133 M N-acetyl lysine is incubated with 1 mM DOPA and iron at 37 C for 72 h.

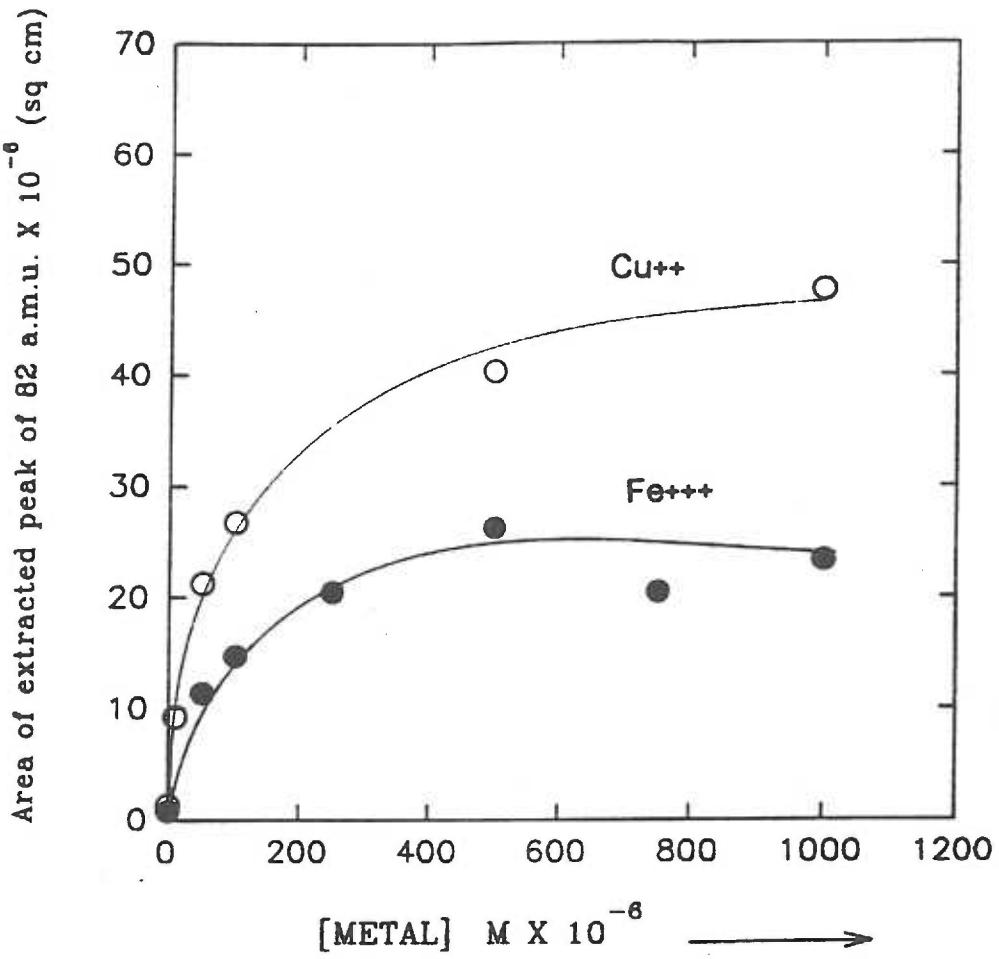


Figure 29 : Effect of different concentrations of copper and iron on the formation of N-acetyl dehydropipecolic acid by oxidative deamination when 0.133 M N-acetyl lysine is incubated with 1 mM DOPA and metal at 37 C for 72 h.

oxidized form Cu(II). This is because its reduction potential is not very much greater than hydrogen (E°/V = 0.000).

Conclusion:

Incubating N-*α*-acetyl-L-lysine with DOPA at a concentration of 1 mM, in the presence of 0.3 mM copper (II) achieves a greater amount of the oxidative reaction than when the substrate is incubated with DOPA (1 mM) in presence of 0.3 mM of iron (III)

3. EFFECT OF THE NATURE OF ORTHO-QUINONE ON OXIDATIVE DEAMINATION

Introduction

The most important requirement for oxidative deamination is the ortho-quinone structure, however the rest of the molecule can have an indirect effect on the reaction. The ability of the quinone to autooxidize and convert to a quinol form, reversibly, is an important requirement. Most quinones have this redox capability. An undesirable property would be a side-reaction that competes for the ortho-quinone group, or one that converts it to a different form.

The following experiment is designed to test the efficiency of the oxidative deamination of different ortho-quinones. The ortho-quinones tested were:

(i) 3,4-Dihydroxyphenylalanine (DOPA)

(ii) 2,4,5-Trihydroxyphenylalanine (TOPA)

(iii) tert-Butyl catechol (tBC)

(iv) Pyrroloquinoline quinone (PQQ)

Experimental

7 ml volumes of N-*α*-acetyl-L-lysine at a concentration of 25 mg/ml in PBS was incubated at 37°C for 72h, in the presence of 1 mM ortho-quinone with 0.3 mM copper(II)chloride. The controls were the 1 mM solution of the corresponding

ortho-quinone in PBS incubated under the same condition. A common control was N-*α*-acetyl-L-lysine incubated in PBS in the absence of an ortho-quinone at 37°C for 72h.

The samples were acidified and ethyl acetate extracted as given in Methods section. BSTFA derivatisation and GC-MS analysis was done as stated in the Methods section.

Results:

Figure 30 is a comparative plot showing the amount of cyclic compound formed as a function of the type of ortho-quinone used. As can be seen from the figure, DOPA has the least efficiency for carrying out oxidative deamination and PQQ has the highest. The order of efficiency of oxidative deamination is:

DOPA < TOPA < tBC < PQQ

Discussion:

The above experiment proves that these ortho-quinones can cause oxidative deamination of lysines. The efficiency of oxidation is depended on the nature and properties of the ortho-quinone. Figure 31 shows the chemical structures of ortho-quinones used.

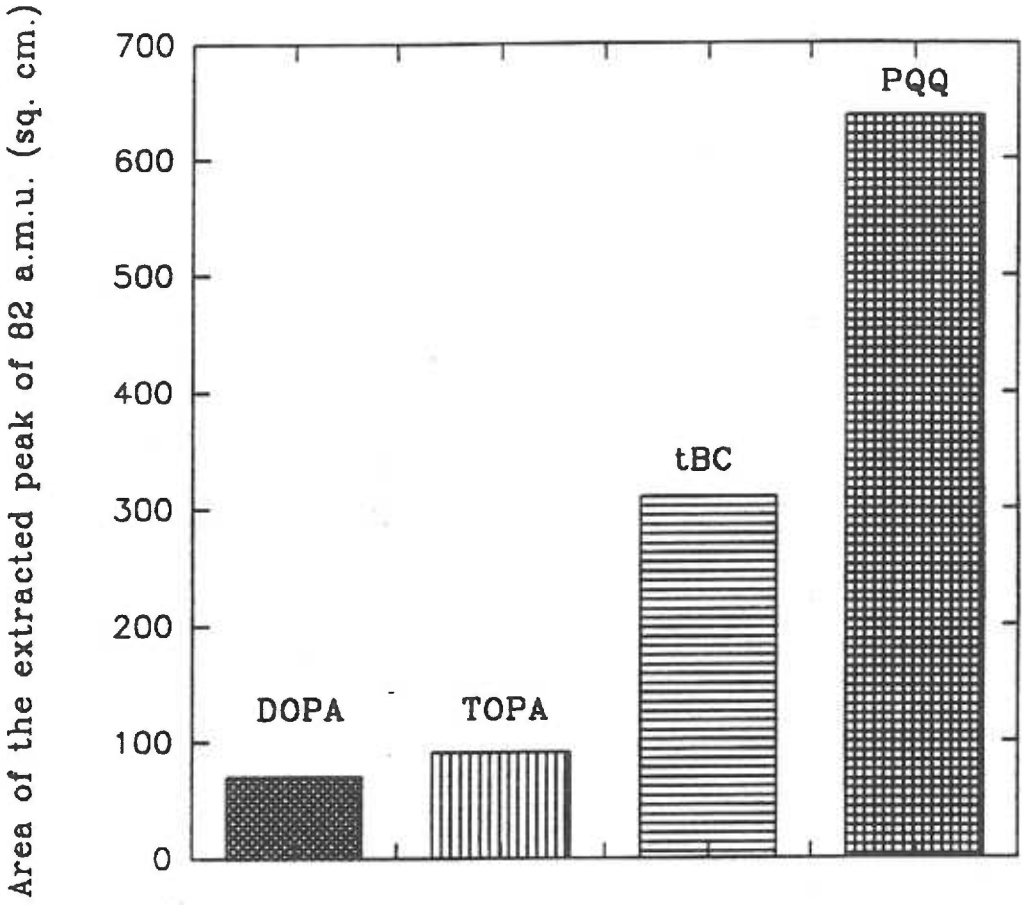
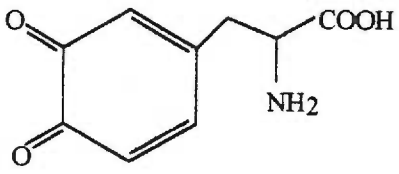
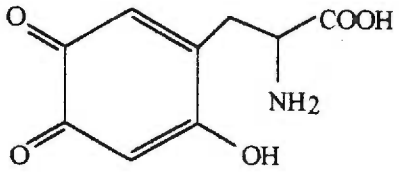


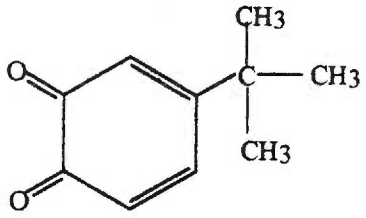
Figure 30 : Effect of different orthoquinones on the formation of N-acetyl dehydropipecolic acid by oxidative deamination when 0.133 M N-α-acetyl lysine is incubated with 1 mM of orthoquinone and 300μM of copper at 37 ℃ for 72 h.



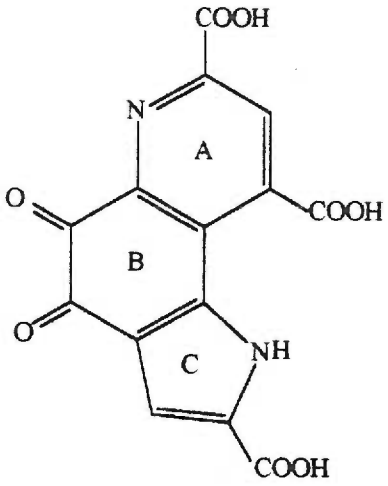
quinone of Dihydroxyphenylalanine
(DOPAquinone)



o-quinone tautomer of Trihydroxyphenylalanine
(TOPAquinone)



4-tert-BUTYLCATECHOL (tBC)



PYRROLOQUINOLINE QUINONE (PQQ)

A - Pyridine ring
B - Quinone ring
C - Pyrrole ring

Figure 31 : Chemical structures of some ortho-quinones.

DOPA and TOPA are very similar to each other in structure and property. Both can undergo auto-polymerization. DOPA polymerizes to a greater extent than TOPA and this might be because there is less steric hindrance in case of DOPA to form the indole compound, which further polymerizes via a nucleophilic addition. DOPA has three positions on the ring: carbons-2,5 and 6 for prospective nucleophilic addition, TOPA however has only two sites for nucleophilic addition: ring carbon-3 and 6.

Tert-Butyl catechol (tBC) cannot form three dimensional polymers, but it can form colored linear polymers via 1,4-addition.The deep yellow coloration is due to the conjugated structure. Nucleophilic addition can take place only at ring C-6. Nucleophilic addition at ring carbons 2 and 5 are not possible due to steric hindrance from the bulky tert-butyl group.

Pyrroloquinoline quinone (PQQ) is an ortho-quinone which cannot polymerize. Pyrroloquinoline quinone is made of three rings: a pyridine ring, a quinone ring and a pyrrole ring, labelled A, B and C, respectively. In PQQ, the central quinone ring is fused on either sides by the pyridine and pyrrole ring, because of which all the six carbons on the quinone ring lack any hydrogen and there are no sites for nucleophilic addition on the quinone ring. The quinone ring is thus unable to undergo any nucleophilic reaction. It is no surprise then, that PQQ shows the maximum oxidative capability.

DOPA has the least activity because a vast majority of it is wasted as the polymer, any small amount that is left for catalytic action is constantly diminishing

since the ability to polymerize is greater than the ability to catalyze the oxidative reaction. TOPA is a little better than DOPA probably because the auto-polymerization reaction is slower. Tert-butyl catechol is much better than DOPA and TOPA because of its incapability of forming extensive three dimensional networks of polymer which eventually precipitate out of solution and become useless, as in case of TOPA and DOPA. PQQ is one quinone that does not undergo any other nucleophilic addition or polymerization reaction. It's quinone structure is locked in its oxidized quinone form due to the unique triple fused ring form, thus it is relatively more stable than the quinones of the other three catechols used. It is my proposal that once the PQQ oxidizes the lysine, and itself is reduced to the aminoquinol form, it is reoxidized by the copper ions in solution into a form that is again usable for catalytic reaction. Thus, all of the PQQ is available solely for the oxidative deamination reaction.

Conclusion

The efficiency of the different o-quinones to oxidatively deaminate lysines is:

DOPA < TOPA < tBC < PQQ

4. EFFECT OF TIME ON OXIDATIVE DEAMINATION.

Introduction

In any typical reaction with N-*α*-acetyl-L-lysine given above the duration of the experiment has been for about 72 hours. Before the author proposed the hypothesis of oxidative deamination, it had been noticed that incubation of collagen with DOPA for about 72 hours resulted in maximum stabilization of fibrils. However, fibrillar stabilization is different from oxidative deamination, in that stabilization via formation of crosslinks occurs after the generation of the aldehydes. Therefore, oxidative deamination should occur much before the stabilization process.

The process of autoxidation of DOPA, to the quinone state is almost instantaneous on exposure to light and oxygen. The process of polymerization is also relatively fast and precipitates of melanin can be seen by 2-3 hours, depending on the amount of exposure to light.

In the following experiment two different ortho-quinones: DOPA and PQQ have been incubated with substrate and copper for varying amount of time. This experiment would give information about the effect of time on the oxidative deamination. It has been shown in the last section that PQQ is the most efficient and DOPA is the least efficient in oxidatively deaminating free lysines.

Experimental:

Two different sets of reactions samples were set up. Set I was N-α-acetyl-L-lysine incubated with DOPA for varying amounts of time. Set II was N-α-acetyl-L-lysine incubated with PQQ for varying amounts of time.

Sample preparation: 7 mls of N-α-acetyl-L-lysine at a concentration of 25 mg/ml were incubated in triplicates, along with 1 mM of DOPA or PQQ and 0.3 mM copper (II)chloride in PBS. The controls for the reaction were N-α-acetyl-L-lysine at 25 mg/ml and the quinone DOPA and quinone PQQ at 1 mM in PBS. The samples were incubated in a water bath maintained at 37°C for specific periods of time.

At the end of each time period, three sample solutions of 7 mls each were removed from the water bath, pooled together and acidified to pH~1 with conc.HCl. A total of 20 ml was extracted with ethyl acetate, rotary evaporated and dried under nitrogen. The dried samples were derivatized with BSTFA-TMCS and analyzed using GC-MS.

Results:

The plot between the duration of incubation and the amount of cyclic compound formed when N-α-acetyl-L-lysine is incubated with DOPA is given in Figure 32, and that of N-α-acetyl-L-lysine incubated with PQQ is given in Figure 33.

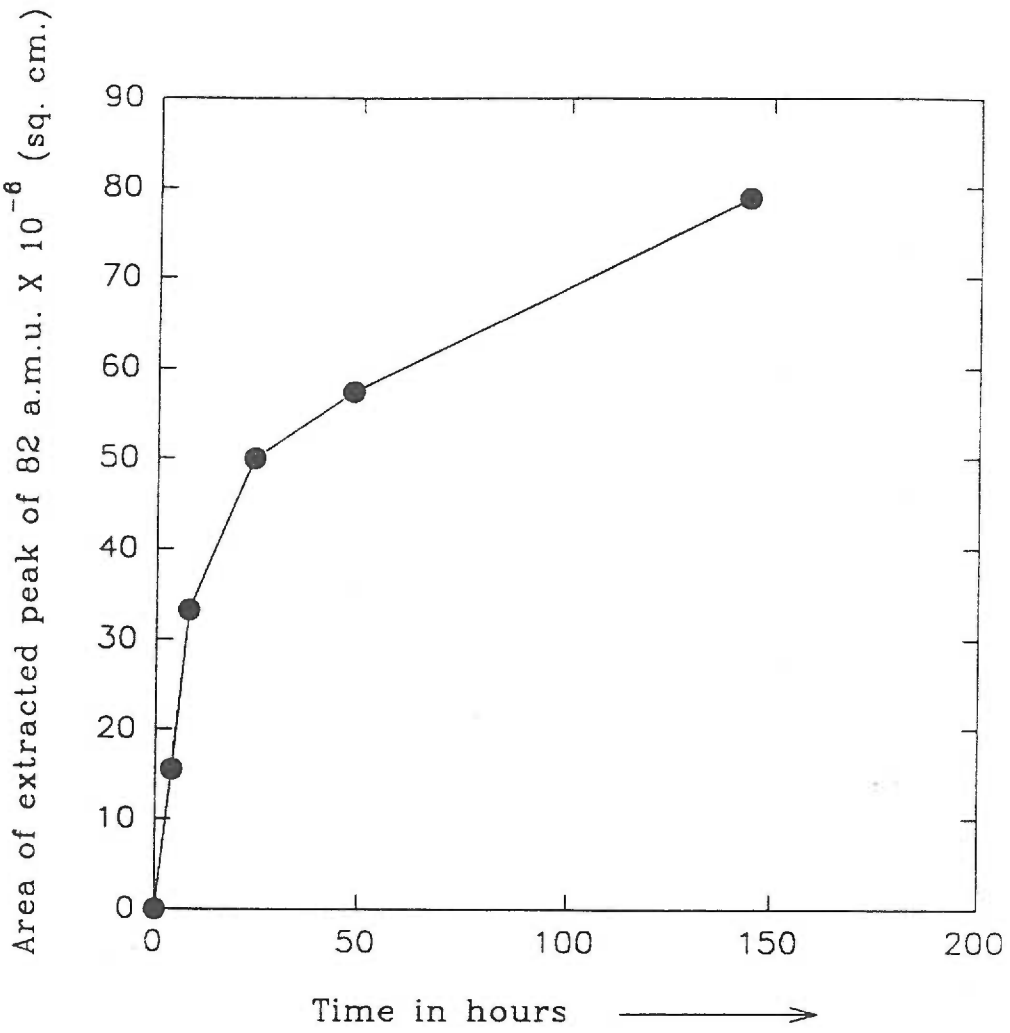


Figure 32 : Effect of duration of incubation at 37°C of 0.133 M N-α-acetyl lysine incubated with 1 mM DOPA and 300 μM copper on the formation of N-acetyl dehydropipecolic acid.

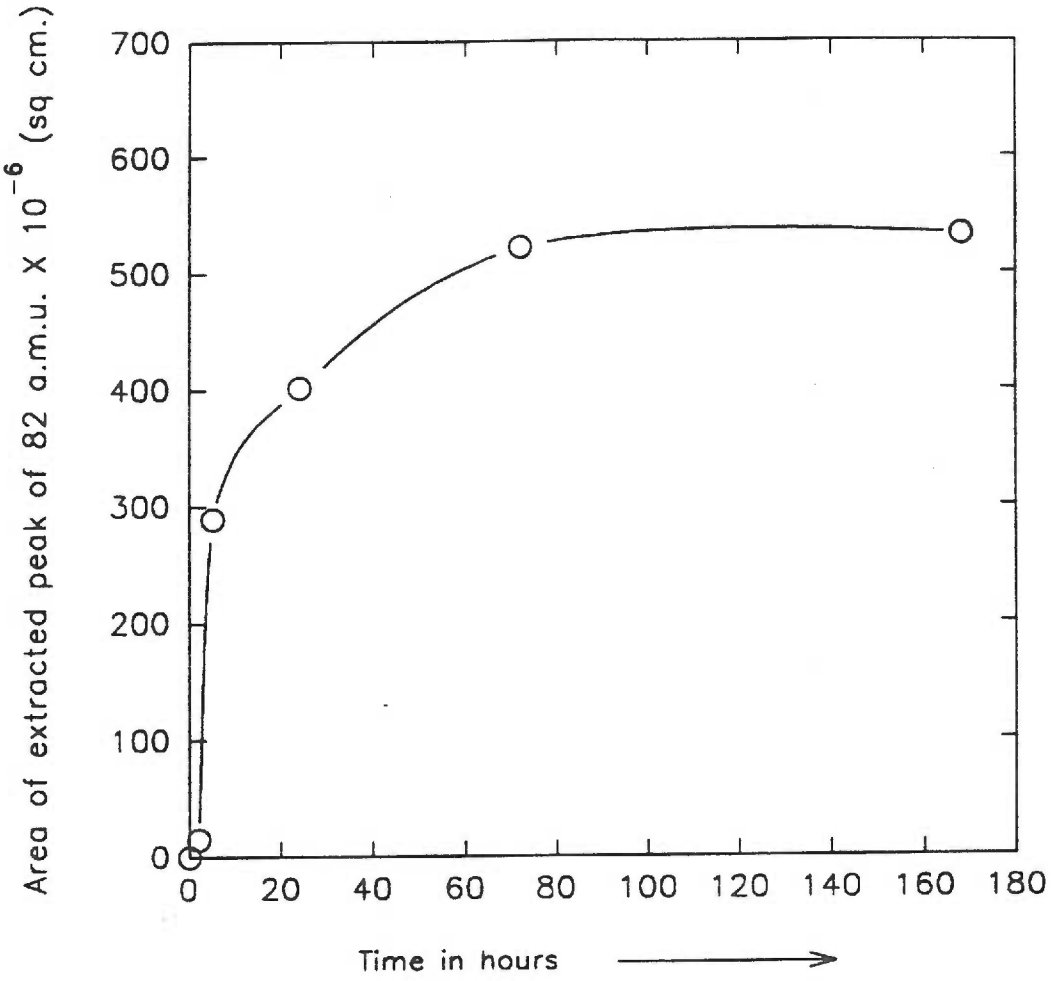


Figure 33 : Effect of duration of incubation at 37°C of 0.133 M N-α-acetyl lysine incubated with 1 mM PQQ and 300μM copper on the formation of N-acetyl dehydropipecolic acid.

The samples of DOPA and N-*α*-acetyl-L-lysine incubated for varying times shows a hyperbolic increase (Figure 32). There is a rapid increase in the formation of the cyclic compound for the first 10 hours followed by a gradual increase and even at 144 hours of incubation saturation is not reached.

The samples of PQQ and N-*α*-acetyl-L-lysine also show a hyperbolic increase in the formation of the product, shown in Figure 33. There is a steep increase in the formation of the cyclic compound for the first 5 hours, after which the increase is gradual between 5-60 hours. After 60 hours there is no further formation of the cyclic compound and a plateau is observed in the plot.

A comparative plot between the duration and incubation and the amount of cyclic compound formed when N-*α*-acetyl-L-lysine is incubated with DOPA or PQQ is shown in Figure 34. The formation of the cyclic compound with PQQ is at least 8X more than with DOPA.

Discussion

Comparing the oxidative deamination of N-*α*-acetyl-L-lysine by DOPA and PQQ, it is obvious that PQQ is much more efficient than DOPA. This is not surprising, because the total 'catalytically-active' concentration of DOPA taking part in the oxidative reaction is much less than the total 'active' amount of PQQ, because the polymerization of DOPA is a major competing side reaction.

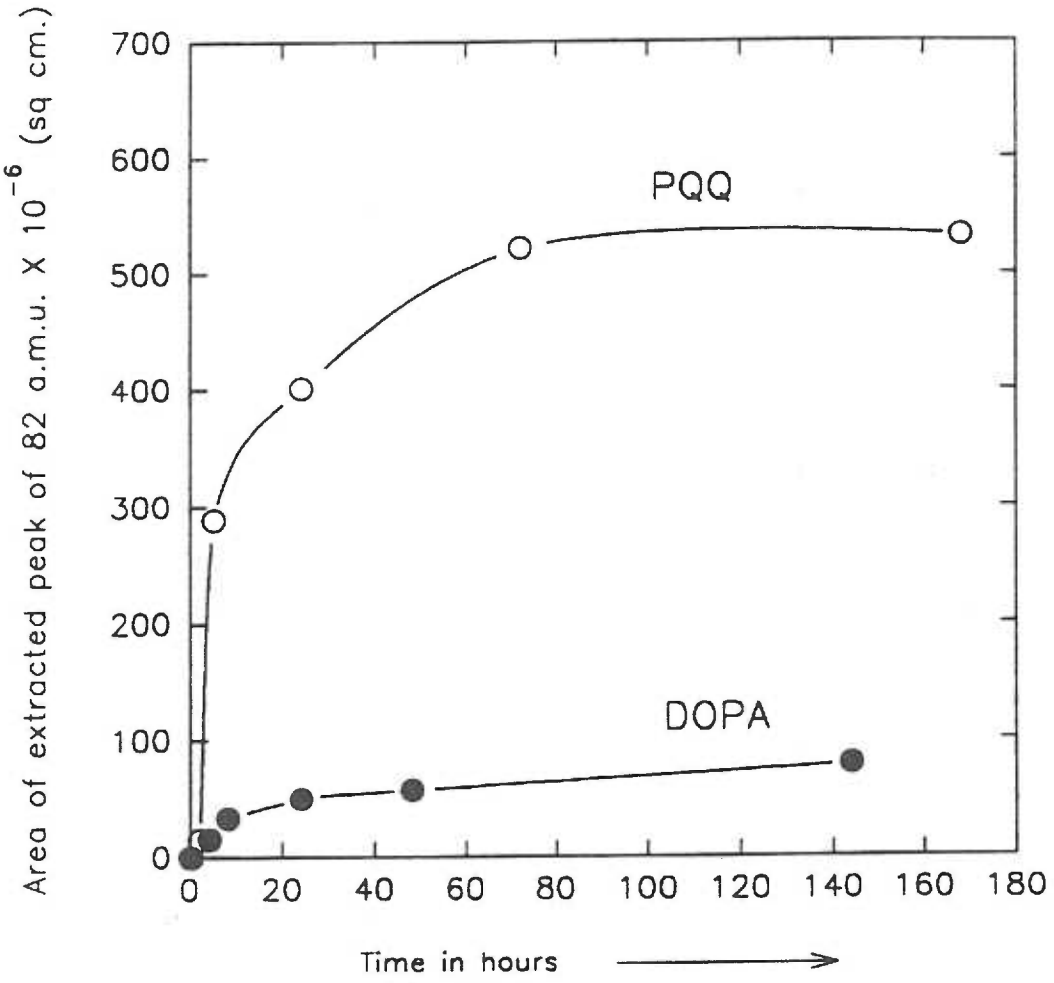


Figure 34 : Effect of duration of incubation at 37°C of 0.133 M N-α-acetyl lysine incubated with 1 mM PQQ or DOPA and 300 μM copper on the formation of N-acetyl dehydropipecolic acid.

In the presence of oxygen, DOPA is converted to its quinone form and this reaction is faster in alkaline media and in the presence of metal ions. Once Dopaquinone is formed it may proceed to carry out the oxidative deamination of N-*α*-acetyllysine. Of course, polymerization is a hindering factor here, and that explains the formation of much smaller amounts of the cyclic compound when compared with PQQ samples. Even up to 140 hours, there seems to be a slight but continuous increase in the amount of product formed. This is because, unlike PQQ, the sample solution is far from saturated with the oxidized product. Polymerization is a continuous process as is the process of oxidation of aminoquinol to DOPAquinone. A fraction of DOPAquinone formed from reoxidation of aminoquinol (by copper) is available for the oxidative reaction, even though a major portion of it goes to polymerization.

The hyperbolic curve obtained with PQQ and N-*α*-acetyl-L-lysine samples also supports the proposed concepts. The larger increase in the formation of the cyclic compound is due to the presence of ample PQQ for oxidative deamination especially since there is no other competing reaction that interferes and consumes PQQ. Copper oxidizes the aminoquinol back to the active quinone form.

It can be seen from Figure 32, that the amount of cyclic compound formed when N-*α*-acetyl-L-lysine is incubated with DOPA is extremely small. It is thus not surprising that we were unable to detect the reduced aldehyde in the reaction mixture containing N-*α*-acetyl-L-lysine and DOPA+copper, GC-MS. But it is clear from the Figure 33 that incubating N-*α*-acetyl-L-lysine with PQQ (+ copper)

results in a much larger amounts of the cyclic compound. In the next experiment, the sample solution containing N-*α*-acetyl-L-lysine and PQQ (+ copper) is probed for the presence of the aldehyde.

Conclusion

The magnitude of the plot between duration of incubation and the extent of oxidative deamination is at least 8 times greater for PQQ than DOPA.

DETECTION OF THE REDUCED ALDEHYDE IN THE PQQ-N-α-ACETYL-L-LYSINE SAMPLES

Introduction

All attempts to isolate the aldehyde from DOPA incubated N-α-acetyl-L-lysine failed, even though a radioactive peak corresponding to the reduced allysine "HNL" was seen on borohydride reduction on the chromatographic profile. The radioactive plot did not show the presence of any crosslinks like "LNL", either. The reduced aldehyde could not be detected by mass spectroscopy. Instead, a cyclic product was isolated; formed as a consequence of the reaction between the aldehyde and the lysine immediately followed by an elimination reaction. Because this cyclic compound cannot be reduced by sodium borohydride, it cannot be labelled and detected as a radioactive peak by chromatography. The aldehyde is normally very reactive and its life-span in solution is limited. The sodium borohydride is a well known reducing agent for aldehydes and ketones. Since a very high specific activity of tritiated borohydride is used in the reaction, it is no surprise that a peak shows up on the radioactive profile, but is not abundant enough for its presence to be picked up by GC-MS as a signal over the noise level.

The case with PQQ and N-α-acetyl-L-lysine is different, for the reaction is at least eight times more efficient with PQQ than with DOPA. I proposed that the basal level of aldehyde at any instant would also be greater by about the same

order of magnitude and may be detectable by mass spectroscopy. The following experiment details the detection of the reduced aldehyde of N-*α*-acetyl-L-lysine with PQQ.

Experimental:

7 ml volumes of N-*α*-acetyl-L-lysine at a concentration of 25 mg/ml in PBS was incubated at 37°C for 72h, in the presence of 1 mM PQQ with and without 0.3 mM copper(II) chloride. The two controls were N-*α*-acetyl-L-lysine at 25 mg/ml and 1 mM of PQQ in PBS, both incubated at 37°C for 72h. The samples were reduced with sodium borohydride as indicated in the Methods section, in order to reduce any aldehydes or Schiff bases to stable compounds. The samples were then acidified to pH~1 with conc. HCl. 7mls of each sample was poured onto a chem-elut (5 ml capacity) column. After 5 minutes of equilibration the column was eluted three times with 10 ml volumes of ethyl acetate.

Since the samples were reduced with borohydride prior to acidification and extraction, considerable amounts of boric acid was extracted into ethyl acetate. It was important to remove the excess boric acid from the mixture before GC analysis. This was done by derivatizing the dried ethyl acetate extract with 0.15 ml of BSTFA-TMCS, and evaporating off the derivatized boric acid which is volatile. The remaining extract was redissolved in more BSTFA-TMCS (0.075 ml) and analyzed by GC-MS.

The GC chromatographic elution of the trimethylsilylated derivative of the ethyl acetate soluble fractions of N-α-acetyl-L-lysine samples along with the relevant controls were compared. Samples were prepared and labelled as follows:

Sample No.	Concentration of		
	N-α-acetyl-L-lysine (mg/ml)	PQQ (mM)	Copper (mM)
A (control)	0	1	0
B (control)	25	0	0
C	25	1	0.3

Results:

The GC profiles of controls DOPA only, N-α-acetyl-L-lysine only and N-α-acetyl-L-lysine incubated with PQQ with 0.3 mM copper, respectively, are shown in the top panels of Figure 35 A, B and C.

The mass spectra of the species eluting at retention time ~20.1 min. is shown in the bottom panels of Figure 35 A, B and C. The mass spectrum of sample C shows a peak at retention time of 20.157 min. with a (M-15) peak of 318 and proposed molecular ion of 333, which corresponds well with the sodium borohydride reduced di-TMS derivative N-acetylallysine. Neither the molecular

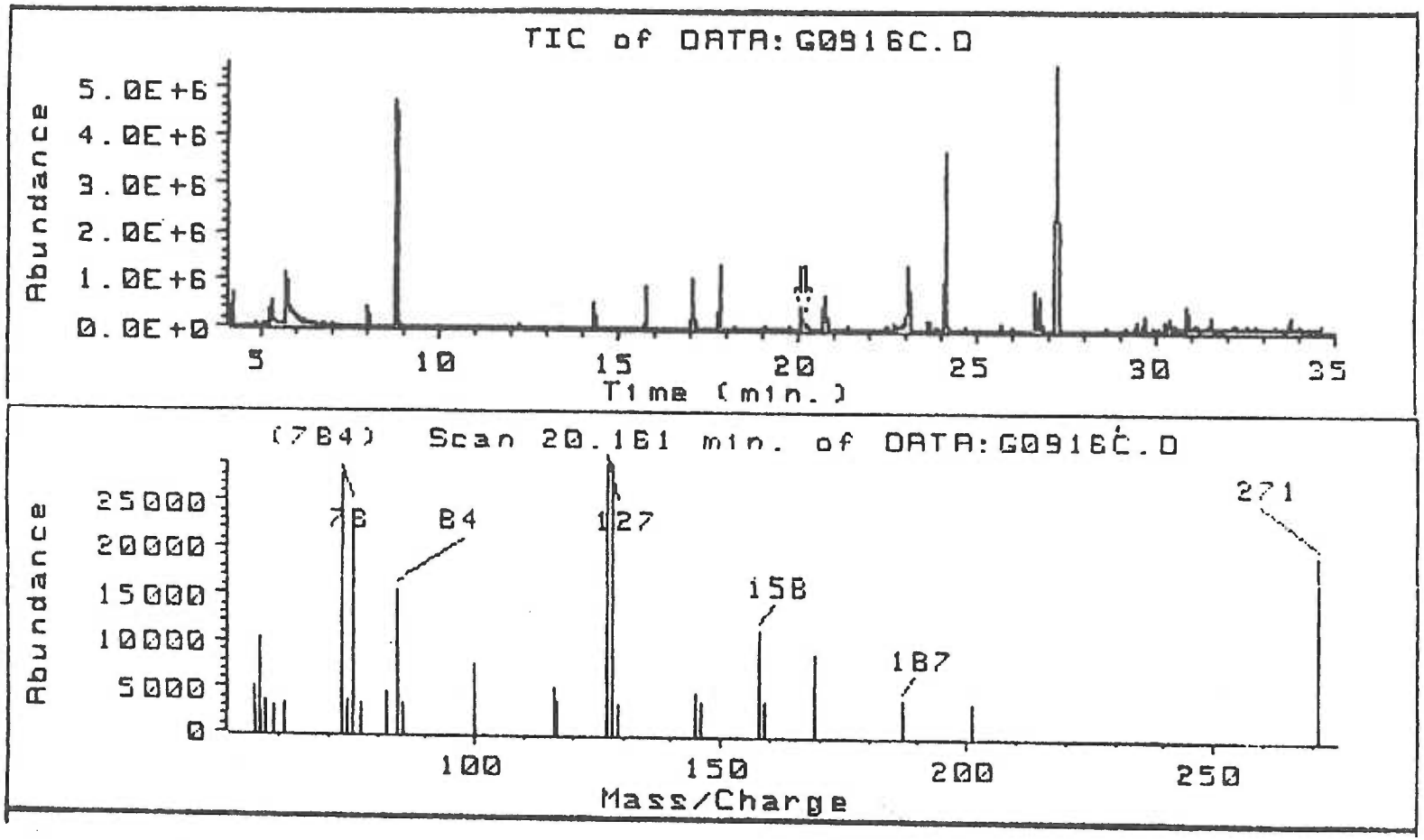


Figure 35B: The top panel shows the GC-profile for the control sample B (TMS derivatized N-α-acetyllysine). Bottom panel shows the mass spectrum of the species eluting at retention time 20.161 min.. Absence of m/z 333 or (M-15) 318 ions indicate that reduced aldehyde is probably not present.

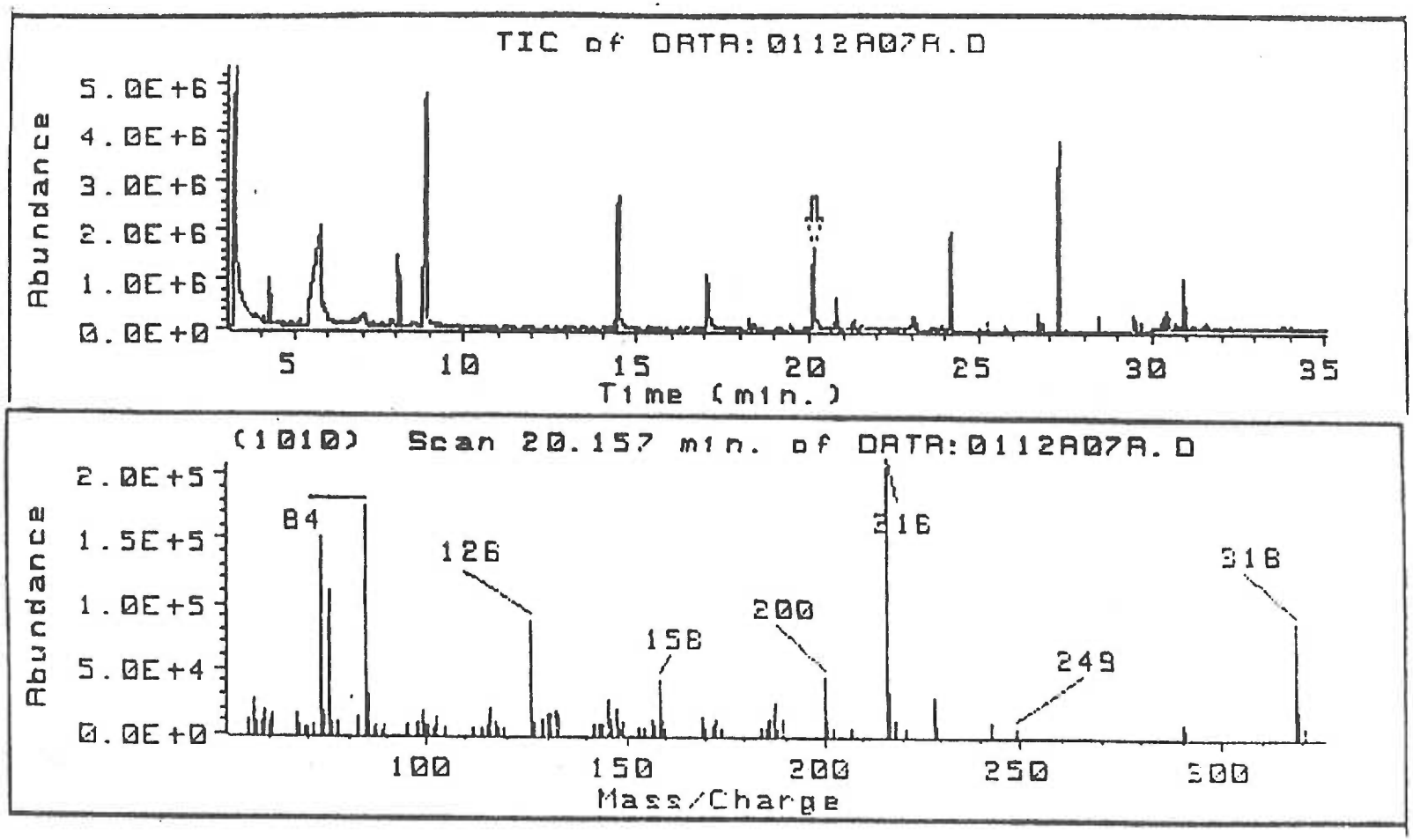


Figure 35C: The top panel shows the GC-profile for the sample C (TMS derivatized Nα-acetyllysine with PQQ and copper). Bottom panel shows the mass spectrum of the proposed derivatized reduced aldehyde of N-α-acetyllysine (m/z = 333) eluting at retention time 20.157 min..

ion 333 nor the (m-15) ion 318 could be detected in samples A or B.

The molecular ion of 333 corresponds to the reduced di-TMS derivatized N-acetyl allysine. Some of the other fragments can also be related to the proposed structure. The fragments obtained were 333, 318, 216, 200, 158, 126, 84 and 73.

Another confirmation for the formation of the aldehyde product is shown in Figure 36. Figure 36 is the GC-profile of sample C that had been left at 4°C for 1 week. At the end of the 7 days, the sample was reinjected into the GC-MS and chromatogram recorded. It was observed that at this extended incubation period, the more difficult hydrogen to be derivatized; namely the proton of the imino group ($N\underline{H}COCH_3$) of the α-carbon also gets derivatized. In Figure 36, the ion of a.m.u. 390 is the M-15 ion for the species of molecular mass 405, which corresponds well with the tri-TMS derivative of the reduced aldehyde of N-α-acetyllysine.

Conclusion:

PQQ oxidatively deaminates N-α-acetyl-L-Lysine to the reduced aldehyde, hydroxynorleucine (HNL) which is identified by mass spectroscopy.

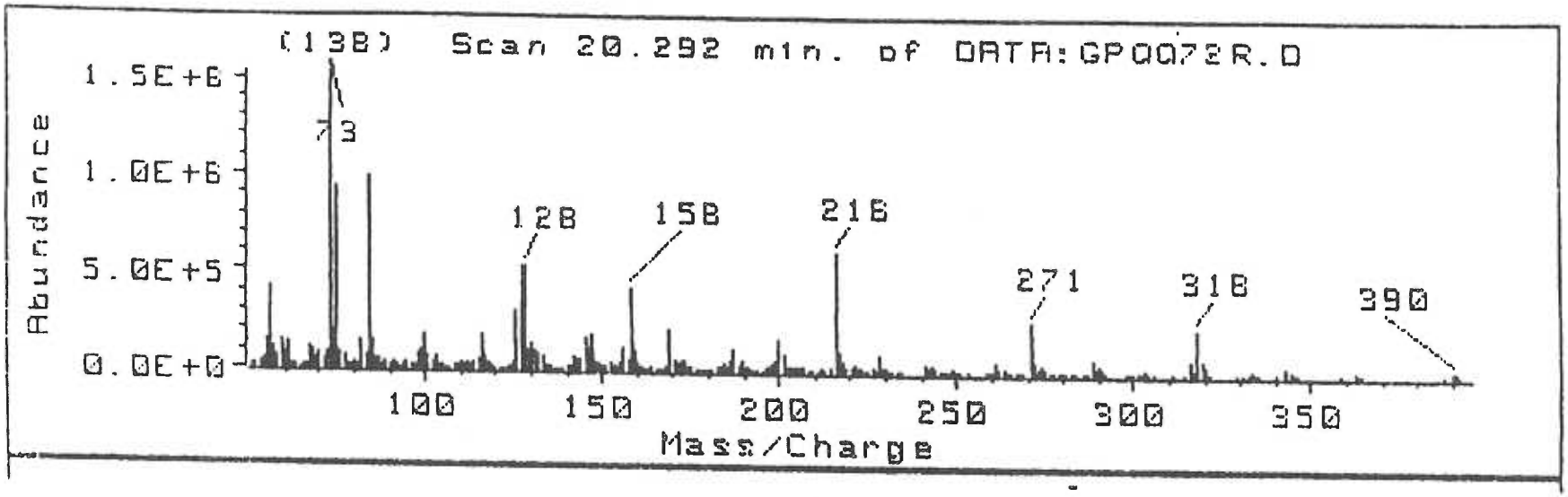


Figure 36: The mass spectral fragmentation pattern for the compound of m/z 405 showing an (M-1) fragment of 390 is proposed to be the tri-trimethylsilyl derivative of the reduced aldehyde of N-α-acetyllysine. Also seen in the spectra is the ion of m/z=318 which is the M-15 fragment of the di-trimethylsilyl derivative of the reduced aldehyde of N-α-acetyllysine.

VI. SUMMARY:

It is conclusively proved that ortho-quinones are capable of oxidatively deaminating free and peptide-bound lysines. DOPA was capable of crosslinking telopeptide intact collagen more strongly than telopeptide-removed collagen. Thus, the lysyl residues on the "telopeptide" must be easily accessible for oxidative deamination and further crosslinking. Upon reacting N-*α*-acetyl-L-lysine with DOPA, the presence of the reduced aldehyde could be shown by radioactive measurements but not by mass spectroscopy. However, a cyclic compound was isolated by mass spectroscopy. This cyclic compound, N-acetyl-dehydropipecolic acid is very stable and has a unique base peak of 82 a.m.u., which is a useful product that can be accurately assayed in order to estimate the extent of oxidative deamination. It was shown that multivalent metals enhance the rate of reaction and a mechanism for this enhancement is proposed in Figure 27. Copper(II) is better than iron(III) compounds. The nature of the ortho-quinones also affected the rate of the reaction, the order of increasing extent of reaction being DOPA < TOPA < tBC < PQQ. With PQQ, the extent of reaction was at least eight times more than with DOPA. The reduced aldehyde was identified by incubating N-*α*-acetyl-L-lysine with PQQ and analyzing the mixture with GC-MS. A reduced aldehyde was also detected when 6-aminocaproic acid, an analog of lysine, was reacted with DOPA.

The proposed overall reaction mechanism by which DOPA is involved in

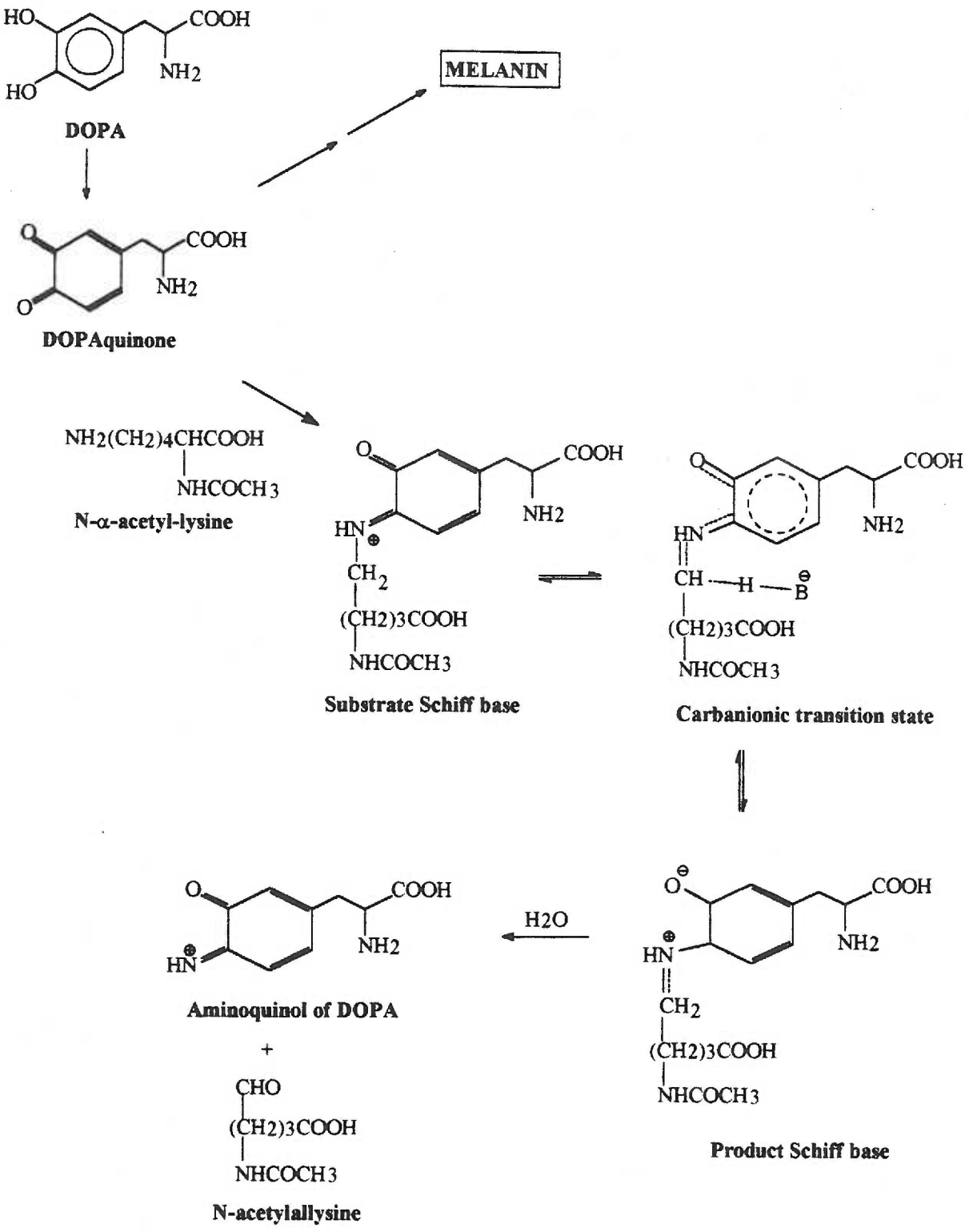


Figure 37: A proposed mechanism for the oxidative deamination of N- α-acetyl-L-lysine by the orthoquinone, DOPA. DOPA is reduced to an aminoquinol form during the oxidative reaction.

the deamination is given in Figure 37. DOPA oxidizes to Dopaquinone in the presence of oxygen. DOPAquinone may then proceed into two pathways. It may polymerize in the classic way to form melanin, or it may convert N-α-acetyl-L-lysine to N-acetylallysine. For the latter, I propose that N-α-acetyl-L-lysine forms a Schiff base with one of the carbonyls of the o-quinone group. Since the double bond is between the lysine ϵ-amino group and the quinone carbonyl group, it is named the 'substrate Schiff base'. In the presence of base, which can abstract the labile hydrogen of the epsilon-carbon of the bound lysine, an internal shift of electrons results in a rearrangement of the double bond. The result is a 'product Schiff base'. Because the aromatic ring tends to pull the lone pair of electrons on nitrogen towards itself, the product Schiff base is easily hydrolyzed. The result is the formation of aminoquinol and allysine. The aminoquinol, which is catalytically nonfunctional, could be oxidized to DOPAquinone by metals like copper(II) ions. Copper(II) oxidizes the aminoquinol to a semiquinone (since it is a 1-e oxidation) and finally to DOPAquinone. In the process copper(II) first gets reduced to copper(I) state. The copper(I) might be oxidized back to the copper(II) state by atmospheric oxygen. The byproducts of the reaction include hydrogen peroxide (reduced oxygen) and ammonia. A similar mechanism has been proposed for amine oxidases (105).

Polymerization is the major pathway for DOPA and to a lesser extent for TOPA. Because of this the amount available for the oxidative deamination reaction is decreased. The reaction is also governed by the rate of reoxidation of

the aminoquinol which depends upon the amount of metal ions, the nature of metals ions and probably other environmental factors. For tBC, pathway 1 is limited to the generation of linear concatomers. With PQQ the major reaction is oxidative deamination, as has been discussed earlier. It is for this reason that even small doses of PQQ are highly efficient.

PQQ has been fed to lathyritic rats which resulted in an increase in the tensile strength of their skin (23). The use of PQQ as a drug is under trial and might soon be marketed by Merck.

Because the pathochemistry of the majority of the collagen crosslinking defects is due to a deficiency in lysyl oxidase and/or other deficiencies, the use of ortho-quinones as a prospective remedial drug for improving collagen crosslinking is very attractive. In this respect, DOPA is in fact a normal metabolite in mammals (106) and therefore is non-toxic. It is already being used to treat Parkinson's disease. In this case, rather high doses of DOPA have been used due to the limited movement of DOPA across the blood-brain barrier (107). This property would be rather advantageous for its proposed application for inducing collagen crosslinking, since penetration through the blood brain barrier is not required. There is, however, at least one report that claims that DOPA may be converted to TOPA non-enzymically (108). TOPA is a known neurotoxin (109).

Pyrroloquinoline quinone, PQQ, which exhibits superior oxidizing capacity, would be an even better candidate drug. PQQ is already known to have very desirable effects on certain diseased conditions of the central nervous system.

Jensen et al. (110) showed that PQQ can behave as a neuroprotector when systemically administered either before or after induction of hypoxia/ischemia brain damage in rats. On administration, PQQ was shown to have anticonvulsant effects, in-vivo. The investigators injected between 10 or 20 mg/kg of PQQ into the rat after an induced stroke (110).

Other investigators (111) have postulated that PQQ acts as a cofactor of complex 1 in mitochondrial oxidative phosphorylation.

PQQ has also been proposed to have a major role as a free radical scavenger and as an anti-oxidant (112), in-vivo. Hultquist et al. have studied the overall anti-oxidant effect of PQQ and have summed up their observations as follows:

(i) $PQQH_2$ reduces higher oxidative states of hemeproteins.

(ii) PQQ protects heart, brain, lungs and muscles against oxidative injury

(iii) A cytosolic PQQ reductase is present in mammalian tissues

(iv) Tissue protection by PQQ involves PQQ reductase.

The above summarized neuroprotectant and antioxidant role for free PQQ present in the human system greatly strengthens the role for free ortho-quinones in the oxidative deamination of lysine ϵ-amino groups proposed in this thesis.

The present study is of much significance clinically. Ehler-Danlos syndrome and other collagen diseases are examples of genetic and acquired defects which to-date have no therapeutic treatment. As discussed before, a common

manifestation of most of these diseases is the development of extremely fragile skin due to defective collagen crosslinking. PQQ and ortho-quinones if administered orally might increase the strength of these defective tissue by oxidatively deaminating lysyl/hydroxylysyl residues and ultimately crosslinking them. Extensive in vivo studies need to be carried out to study the capacity of free ortho-quinones in crosslinking fragile tissues of animals that have lack optimum crosslinks.

Ortho-quinones like DOPA and PQQ are non-toxic 'tanning agents' of collagen. Before this discovery, all the other tanning agents used like formaldehyde, hydroquinone etc. are extremely toxic. Implants made of collagen had to be crosslinked for reasons discussed earlier in the introduction. However, it is important that the crosslinking agents used must be non-toxic, for leaching out of toxic breakdown products and catalysts is the primary cause for biological incompatibility(141). PQQ, especially, is very attractive as a crosslinking agent for collagen, for use as bioprosthesis, for it is both non-toxic and a very efficient crosslinking agent.

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